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Nature of TSST-1 Production by *Staphylococcus aureus* in Aberrant Vaginal Conditions

(Spine Title: Toxin Production by *S. aureus* in Aberrant Vaginal Conditions)

(Thesis Format: Monograph)

by

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Graduate Program in
Microbiology and Immunology

Thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

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Nature of TSST-1 Production by *Staphylococcus aureus* in Aberrant Vaginal Conditions

is accepted in partial fulfilment of the
requirements for the degree of

Master of Science

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Chair of the Thesis Examination Board

ABSTRACT AND KEYWORDS

Menstrual-toxic shock syndrome (TSS) is a serious illness that afflicts women of pre-menopausal age worldwide, and arises from vaginal infection by *Staphylococcus aureus* and concurrent production of toxic shock syndrome toxin-1 (TSST-1). The aim of this study was to investigate the influence of aberrant vaginal states and indigenous vaginal bacteria on production of TSST-1 and identify women who are most susceptible to menstrual-TSS. A TSST-1 reporter strain of *S. aureus* was grown in the presence of vaginal swab contents collected from women in London, Ontario with healthy and aberrant vaginal states. Gene expression assays were also conducted to monitor toxin production in response to indigenous vaginal bacteria. This study found that *Streptococcus agalactiae* significantly induces TSST-1 production, while resident *Lactobacillus* spp. suppress production. Results also indicate that women with aerobic vaginitis, but not bacterial vaginosis, may be more susceptible to menstrual-TSS and would benefit most from prophylactic treatment.

Keywords: Menstrual-toxic shock syndrome, *Staphylococcus aureus*, toxic shock syndrome toxin-1, lactobacilli, aerobic vaginitis, bacterial vaginosis, *Streptococcus agalactiae*, vaginal microbiota

DEDICATION

For my parents, Duncan and Elaine, and brothers, Stuart and Cameron, who have always provided me with love and support.

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TABLE OF CONTENTS

TITLE PAGE i

CERTIFICATE OF EXAMINATION ii

ABSTRACT AND KEYWORDS iii

DEDICATION iv

ACKNOWLEDGMENTS v

TABLE OF CONTENTS vi

LIST OF FIGURES x

LIST OF TABLES xi

LIST OF ABBREVIATIONS xii

CHAPTER 1- INTRODUCTION 1

1.1. Focus of Thesis 2
1.2. Vagina 2
1.2.1. Anatomy and Physiology 2
1.2.2. Development 3
1.2.3. Menstrual Cycle 4
1.2.4. Immune and Host Protective Systems 6
1.2.5. Urogenital Infections 11
1.3. Menstrual-TSS 12
1.3.1. Pathogenesis 12
1.3.2. TSS Subtypes 13

1.3.3. Epidemiology 14

1.3.4. Treatment 15

1.3.5. Disease Progression 17

1.3.5.1. Course of Infection 17
1.3.5.2. *S. aureus* Colonization 19
1.3.5.3. TSST-1 Production 21
1.3.5.4. TSST-1 Translocation 26

1.3.6. Antibody Titres to TSST-1 27

1.3.7. The Tampon Hypothesis 28

1.4. Vaginal Microbiota 31
1.4.1. Definition 31

1.4.2. Techniques used to decipher the vaginal microbiota 34

1.4.3. Aberrant States 36

1.4.3.1. Bacterial Vaginosis 36
1.4.3.2. Aerobic Vaginitis 39

1.5. Rationale for studying *S. aureus* TSST-1 production in aberrant states 41
1.6. Hypothesis and Objectives 42

CHAPTER 2- MATERIALS AND METHODS 44

2.1. Denaturing Gradient Gel Electrophoresis 45
2.1.1. Clinical Samples 45

2.1.2. DNA Extraction and Amplification 45

2.1.3. Denaturing Gradient Gel Electrophoresis 48

2.1.4. DNA Re-amplification and Purification 51

2.1.5. Sequencing 51

2.2. Clinical Study of Vaginal Microbiota of Premenopausal Women 52

2.2.1. Ethics Statement 52

2.2.2. Study Population and Recruitment 52

2.2.3. Sample Collection 53

2.3. Bacterial DNA Preparation for Illumina Sequencing 57
2.4. Luminescence Assay 58
2.5. Supernatant Challenge Assay 60
2.5.1. Bacterial Cultures and Conditions 60

2.5.2. SDS-PAGE and Western Blot 64

2.5.3. Real-Time PCR 67

2.5.3.1. *S. aureus* RNA Extraction 67
2.5.3.2. Quantification of *tst* Expression 71
2.6. Statistical Analysis 72
CHAPTER 3- RESULTS 73

3.1. DGGE versus 16S rRNA Sequencing by Illumina 74
3.2. Vaginal Microbiota of Pre-menopausal Women With and Without Bacterial Vaginosis in London, ON 78
3.3. Abundance of *Staphylococcus aureus* in Pregnant Women in Toronto, ON 84
3.4. Expression of *tst* in *S. aureus* in Women With and Without Bacterial Vaginosis 87
3.5. TSST-1 Production in Response to Vaginal Bacteria Associated with Bacterial Vaginosis, Aerobic Vaginitis and a Healthy Microbiota 91
CHAPTER 4- DISCUSSION 99

4.1. Induction and Inhibition of TSST-1 by Vaginal Bacteria 100
4.2. *S. aureus* TSST-1 Production in Aberrant States 106
4.3. Prevalence of *S. aureus* and Other Bacteria Associated with Aberrant States 108
4.4. Future Directions 113
4.5. Conclusions 115
CHAPTER 5- REFERENCES 119

Appendix 1- UWO HSREB Full Board Submission Form 143

Appendix 2- Recruitment Poster 167

Appendix 3- Letter of Information and Consent 168

Appendix 4- Study Questionnaire 173

Appendix 5A- Luminescence Data 175

Appendix 5B- Growth Curves for Luminescence Assay 178

Appendix 6- Growth Curves of *S. aureus* MN8 in Response to Supernatant Challenge 182

Appendix 7 – Publications During Thesis 184

Curriculum Vitae 186

LIST OF FIGURES

Figure 1.1. Pathogenesis of menstrual-TSS 18

Figure 1.2. Regulation of the *agr* system in *S. aureus*....................................... 24

Figure 2.1. Sample collection of clinical study in London, ON. 54

Figure 2.2.Vaginal swab processing of the ESwab transport system 59

Figure 3.1A. DGGE analysis of subjects of healthy and intermediate status...... 75

Figure 3.1B. DGGE analysis of subjects of intermediate and BV status............. 76

Figure 3.2. Comparison of DGGE and sequencing by Illumina. 77

Figure 3.3A. Vaginal smear of healthy subject from London, ON 79

Figure 3.3B. Vaginal smear of intermediate subject from London, ON 80

Figure 3.3C. Vaginal smear of subject with BV from London, ON 81

Figure 3.4. Vaginal microbiota of subjects recruited from London, ON............... 83

Figure 3.5A. Vaginal microbiota of subjects recruited from Toronto, ON............ 85

Figure 3.5B. Abundance of *Staphylococcus* spp. in clinical samples. 86

Figure 3.6A. Expression of *tst* in response to vaginal clinical samples............... 88

Figure 3.6B. Sustained *tst* expression in response to clinical samples............... 89

Figure 3.6C. Sustained *tst* expression in response to clinical samples 90

Figure 3.7. Production of TSST-1 in response to vaginal isolates 92

Figure 3.8A. Expression of *tst* in response to lactobacilli.................................... 94

Figure 3.8B. Expression of *tst* in response to AV-associated bacteria 95

Figure 3.8C. Expression of *tst* in response to BV-associated bacteria 96

Figure 3.9. Total protein expression of bacteria used to challenge *S. aureus* 98

Figure 4.1. TSST-1 production by *S. aureus* in AV environment 117

LIST OF TABLES

Table 2.1. PCR Master Mix for DGGE. 47

Table 2.2. Composition of denaturant solutions used for DGGE. 49

Table 2.3. Nugent score criteria for BV diagnosis. 56

Table 2.4. Bacterial strains used for supernatant challenge assay. 62

Table 2.5. Grouping of bacteria for supernatant challenge assay 63

Table 2.6. Composition of polyacrylamide gels for SDS-PAGE 66

Table 2.7. Master Mix components for MultiScribe Reverse-Transcription. 70

LIST OF ABBREVIATIONS

σ^B	alternative sigma-factor B
agr	accessory gene regulator locus
AHL	acylated homoserine lactones
AI	auto-inducers
AIP	auto-inducing peptides
APC	antigen-presenting cell
AV	aerobic vaginitis
BCA	bicinchoninic acid
BHI	brain heart infusion media
BV	bacterial vaginosis
CcpA	catabolite protein A
CDC	Centers for Disease Control and Prevention
CPS	capsular polysaccharide
cre	catabolite-responsive elements
DAB	diaminobenzidine
DGGE	denaturing gradient gel electrophoresis
DPD	4,5-dihydro-2,3-pentanedione
ECM	extracellular matrix
EPS	extracellular polymeric substance
FDA	food and drug administration
FnBP	fibronectin-binding protein
FSH	follicle-stimulating hormone

GAS	group A *Streptococcus*
GBS	group B *Streptococcus*
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HPV	human papilloma virus
HRT	hormone replacement therapy
HSV	herpes simplex virus
ica	intercellular adhesion locus
IFN	interferon
IgA	immunoglobulin A
IL	interleukin
IVIG	intravenous immunoglobulin
LAB	lactic acid bacteria
LH	luteinizing hormone
LPS	lipopolysaccharide
LTA	lipoteichoic acid
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MMP	metalloproteases
MRS	de Man, Rogosa and Sharpe media
MRSA	methicillin-resistant *Staphylococcus aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction

PEC pyrogenic exotoxin type C

PRR pattern recognition receptor

PTB preterm birth

QS quorum sensing

RAP RNAIII-activating protein

RFLP restriction fragment length polymorphism

RIP RNAIII-inhibiting protein

Rot repressor of toxins

SAG superantigen

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE staphylococcal enterotoxin

SLPI secretory leukocyte peptidase inhibitor

STI sexually-transmitted infection

TCA trichloroacetic acid

TCR T-cell receptor

TCRS two-component regulatory system

TEMED *N*,*N*, *N*′,*N*′-tetramethylethylenediamine

TGF transforming growth factor

TLR toll-like receptor

TNF tumor necrosis factor

TSS toxic shock syndrome

TSST-1 toxic shock syndrome toxin-1

VDMP vaginally-defined medium + 0.5% proteose peptone

VVC vulvovaginal candidiasis

CHAPTER 1

INTRODUCTION

1.1. Focus of Thesis

The purpose of this study is to investigate the relationship between the vaginal microbiota and *Staphylococcus aureus* in the context of menstrual-Toxic Shock Syndrome (TSS). This section will cover information on the site of infection, as well as the nature of *S. aureus* and key information that has yet to be uncovered. In order to appreciate the dynamic nature of menstrual-TSS, including its pathogenesis and associated risk factors, it is important to first consider the physiology of the vagina.

1.2. Vagina

1.2.1. Anatomy and Physiology

The female genital tract is a system of several structures that function together in reproduction. The tract as a whole consists of the ovaries, fallopian tubes, uterus, vagina and vulva. The vagina in particular is important as it is exposed to the external environment and is thus vulnerable to invasion by external agents such as bacteria, viruses and fungi. The vagina is an elastic muscular canal with a length of approximately 7-9 cm that extends from the cervix to the external vulva. The vaginal wall is lined with a non-keratinized stratified squamous epithelium attached to underlying stromal cells and a basement membrane. The vaginal wall lacks glands and thus does not produce mucus, which instead comes from the cervical mucosa and transudate through the vaginal epithelium (Paavonen, 1983). Glycogen is present in the middle and superficial layers of the epithelium and is dependent upon the amount of

circulating estrogen, and thus fluctuates throughout the menstrual cycle. During mid-cycle, the vagina is under anaerobic conditions relative to atmospheric gas levels; oxygen is at a partial pressure of 3 mm Hg (versus 100-140 mm Hg atmospheric) and carbon dioxide at 64 mm Hg (versus 5 mm Hg atmospheric) (Wagner *et al.*, 1984). The nature of the vagina makes it a unique habitat for microorganisms.

1.2.2. Development

The vagina of a newborn is influenced by estrogen from the mother, and includes a stratified squamous epithelium high in glycogen content, as well as the presence of lactic-acid-producing bacteria and an acidic pH. In early childhood, however, the effects from maternal hormones are lost and there is a thinning of the vaginal epithelial layer, loss of glycogen and subsequent neutral pH and a reduced population of lactic-acid-producing bacteria (Gerstner *et al.*, 1982; Hill *et al.*, 1995). The event of puberty, typically occurring from the age of 8-13 years, initiates changes in the vagina due to increased circulation of adrenal and gonadal hormones, including estrogen (Farage & Maibach, 2011). Menarche, the first menses experienced in an individual, typically begins at the age of 12-13 years, and it takes around six years to establish regular menstrual cycles with predictable occurrences of menses (Flug *et al.*, 1984; WHO 1986; Boynton-Jarrett *et al.*, 2011). Following puberty, the vaginal wall again begins to thicken through the proliferation of stratified squamous epithelial cells, which results in increased glycogen content in response to elevated estrogen levels (Hammerschlag *et al.*, 1978). In particular, circulating estrogen triggers

underlying stromal cells to release soluble factors to the neighboring epithelial cells and induce cellular changes (Cunha & Young, 1992). Glycogen deposits in the vaginal epithelium and is metabolised anaerobically primarily by lactic-acid bacteria (LAB), and to a lesser degree by vaginal epithelial cells (Boskey *et al.*, 2001). Metabolism of glycogen leads to lactic acid production through fermentation of glucose which lowers the pH of the vagina. Several investigators over the years have reported varying pH values for the healthy vagina, but it tends to range from about 3.5 to 5.0 (Owen & Katz, 1999). At the onset of menopause comes a drop in estrogen levels, which causes a thinning of the vaginal epithelium, loss of glycogen content and subsequent loss of lactobacilli. This in turn leads to an increased pH from 4.5 to about 6.0 (Roy *et al.*, 2004). This effect has been shown to be reversible by hormone replacement therapy (HRT) (Heinemann & Reid, 2005).

1.2.3. Menstrual Cycle

The human menstrual cycle consists of a series of events dictated by fluctuating hormone levels. The cycle can be broken down into three phases: the follicular phase (or proliferative phase), ovulation and the luteal phase (or secretory phase). The follicular phase typically marks days 1-13 of the cycle, and is characterized by a rise in follicular-stimulating hormone (FSH), released by the anterior pituitary gland. FSH stimulates development of ovarian follicles, which in turn begin to secrete estradiol. As this phase progresses, elevated estradiol suppresses FSH production, leading to the atrophy of all but the most dominant follicle, subsequently referred to as the tertiary follicle. Estradiol also contributes

to endometrial remodeling and proliferation of the uteral wall at this time, as well as revascularization of the tissue. Initial triggering of this event seems to be independent of estradiol and is still not fully understood (Lecce *et al.*, 2001; Maybin & Critchley, 2011), but recent studies have shown that progesterone withdrawal that occurs at the end of the luteal phase seems to trigger repair-associated molecules such as fibronectin (Cao *et al.*, 2007). Ovulation occurs when levels of estradiol reach a threshold that triggers a surge in luteinizing hormone (LH), an event which in turn triggers the release of the ovum from the tertiary follicle. This phase typically occurs from day 13-16 of the cycle, and the LH surge can last for 48 hours. The luteal phase typically marks day 16-28 of the cycle, and is marked by increased production of progesterone from the corpus luteum, an endocrine structure that remains following the rupture of the tertiary follicle. Progesterone, together with estradiol, is responsible for the thickening of the uteral endometrium in preparation of embryo implantation; in particular, a network of reticular fibers containing type III and type I collagen is built up in the extracellular matrix (ECM) of the endometrium. The proliferation of the surface endometrial cells is dependent upon hormonal stimulation of the underlying stromal cells (Cunha & Young, 1992).

Progesterone also triggers release of estradiols from the adrenal glands, both of which suppress LH and FSH. If fertilization of the ovum does not occur, the corpus luteum atrophies and progesterone and estradiol levels drop, leading to menstruation. This event marks day 1 of the cycle, and results in sloughing of the uteral endometrium and blood flow through the vaginal canal that typically

lasts for 3-5 days. Breakdown of the endometrial extracellular matrix occurs through activity of matrix metalloproteases (MMPs), which are produced by endometrial cells and leukocytes in response to a drop in progesterone (Marbaix *et al.*, 1996; Salamonsen *et al.*, 1997). Several *in vitro* studies have also demonstrated an involvement of an inflammatory process in the upregulation of MMPs during menses, including production of transforming growth factor (TGF)-β, tumor necrosis factor (TNF)- α, interleukin (IL)-1, and other inflammatory markers (Chegini *et al.*, 1992; Hunt *et al.*, 1992; Kauma *et al.*, 1990; Salamonsen & Woolley, 1998). Some of these pro-inflammatory molecules are suppressed by progesterone, and thus shedding of the endometrium seems to result from MMP production both by a loss of direct suppression by progesterone, as well as by a subsequent trigger of a pro-inflammatory process (Hunt *et al.*, 1997). It is during the menses phase that organisms such as *S. aureus* can potentially cause problems for the host, as will be discussed below.

1.2.4. Immune and Host Protective Systems

The vagina is continuously exposed to a complex community of bacteria, some of which can be pathogenic, and so it utilizes the innate and adaptive immune systems to help prevent infection. A critical component in innate immune defense is the group of transmembrane signaling proteins referred to as Toll-like receptors (TLRs) present on the epithelial cells in both the upper and lower female genital tract (Nasu & Narahara, 2010). TLRs are part of the pattern-recognition receptor (PRR) family on antigen-presenting cells (APCs) and epithelial cells that recognize distinct molecular patterns on the surface of

microbial cells called pathogen-associated molecular patterns (PAMPs) and trigger a subsequent inflammatory reaction to neutralize the recognized pathogen. The TLR family is made up of ten currently identified members that are collectively able to recognize bacteria, viruses, fungi and protozoa. The binding of a TLR to its ligand triggers a signaling transduction pathway which ultimately activates the NF-κB pathway and expression of genes encoding pro-inflammatory cytokines and co-stimulatory molecules in APCs and epithelial cells (McGowin *et al.*, 2009). One study investigated the presence of these receptors in the female genital tract and found TLRs 1-9 to be expressed in the vaginal epithelial cells, with TLR-2, -3, -5 and -6 being the most abundantly expressed (Herbst-Kralovetz *et al.*, 2008). These TLRs were also shown to respond to their respective bacterial and viral PAMPs which elicited strong cytokine responses. The presence of these receptors is especially apparent during times of microbial invasion of pathogenic bacteria. For instance, a study looked at cytokine levels in the lower genital tract of healthy women versus women with bacterial vaginosis (BV), a condition characterised by depletion of protective lactobacilli and an abundance of pathogenic anaerobes (Mattsby-Baltzer *et al.,* 1998). This study found elevated levels of the pro-inflammatory cytokine IL-1β and IL-6 associated with BV, and linked this to the presence of endotoxins characteristic of Gram-negative bacteria. These endotoxins would be detected in large part by TLR-4, leading to the production of these cytokines.

The vaginal epithelial cells play an important role as a physical barrier to the intrusion of bacteria, but studies indicate that they also play an active role in

vaginal immunity. One study investigated immunological markers from the immortalized VK2 vaginal cell line and found that these cells produced a vast array of cytokines, chemokines, and surface receptors (Fichorova & Anderson, 1999). This cell line constitutively expressed macrophage colony-stimulating factor (M-CSF) (development of macrophages and neutralizing intracellular viral infection), transforming growth factor beta 1 (TGF-β) (development of T-cells), IL-8 (attracts neutrophils), and secretory leukoproteinase inhibitor (SLPI) (an anti-microbial and anti-protease peptide). These cells can also be stimulated by IFN-γ and TNF-α to produce IL-6 (pro- and anti-inflammatory cytokine), CCL5 (recruitment of leukocytes) and the expression of major histocompatibility complex (MHC) class II antigens. Vaginal epithelial cells are also able to secrete elafin, a serine protease inhibitor with demonstrated antimicrobial activity against *S. aureus* (Simpson *et al.*, 1999) as well as protection against HIV infection (Ghosh *et al.*, 2010). Thus, vaginal epithelial cells can actively contribute to an inflammatory response by detecting pathogens with such receptors as TLRs and MHC class II antigens, and response with a number of different cytokines, chemokines and other anti-microbial agents.

Endogenous members of the vaginal microbiota are able to interact with, and stimulate, TLRs which can elicit the release of inflammatory markers to combat pathogenic microorganisms. One study found that stimulation of TLR-2 with lipoteichoic acid (LTA), a major component of the cell wall of Gram-positive bacteria, and stimulation of TLR-4 with lipopolysaccharide (LPS), found in the outer-membrane of Gram-negative bacteria, leads to production of IL-8 and

interferon (IFN)-β and protects against human cytomegalovirus (HCMV) infection (Harwani *et al.*, 2007). In this way, endogenous bacteria of the vagina maintain a state of immunological alertness which helps protect against invading pathogens.

Another key feature of vaginal immunity is the presence of immunoglobulin A (IgA), a component of mucosal immunity in general (Woof & Kerr, 2006). IgA-producing plasma cells, producing both IgA1 and IgA2 isotypes, have been found in the sub-epithelial layer of female genital tissue, including the vagina (Kutteh & Mestecky, 1994), and indigenous bacteria appear necessary for the production of these immunoglobulins (Benveniste *et al.*, 1971; Moreau *et al.*, 1978). Functions of IgA are vast and include neutralization of pathogenic bacteria and exotoxins, as well as prevention of mucosal penetration of indigenous bacteria into the basolateral side of the mucosal epithelial layer (Johansen *et al.*, 1999; Lycke *et al.*, 1987).

An important aspect of the vaginal microbiota is its ability to persist without inducing a prolonged pro-inflammatory response. Various mechanisms of mucosal immune tolerance to indigenous bacteria have been uncovered, and two prominent theories have been established (Sansonetti, 2011). The first theory suggests that modifications of PAMPs on commensal bacteria exist to prevent or even antagonize interaction with host cell PRRs. For instance, the lipid A component of LPS of *Porphyromonas gingivalis*, a member of the oral microbiota, has been shown to antagonize TLR-4 due to its tetra-acylated structure, thus preventing release of pro-inflammatory cytokines upon interaction with the host cells (Zhang *et al.*, 2008). In contrast, TLR-4 recognizes hexacylated lipid A

structures and, upon recognition of this PAMP, elicits pro-inflammatory cytokines (Takahashi *et al.*, 1987). The classes α- and ε-*Proteobacteriaceae*, which include the commensal *Helicobacter*, have flagellin with an altered amino acid sequence in the TLR-5 recognition site, which prevents host recognition and an inflammatory response (Andersen-Nissen *et al.*, 2005).

The second theory of immune tolerance to commensal bacteria is that some of these bacteria release effector molecules that either directly suppress pro-inflammatory or induce anti-inflammatory signals. A study found that the supernatant of *L. rhamnosus* GR-1, a probiotic strain frequently used for improving vaginal health, was capable of dampening the pro-inflammatory response of TLR-4 stimulated with LPS (Yeganegi *et al.*, 2009). Specifically, the supernatant suppressed TNF-α and increased IL-10, an anti-inflammatory cytokine. Another study found that *L. casei* is able to dampen the pro-inflammatory response to *Shigella flexneri* by way of stabilizing I-κBα, an inhibitor of NF-κB (Tien *et al.*, 2006). Much research has also been conducted on immune effects induced by bacteria in the gut, which is where most vaginal bacteria originate. *Bacteroides thetaiotaomicron*, a gut commensal, is able to antagonize NF-κB through a unique mechanism in which it triggers the nuclear export of RelA, a subunit necessary for proper activity of NF-κB (Kelly *et al.*, 2004). Another species of this genus and of the gut commensal population, *B. fragilis*, has been shown to suppress pro-inflammatory IL-17 and trigger IL-10 release from $CD4^+$ T-cells, which neutralizes the inflammatory response from *Helicobacter hepaticus* (Mazmanian *et al.*, 2008). Therefore, a prominent theory

of immune tolerance to commensal bacteria is that many members suppress inflammatory responses thereby counteracting pro-inflammatory stimulation by other members, leading to an immunologically homeostatic environment at the mucosa.

1.2.5. Urogenital Infections

Despite the protective barriers present in the vagina, opportunities arise in which exogenous microorganisms enter the vagina and cause an infection. These include sexually-transmitted infections (STIs) caused by bacteria (*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Treponema pallidum*) (Biro *et al.*, 1995) and viruses, as well as fungal infections and some associated with parasites and protozoa. Vulvovaginal candidiasis (VVC), referred to clinically as common yeast infection, occurs most often through infection with the fungus *Candida albicans*. Viruses known to enter the vagina and cause disease include herpes simplex virus (HSV), human immunodeficiency virus (HIV) and human papilloma virus (HPV). A protozoan known to infect the vagina is *Trichomonas vaginalis*, causing trichomoniasis (Graves & Gardner, 1993).

Not all infections originating in the vagina are linked to sexual activity. Some arise at opportunistic times, such as during menses when blood levels are high or in mid-cycle when estrogen levels peak. A main reason for this is due to a change in the composition and abundance profiles of the vaginal microbiota, such as following exposure to antibiotics and spermicides.

It is not clear if *Staphylococcus aureus* take advantage of a disrupted or aberrant microbiota to multiply and cause menstrual-toxic shock syndrome (TSS). This disease remains a concern in today's society, yet there is still no means to predict its onset. If the risk of menstrual-TSS could be associated with a diagnosable change in the vaginal microbiota, such as onset of bacterial vaginosis (BV) or aerobic vaginitis (AV), this would potentially provide a method of early detection and intervention.

1.3. Menstrual-TSS

1.3.1. Pathogenesis

Toxic shock syndrome (TSS) is a systemic illness characterized by extensive T-cell proliferation throughout the body. The condition arises from bacterial infection and subsequent release of exotoxins known as superantigens (SAGs). These SAGs are unique in that they bind directly to the β-chain variable region of the T-cell antigen receptor (TCR) (Gascoigne & Ames, 1991), as well as the α-chain and sometimes β-chain of MHC class II molecules of antigen presenting cells (APCs) (Jardetzky *et al.*, 1994; Li *et al.*, 1999). This makes them potent activators of $CD4^+$ and $CD8^+$ T-cells as well as APCs (Fleischer & Schrezenmeier, 1988; White *et al.*, 1989). These toxins bypass the need for APCs to process and present antigen peptides to T-cells, resulting in excessive production and release of pro-inflammatory cytokines into the bloodstream - namely IFN-γ, IL-1, IL-2, TNF-α and TNF-β (Fast *et al.*, 1989; Hackett & Stevens,

1993; Ikejima *et al.*, 1984). This, in turn, can lead to hypotension, rash formation, multiple organ failure, shock and death.

1.3.2. TSS Subtypes

TSS has traditionally been associated with infection by *Staphylococcus aureus*, but cases have been reported since 1987 due to *Streptococcus pyogenes* (also known as group A *Streptococcus*, GAS) (Cone *et al.*, 1987; Stevens *et al.*, 1989). There are two groupings of TSS: staphylococcal TSS and streptococcal TSS. Staphylococcal TSS can be further broken down into two subtypes: menstrual- and non-menstrual -TSS. Menstrual-TSS is a unique form characterized by *S. aureus* infection in the vagina, which typically occurs around the time of menses. Non-menstrual-TSS, on the other hand, occurs in both men and women of any age, and can arise from various forms of *S. aureus* infection, including after surgical complications (Bartlett *et al.*, 1982), burn wounds (Bacha *et al.*, 1994), recalcitrant erythematous desquamating syndrome (Cone *et al.*, 1992) and influenza (MacDonald *et al.*, 1987). Approximately half of all cases of non-menstrual-TSS are caused by the SAG toxic shock syndrome toxin-1 (TSST-1), with the remainder mostly due to staphylococcal enterotoxins (SEs) B and C (Bohach *et al.*, 1990). Menstrual-TSS, however, is predominantly associated with TSST-1 as it is the only SAG capable of crossing the vaginal epithelium into the bloodstream (Schlievert *et al.*, 2000), a key step in the pathogenesis of this condition. TSST-1, encoded by the *tst* gene, was first identified in 1981 by two unrelated groups of researchers, and was initially given the names SEF (Bergdoll

et al., 1981) and pyrogenic exotoxin type C (PEC) (Schlievert *et al.*, 1981) before an agreement was made to coin the name TSST-1.

1.3.3. Epidemiology

The incidence of menstrual-TSS has been routinely monitored in the United States, but is largely unknown in other parts of the world. Monitoring of this condition started in 1980 when a spike in TSS cases was seen, and was strongly linked to menstruating women using tampons, in particular a newly-introduced Rely® high-absorbency tampon from Procter & Gamble (Reingold *et al.*, 1982; Schlech *et al.*, 1982). In 1980, the incidence of menstrual-TSS was found to be 6-12 per 100,000 premenopausal women, with a higher incidence occurring in women younger than 30 years of age (Davis *et al.*, 1980; Latham *et al.*, 1982). By 1986, the incidence of menstrual-TSS decreased to 1.05 per 100,000 women in the age range of 15-44 years and 1.52 per 100,000 women in the age range of 15-19 years, as determined through the surveillance of 6 states in America (Gaventa *et al.*, 1989). This drop followed removal of highly absorbent tampons from the market. Menstrual-TSS cases accounted for 91% of all TSS cases in 1970-80, and declined to 71% during 1981-1986 and 59% during 1987-1996 (Hajjeh *et al.*, 1999). Since 2000, rates of menstrual-TSS have been harder to gauge, but the number of cases for non-streptococcal TSS, a portion of which would include menstrual-TSS, has fallen up to 2009, as reported by the Centers for Disease Control and Prevention (CDC): the cases in 1984 numbered 482, declining to 192 by 1994, 95 in 2004 and 74 in 2009 (CDC, 2011). The reasons for this drop in occurrence include removal of highly absorbent tampons from the

market, as well as a better awareness for the condition and the requirement for standardized labeling by the U.S. Food and Drug Administration (FDA) on tampon products. Approximately 70% of women in North America and Western Europe use tampons regularly (Shands *et al.*, 1980), so although the incidence of menstrual-TSS is decreasing, there is much interest in better understanding this condition and preventing its recurrence.

1.3.4. Treatment

Treatment of TSS in general tends to include antibiotics and treatment of symptoms, and cessation of tampon use in the case of menstrual-TSS. Investigators discovered that clindamycin, despite being a bacteriostatic antibiotic to *S. aureus*, was able to significantly suppress exotoxin production in this organism, thus serving as an effective candidate in the treatment of staphylococcal-TSS (Dickgiesser & Wallach, 1987; Schlievert & Kelly, 1984). However, a source for concern was this antibiotic's failure to reduce bacterial levels in an infection of high bacterial load. A combination of flucloxacillin, a bactericidal β-lactam antibiotic, with gentamicin, a protein synthesis-inhibiting aminoglycoside antibiotic, was tested for reduction in TSST-1 production by *S. aureus* (van Langevelde *et al.*, 1997). It was discovered that flucloxacillin, both alone and in combination with gentamicin, reduced TSST-1 production similarly to clindamycin at logarithmic phase (approximately 95%) but less so at stationary phase (30% and 75%, respectively). Thus, this antibiotic combination provides a valuable alternative to clindamycin if reducing bacterial numbers in the patient is of high priority. In more recent times, the treatment of staphylococcal-TSS has

included the combination of clindamycin with bactericidal antibiotics such as vancomycin.

Linezolid, the first commercially-approved drug of the oxazolidinone family of antibiotics, has recently been of interest for treating patients suffering from TSS. This family of antibiotics is bactericidal towards Gram-positive bacteria, including methicillin-resistant *S. aureus* (MRSA), and acts by inhibiting protein synthesis. The method of protein synthesis inhibition is unique in that it binds to the 23S rRNA portion of the 50S subunit of the ribosome, preventing formation of the initiation complex, making it particularly effective towards bacteria with resistance to other families of antibiotics (Colca *et al.*, 2003). Two groups studied the effect of linezolid on *S. aureus* exotoxin production, and found the drug to be effective in significantly reducing production of toxins such as SEA, SEB, protein A, autolysin and hemolysins, while also decreasing growth in a dose-dependent manner (Bernardo *et al.*, 2004; Gemmell & Ford, 2002). Another group recently isolated a TSST-1-producing strain of *S. aureus* from a patient suffering from staphylococcal-TSS and found that TSST-1 production was completely suppressed *in vitro* by linezolid and clindamycin (Stevens *et al.*, 2006). In today's clinical setting, antibiotics that are currently used for the treatment of TSS (including menstrual-TSS) include clindamycin and linezolid, and occasionally vancomycin, daptomycin, dalbavancin and tigecycline which are all generally effective against Gram-positive bacteria unless resistance has been developed.

Pooled intravenous immunoglobulin G (IVIG) containing antibodies to several of the staphylococcal SAGs, including TSST-1, significantly inhibited

human peripheral blood T-cell activation by these toxins through interfering with SAG binding to the T-cells (Takei *et al.*, 1993). Despite this *in vitro* evidence, there are no case-control studies to measure its effectiveness in patients. Nevertheless, some physicians implement IVIG in the treatment regime for staphylococcal TSS (Schlievert, 2001). Case-control studies have been conducted for IVIG usage in the treatment of patients suffering from streptococcal-TSS, with one showing a significant improvement in 30-day survival, as well as a reduced T-cell production of IL-6 and TNF-α (Kaul *et al.*, 1999). Many physicians are therefore implementing both antibiotic treatment as well as IVIG to try and neutralize the pro-inflammatory effects of the SAGs in the bloodstream.

1.3.5. Disease Progression

1.3.5.1. Course of Infection

In order for menstrual-TSS to occur, three fundamental steps must take place: (1) a strain of *S. aureus* must colonize the vaginal tract, (2) the strain must produce TSST-1, and (3) the TSST-1 must be able to penetrate or translocate across the vaginal epithelial wall into the blood (Figure 1.1).

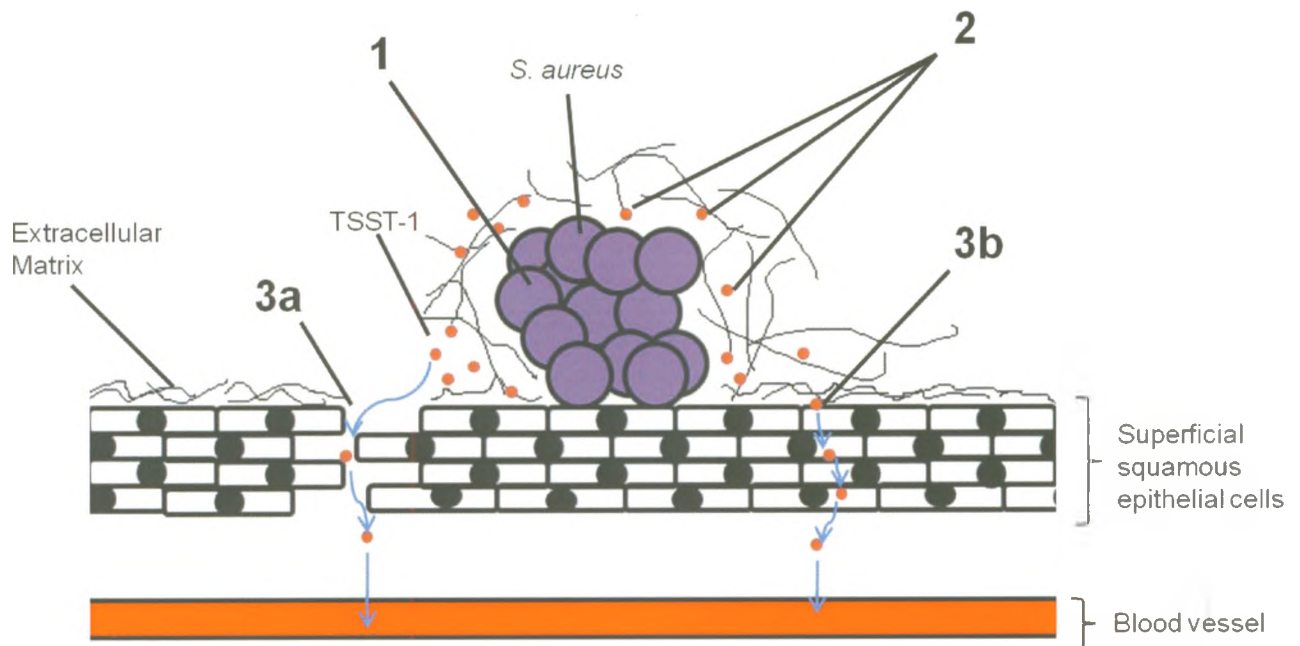

Figure 1.1. Simplified depiction of the course of disease progression in menstrual-TSS. (1) *Staphylococcus aureus* colonize the vaginal epithelium within a network of extracellular matrix; (2) The bacteria secrete TSST-1; (3) The toxins pass through leaky regions of the epithelial layer affected by inflammation (3a) or bind directly to surface epithelial cells and undergo receptor-mediated endocytosis (3b), after which they enter the blood.

1.3.5.2. *S. aureus* Colonization

The microenvironment of the vaginal epithelium consists of exposed epithelial cells with surrounding extracellular matrix (ECM) and vaginal secretions containing proteins, polysaccharides, organic acids, glycerol and urea (Owen & Katz, 1999). The main mechanism of *S. aureus* attachment to mucosal surfaces, and thus the initial step to tissue colonization, is binding to epithelial cell receptors and components of the ECM. These interactions are mediated by a family of cell surface adhesins on *S. aureus* that bind to the extracellular matrix (ECM), which are collectively known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Patti *et al.*, 1994). *Staphylococcus aureus* utilizes a collection of MSCRAMMs to bind multiple components of the ECM, including fibronectin (Espersen &Clemmensen, 1982; Flock *et al.*, 1987), collagen (Holderbaum *et al.*, 1985; Patti *et al.*, 1992), laminin (Vercellotti *et al.*, 1985), vitronectin (Chhatwal *et al.*, 1987; Paulsson *et al.*, 1992), and elastin (Park *et al.*, 1991). Fibronectin, a glycoprotein that is bound to integrin on epithelial cells and is a common component of ECM, has been extensively studied as a ligand for *S. aureus* attachment (Kuusela, 1978; Proctor *et al.*, 1982; Rydén *et al.*, 1983).Today, two proteins in particular, fibronectin-binding proteins A and B (FnBPA and FnBPB, respectively) have been found to effectively bind fibronectin, fibrinogen and elastin (Jonsson *et al.*, 1991; Roche *et al.*, 2004; Wann *et al.*, 2000). These MSCRAMMs, along with various polysaccharide adhesins, are also responsible for the initial stages of biofilm formation, which appears to aid vaginal colonization (Costerton, 2005; Gotz,

2002; Veeh *et al.*, 2003). Thus, *S. aureus* is capable of attaching itself to multiple components of the vaginal environment, including epithelial cells and the ECM, and can at times persist for longer periods through the generation of biofilms.

In a study of 600 women, with the majority at ages 16-35 years (Linnemann *et al.*, 1982), vaginal colonization, including the canal and labia, was seen in 9% of the population, and vaginal colonization of TSST-1-producing *S. aureus* was present in 1%. Interestingly, greater *S. aureus* colonization was seen in black women (14%) compared to Caucasian women (6%), and the subpopulation with the greatest rate of colonization was postpartum (17%).

A more recent study investigated the nature of *S. aureus* colonization at various body sites in premenopausal women, including the nares, vagina and anus, with colonization rates near identical to that found by Linnemann *et al.* (1982) (Parsonnet *et al.*, 2005). *Staphylococcus aureus* was found to colonize 26% of the participants, with a total of 9% of women being colonized vaginally. Black women were found to have a higher vaginal colonization rate (14%) than that of Caucasian women (8%). Of all women enrolled, approximately 1% were colonized vaginally with a TSST-1-producing strain of *S. aureus*.

Another study analyzed vaginal-rectal samples from 2,963 pregnant women, and identified *S. aureus* colonization in 17.1% of this population (Chen *et al.*, 2006). They also found that *S. aureus* colonization was significantly associated with colonization with *Streptococcus agalactiae*, or group B *Streptococcus* (GBS), reflected by the finding that 6.4% of this population was

colonized with both organisms. Thus, it appears that *S. aureus* colonizes 9% of premenopausal women vaginally in North America, with a higher rate of colonization seen in black, pregnant and postpartum women.

However, it is not well understood which proportion of the vaginal microbiota within an individual is made up from *S. aureus* when it is present, whether it constitutes a small fraction or acts as the predominant member. This is important when considering the vaginal state during the initial stages of menstrual-TSS progression.

1.3.5.3. TSST-1 Production

Toxic shock syndrome toxin-1 is encoded by *tst*, a gene located on various mobile *S. aureus* pathogenicity islands (SaPIs), namely SaPI1, SaPI2, SaPIbov1, SaPIn1 and SaPIm1 (Novick *et al.*, 2010). The expression of *tst*, along with several other virulence factors in *S. aureus*, is tightly controlled by two families of global regulators: two-component regulatory systems (TCRS) and the DNA-binding SarA protein family (Cheung *et al.*, 2004).

TCRSs are common stimulus-response systems employed by many bacteria to detect environmental conditions. A major virulence regulator under the control of a TCRS is the *agr* (accessory gene regulator) locus which is responsible for controlling the expression of *tst* and other virulence factors. This system takes part in the quorum sensing (QS) of *S. aureus*; that is, the detection of environmental cues and communication with surrounding bacteria. It drives production of adhesion factors during exponential phase, and exotoxins and

other virulence factors during late-exponential phase (Dunman *et al.*, 2001). Thus, when *S. aureus* first comes into contact with the host, *agr* genes encode factors to help with adherence to the tissue. Once bacterial numbers reach a threshold density, toxins are produced that will help the bacteria evade the host defenses and spread to other areas. This locus is made up of the P3 and P2 operon, which drive transcription of *hld* and *agrBDCA*, respectively. The latter encodes a QS system and TCRS, encoded by *agrBD* and *agrAC*, respectively. This operon is responsible for the production and sensing of auto-inducing peptides (AIPs), which are detected by the system's sensory component, AgrC. This, in turn, leads to phosphorylation of the activator, AgrA, which then binds to the P2 and P3 promoters, driving cyclic activation of the system. Initiation of RNAIII transcription (the effector molecule of the system) by the P3 promoter leads to the production of various exotoxins (including TSST-1) and suppression of cell-surface adhesins. Thus, as bacterial density increases, a local build-up of AIPs will lead to concurrent activation of RNAIII. This system not only detects bacterial density but also monitors environmental stresses, and can therefore orchestrate the most appropriate production of virulence factors in response to local stimuli. Other TCRSs shown to regulate the *agr* locus include SrrAB (Yarwood *et al.*, 2001) and ArlRS (Fournier *et al.*, 2000), which both counteract *agr* activity.

SarA is a dimeric DNA-binding protein that, together with a family of homologues, plays a role of virulence regulation in *S. aureus*, including the regulation of *agr*. At high levels, this protein binds to the P2 and P3 promoters of

the *agr* locus and drives transcription of RNAII and RNAIII, respectively (Chien & Cheung, 1998). This protein also binds directly to the *tst* promoter and thus drives *tst* expression both directly and indirectly through increased RNAIII production (Andrey *et al.*, 2010). Rot (Repressor of toxins), a SarA homologue, has anti-*agr* activity and down-regulates secretion of toxins, while up-regulating cell surface proteins (Saïd-Salim *et al.*, 2003).

Other factors shown to affect the *agr* system include the alternative sigma-factor B (σ^B), which is a transcription initiation factor that plays a role in bacterial stress response and demonstrates anti-*agr* activity, reflected by its ability to down-regulate exoprotein expression in *S. aureus* (Nicholas *et al.*, 1999). The TRAP system is a QS system that was believed to regulate *agr*, made up of RNAIII-activating protein (RAP) and RNAIII-inhibiting protein (RIP) which were thought to cause up- and down-regulation of RNAIII, respectively (Balaban & Novick, 1995; Korem *et al.*, 2003). However, recent findings find no link between these two systems (Shaw *et al.*, 2007). The regulation of TSST-1 production in *S. aureus* is therefore a complex process involving many factors (Figure 1.2).

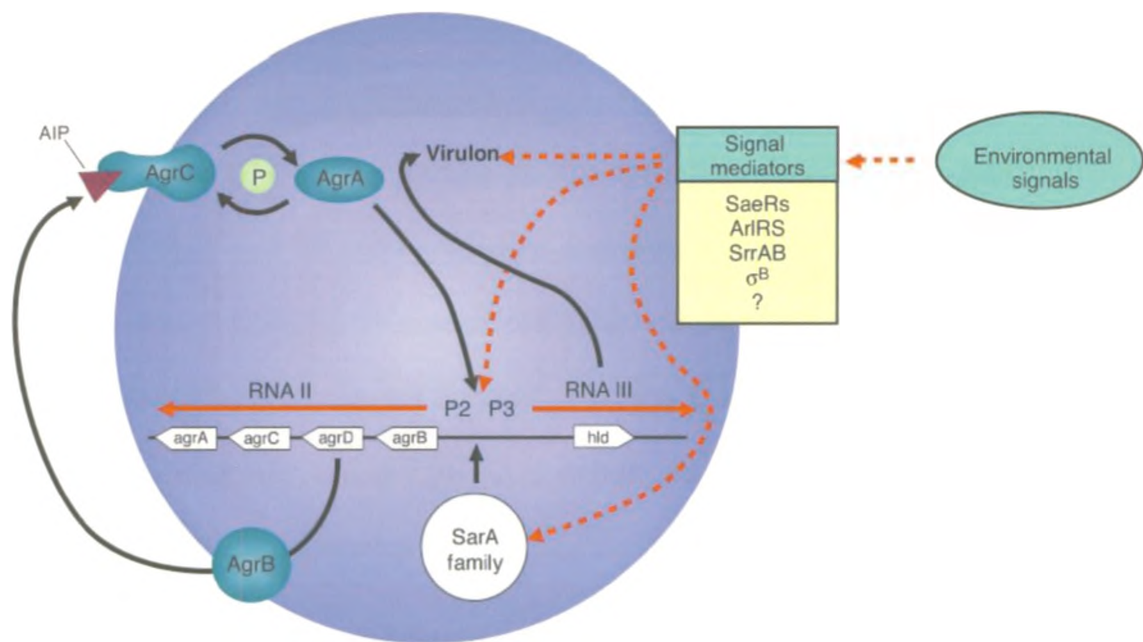

Figure 1.2. Schematic diagram showing the regulation of the *agr* system and exotoxin production in S. aureus. Figure obtained from Yarwood and Schlievert (2003).

A number of environmental factors have been identified that affect the induction of TSST-1 in *S. aureus*, including oxygen and carbon dioxide levels, environmental pH and magnesium ion concentration.

A study by Ross and Onderdonk revealed that TSST-1 production by *S. aureus* was triggered by elevated oxygen levels, and that the presence of both oxygen and carbon dioxide were required for toxin production (Ross & Onderdonk, 2000). Another study tested TSST-1 production across a range of oxygen levels in the presence and absence of carbon dioxide (Yarwood & Schlievert, 2000). In the absence of carbon dioxide, TSST-1 production reached a maximum of 500 ng/mL at 15 mm Hg oxygen and this dropped as oxygen levels increased. However, in the presence of 53 mm Hg carbon dioxide, TSST-1 production was proportional to oxygen content, reaching 1,900 ng/mL at 159 mm Hg oxygen. Therefore, production of TSST-1 is enhanced by elevated oxygen and carbon dioxide levels.

There has also been an established link between TSST-1 production and pH. Cultures of *S. aureus* cultures have been assessed against a range of pH values from 4.0-8.0, with the result that TSST-1 production increases to a maximum at a neutral pH of 6.5-7.0, after which it starts to decrease (Sarafian & Morse, 1987). This phenomenon of optimal TSST-1 production at a neutral pH was confirmed by others (Wong & Bergdoll, 1990). *In vivo* evidence of neutral pH being optimal for toxin production has also been presented: clinical samples of lesions from 4 patients with TSS had physiological characteristics matching

optimal *in vitro* conditions for TSST-1 production, including a pH range of 6.75-7.45 and elevated oxygen and carbon dioxide levels (Todd *et al.*, 1987).

Magnesium ions have been shown to have a suppressive effect against TSST-1 production. The first demonstration of this phenomenon was seen in an experiment testing the effect of tampon fibers on TSST-1 production (Mills *et al.*, 1985). Addition of fibers from the Rely® tampon to a culture of *S. aureus* led to an increase in TSST-1 production, which was linked to the removal of magnesium ions from the medium. Addition of the ion led to a suppression in TSST-1 production. The same group confirmed the suppressive nature of magnesium ion concentration on TSST-1 production in environments of variable oxygen levels and temperature (Kass *et al.*, 1987). Others found that TSST-1 production was highest at a magnesium concentration of 0.4 mM, but then decreased as the metal concentration was further increased (Sarafian & Morse, 1987).

1.3.5.4. TSST-1 Translocation

A vital step in the pathogenesis of menstrual-TSS is translocation of TSST-1 across the vaginal epithelium into the bloodstream. It was initially discovered that TSST-1 binds to epithelial cells and is internalized into the cytoplasm through receptor-mediated endocytosis, after which it is translocated through special transport vesicles different from receptosomes (Kushnaryov *et al.*, 1984). Research has investigated the particular mechanism responsible for toxin binding to the epithelial cells, as well as contributing factors for translocation. In particular, a dodecapeptide region in the amino acid sequence

of TSST-1, F119-D130, has been implicated in transcytosis of this toxin across the vaginal epithelial cells as well as triggering of inflammatory cytokines from these cells (Brosnahan *et al.*, 2008). This latter process is believed to contribute most to TSST-1 translocation as the pro-inflammatory cytokines that are induced (TNF-α, IL-8 and MIP-3α) increase permeability of the epithelial layer (Peterson *et al.*, 2005). *Staphylococcus aureus* also secretes a cytolysin, α-toxin, which has been shown to induce an inflammatory response at the nonkeratinized stratified squamous epithelial surface of the vaginal wall and lead to increased translocation of TSST-1 (Brosnahan *et al.*, 2009). The event of TSST-1 translocation therefore seems to occur in two ways: 1) receptor-mediated endocytosis into vaginal epithelial cells and subsequent transport through the cell, and 2) passive transfer of the toxin across leaky regions of the vaginal epithelium.

1.3.6. Antibody Titres to TSST-1

In the event that *S. aureus* is successful in colonizing the vagina and produces TSST-1, which then translocates across the vaginal epithelium and enters the bloodstream, many women are protected with anti-TSST-1 antibodies (Parsonnet *et al.*, 2005). This study found that 85% of women had a positive TSST-1 antibody titre, defined as a titre with a dilution ratio of 1:32 or lower. Seventy-nine percent of subjects aged 13-15 years (typical age when menstruation begins) had positive antibody titres. More women had a positive titre when colonized with TSST-1-producing *S. aureus* (98%) than when colonized with TSST-1-negative *S. aureus* (84%). Interestingly, when the study

compared carriers of TSST-1-producing *S. aureus,* fewer black women had a positive antibody titre (89%) than Caucasian women (98%). Therefore, evidence suggests that black women may be more susceptible to menstrual-TSS on the basis of increased vaginal colonization of *S. aureus* and decreased positive TSST-1 antibody titres compared to Caucasian women. The reason for this different antibody response is unclear.

Thus, most women of premenopausal age seem to be protected from the effects of systemic TSST-1, including girls at an age when they first use tampons. Studies have confirmed the absence of circulating antibodies to TSST-1 in women who develop menstrual-TSS (Stolz *et al.*, 1985). In this study, 86 of 95 patients (90.5%) with menstrual-TSS were essentially lacking anti-TSST-1 antibodies, highlighting the importance of TSST-1 antibody titres for resistance of menstrual-TSS.

1.3.7. The Tampon Hypothesis

It is widely accepted that menstrual-TSS is linked to tampon use. Menstrual-TSS was first recognized as a result of the introduction of high-absorbency tampons (Schlech *et al.*, 1982), and 98% of patients with TSS report the regular use of tampons (Hajjeh *et al.*, 1999). Studies have also found *S. aureus* biofilms (Veeh *et al.*, 2003) and TSST-1 (Schlievert *et al.*, 2010) on tampon fibers following insertion. It is not completely understood, however, why tampons increase the susceptibility of menstrual-TSS. Two prominent theories

exist which consider the influence of environmental conditions on TSST-1 production.

The first theory considers the finding by Yarwood and Schlievert (2000) that TSST-1 production is proportional to oxygen content. Tampon insertion during menstruation was initially believed to introduce a bolus of oxygen into the vagina, raising oxygen levels to near-atmospheric levels; following tampon insertion, vaginal oxygen levels increased from 3 mm Hg to 130-140 mm Hg (Wagner *et al.*, 1984). A recent study, however, demonstrated that the rise in oxygen was most likely due to gas level detection in the tampon itself, and that oxygen levels in the vaginal canal actually decreased upon insertion (Hill *et al.*, 2005). This research group found that the partial pressure of oxygen decreased from an initial 15-35 mm Hg to 1 mm Hg after insertion (subjects in this study included women who were previously pregnant, and so had a higher partial pressure of oxygen than typically seen). The same study verified oxygen at atmospheric levels in inserted tampons. Therefore, considering that *S. aureus* and TSST-1 have been found on tampon fibers, it is possible that the pathogen colonizes the tampon and gains access to the high levels of oxygen content within the device, rather than being introduced to an overall elevated aerobic environment in the vaginal canal.

Another prominent theory to why tampons contribute to menstrual-TSS is that tampons absorb magnesium ions in the vaginal canal, effectively decreasing its concentration and thereby inducing TSST-1 production. In support of this theory, Mills *et al.* (1985) found that tampon fibers absorbed magnesium ions and

increased TSST-1 production as a result. Further studies validated this suppressive effect of excess magnesium ions *in vitro* (Kass *et al*., 1988; Mills *et al*., 1986). Although this might contribute in part to increased toxin production upon tampon insertion, more *in vivo* work detecting vaginal magnesium ions should be done before making any definite conclusions.

The effects of different materials used to make tampons, as well as their absorbency, on *S. aureus* growth and TSST-1 production have been investigated. Tampons are made from one of three compositions: cotton, cotton-rayon and rayon. A study of the effects of cotton tampons on TSST-1 production found that these fibers prevented production of the toxin while cotton-rayon and rayon induced production, making cotton the safer of the fiber types (Tierno & Hanna, 1994). However, two subsequent studies disproved this theory, showing that neither of the three tampon compositions increased production of TSST-1 (Parsonnet *et al*., 1996; Schlievert, 1995). Another aspect of tampon design of interest was absorbency, considering that menstrual-TSS first emerged from the introduction of a highly-absorbent tampon. Tampons sold in North America are available in five different absorbency strengths, ranging from 6 grams and under (junior absorbency) to 15-18 grams (ultra absorbency) of menstrual fluid absorbance. The study by Parsonnet *et al.* (1996) altered its experimental design to test for effects of absorbency and showed that a super plus absorbency (12-15 grams) lead to slightly higher levels of TSST-1 in a *S. aureus* culture compared to tampons made of the same material but of a lower absorbency rating. The study by Schlievert (1995), as well as a case control study from the late 1980s

(Reingold *et al.*, 1989), also suggests that an increase in tampon absorbency leads to a higher incidence of menstrual-TSS.

Conclusive *in vivo* work is still missing in this field of research, and there is still a need to either revise the design of the tampon or develop other approaches to prevent all cases of menstrual-TSS. As the composition of the tampon seems to have a minimal effect on TSST-1 production, and usage of low-absorbency tampons seems effective at keeping the risk of menstrual-TSS relatively low, investigations might consider oxygen and magnesium ions, and the role of other vaginal microbes.

1.4. Vaginal Microbiota

1.4.1. Definition

The vaginal microbiota is a community of bacteria that reside in the vagina and play a role in disease as well as protection from infection by pathogenic bacteria, viruses and fungi. Most members of the microbiota are thought to derive from migration of bacteria from the rectum to the vagina via the perineum, but bathing, sexual contact and clothing are other potential sources. The predominant bacteria in this population are those from the genus *Lactobacillus*, members of the lactic-acid bacteria (LAB) group (Lamont *et al.*, 2011). Lactobacilli belong to the phylum Firmicutes (low G-C content, Gram-positive bacteria), class *Bacilli* and order Lactobacillales. Recent advancements in molecular, cultivation-independent sequencing methods have shed light on the species and abundance of the microbiota population (Gloor *et al.,* 2010). The

species found most often in the healthy vaginal microbiota are *Lactobacillus crispatus*, *L. jensenii* and *L. iners*, with the latter also abundant in BV (Zozaya-Hinchliffe *et al.*, 2010). Under estrogen influence, glycogen is produced by vaginal epithelial cells, metabolized by lactobacilli and converted to lactic acid through the fermentation of glucose. This in turn maintains the vagina at a pH of approximately 3.8-4.5. The resulting acidic pH serves as a broad protective mechanism against the growth and colonization of pathogenic bacteria in the vagina. Other protective mechanisms include displacement of pathogens (McMillan *et al.*, 2011), immune modulation (Christensen *et al.*, 2002; Sung Kim *et al.*, 2006), production of anti-microbial agents such as hydrogen peroxide (Klebanoff *et al.*, 1991), bacteriocins-like substances (McGroarty & Reid, 1988) and biosurfactants (Reid *et al.*, 1993; Velraeds *et al.*, 1996).

Evidence suggests that the vaginal microbiota varies amongst racial groups. A study by Zhou *et al.* (2007) found only 68% of black women had a *Lactobacillus*-dominated microbiota, as compared to 91% of Caucasian women, which could account for the higher incidence of BV seen in black women (Allsworth & Peipert, 2007). Zhou *et al.* (2010) found *Lactobacillus*-dominated populations in 75% of Japanese women, consisting primarily of *L. iners*, *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. crispatus.* Another study found *Lactobacillus*-dominated vaginal populations in 80.2% and 89.7% of Asian and Caucasian women, respectively, but only 61.9% and 59.6% of black and Hispanic women, respectively (Ravel *et al.*, 2011). Possible reasons for inter-

racial differences in the vaginal microbiota include differences in host genetics as well as cultural and behavioral nuances.

Menses has been shown to temporarily alter the vaginal microbiota. At mid-cycle, estrogen levels and glycogen content peak, and lactobacilli numbers are expected to be at their highest (Farage & Maibach, 2006). In contrast, the end of the menstrual cycle sees a drop in estrogen, as well as the delivery of blood which, at a neutral pH, can significantly alter the microbiota. A recent study utilized quantitative polymerase chain reaction (PCR) to detect changes in members of the microbiota throughout menses of healthy women (Srinivasan *et al.*, 2010). There was an increase in non-*Lactobacillus* spp., including *G. vaginalis*, and reduced amounts of *L. crispatus* and *L. jensenii* associated with menses. Eschenbach *et al.* (2000) reported heavy growth of *Lactobacillus* spp. in 84% of healthy women during the postovulatory phase, which decreased to 70% during menses, while the number of subjects with non-*Lactobacillus* spp. increased from 40% to 72%. In particular, *Prevotella* spp. increased from 28% in subjects at the postovulatory phase to 56% at menses. Subjects with BV saw a slight decrease in *Lactobacillus* spp., from 54% during postovulatory phase to 33% at menstruation, while *Prevotella* spp. and other non-lactobacilli remained relatively constant throughout the menstrual cycle. Thus, menstruation seems to promote fluctuations in the vaginal microbiota, including a reduction in lactobacilli numbers and increased complexity as a result of menses.

1.4.2. Techniques used to decipher the vaginal microbiota

The advancements in identification techniques for the vaginal microbiota made especially over the past 10 years have allowed us to better understand the bacteria that make up this community. Back in 1960, use of phenotypic assays such as sugar fermentation and other biochemical characteristics resulted in *L. acidophilus* being identified as the main constituent of the microbiota (Rogosa & Sharpe, 1960). This led to the belief that *L. acidophilus* was important in vaginal health. However, the sub-division of the *L. acidophilus* group into six different species (*L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. gasseri* and *L. johnsonii*) emphasized the inaccuracy of the belief (Fujisawa *et al.*, 1992). Utilizing whole-chromosomal DNA probes, *L. crispatus* and *L. jensenii* were found to be the predominant members of the vaginal lactobacilli (Antonio *et al.*, 1999). However, this conclusion was based upon isolating and growing the lactobacilli, and thereby it failed to detect fastidious organisms. Also, the use of whole-chromosomal DNA probes requires the user to pre-select the organisms they wish to identify, which makes this method inappropriate for exploratory analysis of identifying any and all bacteria present.

More recent studies have utilized culture-independent molecular techniques to better identify constituents of the vaginal microbiota, the first of which was reported using denaturing gradient gel electrophoresis (DGGE) following PCR amplification of 16S rRNA segments of vaginal bacteria (Burton & Reid, 2002). Methods have also included cloning and sequencing 16S rRNA fragments following PCR to compare to a database for bacterial identification

(Hyman *et al.*, 2005; Verhelst *et al.*, 2004; Zhou *et al.*, 2004). In 2007, use of 16S rRNA gene terminal restriction fragment length polymorphism (RFLP) fingerprinting was reported to identify bacteria in the healthy and BV vagina, with a total of 23 species making up those of BV subjects and only 3 from healthy subjects (Thies *et al.*, 2007). An advantage of DGGE, however, is that it is less time consuming and more cost-effective than cloning, and allows for effective comparisons of bacterial diversity across many samples (Burton & Reid, 2002; Burton *et al.*, 2003; Ferris *et al.*, 2004). A disadvantage of this method is that organisms of low abundance may not be detected, and the abundance threshold below which a species is not detected is of yet unknown.

A recent revolutionary advancement in the identification of vaginal microbiota is the use of metagenomics, a deep-sequencing approach that is able to reveal virtually all bacteria present in a sample, regardless of abundance. A study by Sundquist *et al.* (2007) was the first to analyze vaginal samples using high-throughput pyrosequencing, and established the general protocol for this approach. Spear *et al.* (2008) used pyrosequencing to sequence vaginal bacterial profiles, by collecting samples from BV-positive and BV-negative women infected or not infected with HIV, and was able to detect a total of 35 different taxa (range of 8-18 in BV and 1-4 in healthy). A recent study by our group was the first to use 16S rRNA sequencing by Illumina to examine the impact of antibiotic therapy on the vaginal microbiota (Hummelen *et al.*, 2010). Sequencing results from this study found a total of 60 phylogenetic groups within

the samples, and the method was able to identify to the strain level for some species.

Thus, the techniques used today to unravel the constituents of the vaginal microbiota are culture-independent and are able to detect the presence of bacteria at low abundance. Although it is presumed that techniques such as DGGE are unable to detect bacteria in low numbers, it is unclear exactly how abundant these bacteria need to be in a sample. Before the field can take a confident step towards next-generation sequencing, a comparison should be made between DGGE and high-throughput sequencing using DNA from the same sample pool.

1.4.3. Aberrant States

Changes in the vaginal environment, such as varying glycogen levels in the mucosa, render the vaginal microbiota susceptible to constant fluctuations in its members. Although this is a normal and inevitable process, the microbiota is at risk of falling into certain aberrant states characterised by a depletion of lactobacilli and a subsequent increase in pathogenic bacteria. Two such states are bacterial vaginosis (BV) and aerobic vaginitis (AV).

1.4.3.1. Bacterial Vaginosis

Bacterial vaginosis is the most common aberrant vaginal condition, afflicting an estimated 29% of North American premenopausal women and up to 71% of some populations of sex workers (Ramjee *et al.*, 1998). The incidence of

this condition differs among ethnic groups: BV is present in 23% of Caucasian women, 32% of Mexican Americans, and 51.4% of black women (Koumans *et al.*, 2007). This condition is characterized by a depletion in lactobacilli abundance, with the exception of *L. iners*, and an increased bacterial complexity and abundance of anaerobic bacteria, including *Gardnerella vaginalis, Atopobium vaginae*, *Prevotella bivia* and *Leptotrichia amnionii* (Fredricks *et al.*, 2005; Hummelen *et al.*, 2010). In this condition, the vaginal environment becomes anaerobic and less acidic, with a pH greater than 4.7. Irrespective of symptoms including vaginal discharge, irritation and a fishy odor, BV has been associated with increased susceptibility to sexually transmitted infections (STIs), preterm labour (Holst *et al.*, 1994) and pelvic inflammatory disease (Gomez *et al.*, 2010; Ness *et al.*, 2005). There have been numerous behavioral characteristics linked with BV, including having multiple sex partners, receptive oral sex, sexual activity with a female partner, not using a condom, smoking, using an intrauterine device and douching (Brotman *et al.*, 2008; Schwebke JR *et al.*, 1999; Smart *et al.*, 2004).

The gold standard for diagnosis of BV is the Nugent scoring method, whereby a vaginal swab is smeared onto a glass slide, Gram-stained, viewed under a microscope and scored based on the proportion of Gram-positive rods (lactobacilli), Gram-variable rods (*Gardnerella* spp.) and Gram-negative curved rods (*Mobiluncus* spp.) (Nugent *et al.*, 1991). However, current knowledge of the diverse populations that can make up the BV microbiota indicates that diagnosing this condition based on the presence of *Gardnerella* spp. alone

neglects the possible presence of such bacteria as *A. vaginae*, *Prevotella* spp., *Leptotrichia* spp. and others (Dols *et al.*, 2011). Furthermore, BV-associated *A. vaginae* are Gram-positive rods, and they could be misidentified as lactobacilli thereby causing a BV score to be read as normal (Burton *et al.*, 2004). Another common and more clinically-based diagnosis method is the Amsel criteria, which is based on the presence of a milk-like discharge, odor, a pH greater than 4.5 and the presence of epithelial cells covered in Gram-negative rods (clue cells) (Amsel *et al.*, 1983). Other tests have emerged, such as the BVBlue test which detects the levels of sialidase, a microbial enzyme elevated in BV (Briselden *et al.*, 1992; Myziuk *et al.*, 2003), and the FemExam that detects elevated pH and presence of trimethylamine, the compound responsible for the fishy odor linked with BV (Reid *et al.*, 2004). None of these tests are ideal, and so clinical studies should preferably include two or more differential tests and should at least measure pH and the microbiota composition.

Treatment of BV typically includes the use of 500 mg oral metronidazole or clindamycin twice daily for 7 days and occasionally intra-vaginal metronidazole gel (CDC *et al.,* 2006). However, none of these approaches prevent recurrence of BV, which is a common occurrence. One study treated BV with the typical regime of oral metronidazole and found that 58% of patients experienced recurrences, with 69% having an abnormal vaginal microbiota after 12 months of treatment (Bradshaw *et al.*, 2006). A few recent studies have shown that the use of probiotics, either alone or with antibiotics, can improve the treatment of BV and reduce recurrence rates (MacPhee *et al.*, 2010). Mastromarino *et al.* (2009)

found that giving one tablet containing *L. brevis* CD2, *L. salivarius* subsp. *salicinius* FV2 and *L. plantarum* FV9 (>10^9 CFU each) daily for 7 days significantly improved BV cure rate after 7 and 21 days, compared to giving placebo alone. Another study tested the effectiveness of oral tinidazole with 2 capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (10^9 CFU each) with the resultant significant increase in cure rate of BV compared to the antibiotic alone (Martinez *et al*., 2009). Probiotics have also been shown to prolong the time until a relapse occurs when they are applied vaginally following typical metronidazole treatment (Marcone *et al*., 2008). Therefore, the use of probiotics for BV treatment and prevention is increasingly becoming a viable alternative to the use of antibiotics alone, and could potentially improve the quality of life for these patients.

1.4.3.2. Aerobic Vaginitis

Aerobic vaginitis is also characterized by depletion in members of the *Lactobacillus* genus and an increased pH, but this condition differs from BV in several ways. Colonization by enteric aerobic commensals such as *Escherichia coli*, *Streptococcus agalactiae*, *Enterococcus faecalis* and *Staphylococcus aureus* is one feature (Donders, 2002). The condition is also characterized by a local immunological response, reflected in increased levels of pro-inflammatory cytokines IL-1, IL-6 and IL-8, and the resulting presence of dead epithelial cells when viewed under microscopy. Aerobic vaginitis has been strongly associated with pregnancy complications, including preterm birth (PTB), preterm rupture of membranes, chorioamnionitis, funisitis, miscarriage and low birth weight (Carey &

Klebanoff, 2005; Donders *et al*., 2009; Hay *et al*., 1994; Rezeberga *et al*., 2008). Clinical signs of this condition differ slightly from BV, with patients usually reporting a yellow discharge and a rotten rather than a fishy odor. Patients also experience a burning sensation (rather than itching as experienced in BV) noted in vulvovaginal candidiasis (VVC), as well as dyspareunia (painful intercourse). The vagina often appears red and inflamed, and may have ulcers on the wall. Severe AV can lead to a pH greater than 6.0, compared with a pH of 4.7-6.0 associated with BV.

Diagnostic options for AV are limited, in part because it has only recently been recognised as a condition separate from BV, VVC and trichomoniasis (Donders, 1999). An aberrant vaginal condition is diagnosed as AV with the following microscopic findings: decreased lactobacilli, increased leukocytes, leukocytes with toxic appearance (full of lysosymic granule), presence of cocci or chains, and parabasal-type epithelial cells (resulting from epithelial inflammation).

The most effective treatment for AV has yet to be established, but metronidazole is ineffective (Donders *et al*., 2002), as is expected since this antibiotic is designed for anaerobic bacteria and protozoa. Misdiagnosis of AV as BV is thus a problem, since treatment with metronidazole will be ineffective. Investigators tested the use of two intravaginal antibiotics, kanamycin and meclocycline, in treating AV in non-pregnant women, and found kanamycin to be most effective at alleviating clinical symptoms and normalising the vaginal pH (Tempera *et al*., 2004). However, monitoring of the microbiota and symptoms of these subjects stopped only 9 days following the last treatment, and so the AV

recurrence rate was not assessed. No study with probiotics has yet been performed to restore the vaginal microbiota in AV subjects. Treatment using anti-inflammatory medication should provide some relief, and combined with antibiotics and restoring the vaginal microbiota with lactobacilli, should become a standard approach for management in the future.

1.5. Rationale for studying *S. aureus* TSST-1 production in aberrant states

Given that aerobic bacteria, including *S. aureus*, can colonize the vagina in otherwise healthy women and those with either AV or BV, manufacturers of tampons face an important dilemma. Their products are widely used and extremely effective for managing menses, but they can never be fool-proof at preventing menstrual-TSS from occurring. It is difficult to control oxygen levels or magnesium, and unclear as to how big a difference it would make in preventing menstrual-TSS. However, it could be feasible to attempt to control the vaginal microbiota in a way that reduced the spread and toxin production of *S. aureus*. Clearly, lactobacilli can inhibit the growth of urogenital pathogens *E. coli*, *E. faecalis* and *S. aureus* (Coconnier *et al.*, 1997; Gopal *et al.*, 2001; Reid *et al.*, 1988; Reid, 2001; Velraeds *et al.*, 1996; Voravuthikunchai *et al.*, 2006). More specifically, a vaginal probiotic *L. reuteri* RC-14 has been shown to produce cyclic dipeptides that suppress TSST-1 production in *S. aureus* (Li *et al.*, 2011). This anti-virulence activity holds great potential to prevent TSS from occurring. Meanwhile, studies are needed to assess whether disruption of the vaginal

microbiota, in particular onset of BV and AV, provides *S. aureus* with a window of opportunity to secrete TSST-1 and put the host at risk of menstrual-TSS.

1.6. Hypothesis and Objectives

We hypothesize that aberrant vaginal microbiomes, most notably BV and AV, contribute to increased TSST-1 production in *S. aureus* and an increased susceptibility to menstrual-TSS.

Based upon the healthy vaginal microbiota, consisting of several *Lactobacillus* species, their production of antibacterial compounds, and in some cases cyclic dipeptides (Li *et al.*, 2011), we believe that depletion of these organisms will lead to increased virulence of *S. aureus* in the context of menstrual-TSS.

Objective 1: Compare techniques to effectively determine the composition and abundance of the vaginal microbiota of premenopausal women.

The goal here is to identify the prevalence and abundance of *S. aureus* in clinical samples from healthy women and those with an aberrant vaginal state. In doing so, we wanted to compare DGGE with high-throughput sequencing and determine which technique gives the most reliable and accurate identification power.

Objective 2: Determine the nature of *tst* expression in *S. aureus* in response to aberrant vaginal environments.

We investigated the nature of toxin production by *S. aureus* when this organism is exposed to a lactobacilli-abundant vaginal sample versus conditions

depicting a depletion of lactobacilli. This will help to identify whether some aberrant conditions make a woman potentially more susceptible to developing menstrual-TSS.

Objective 3: Determine the nature of TSST-1 production in response to representative members of the healthy, BV and AV microbiota.

The final step in this study was to perform *in vitro* testing of vaginal bacterial strains against *S. aureus* to identify groups of bacteria with inducing, neutral and inhibitory effects on TSST-1 production. If certain organisms influence TSST-1 production, efforts could be made to detect their co-existence with *S. aureus* in women at risk of menstrual-TSS, and to target interventions that neutralize this threat.

CHAPTER 2

MATERIALS AND METHODS

2.1. Denaturing Gradient Gel Electrophoresis

2.1.1. Clinical Samples

Vaginal samples were collected from HIV+/- and BV+/- women recruited in Tanzania, Africa, as part of another study (Hummelen *et al.*, 2010). Sequencing by Illumina was used to analyse the samples, separate from my involvement. For my project, a total of 22 of these vaginal samples were randomly selected for subsequent DGGE analysis, so that results could be compared to sequencing by Illumina. Of the 22 samples, 8 were healthy, 9 had BV, and 5 had an intermediate Nugent score.

2.1.2. DNA Extraction and Amplification

Samples were obtained by brushing the vaginal wall with a CultureSwab polyester-tipped swab (BD Biosciences) with the aid of a non-lubricated speculum. The swab was placed in a cryovial and stored at -20°C. When DNA extraction was performed, swabs were thawed and agitated in 1 mL PBS (pH 7.5). The solution was centrifuged at 10,000 rpm for 20 min and the supernatant discarded. Next, 200 µL InstaGene Matrix (BioRad #732-6030) was added to the bacterial pellet and incubated for 30 min at 56°C. The solution was vortexed for 10 sec and incubated for 8 min at 100°C, then re-vortexed for 10 sec followed by centrifugation at 10,000 rpm for 30 sec. The supernatant was transferred to a clean microcentrifuge tube and used as DNA template for subsequent polymerase chain reaction (PCR). Prior to use, the solution was centrifuged at 10,000 rpm for 3 min to spin down the InstaGene Matrix component of the

solution. PCR amplification of the DNA was carried out in 50 µl reactions consisting of 1 µL DNA template and 49 µL master mix (Table 2.1).

Table 2.1. PCR Master Mix for DGGE.

PCR Component	Volume added (µL)	Final Concentration
10X PCR Buffer (Invitrogen)	5.0	1X
$MgCl_2$ (50 mM)	1.7	1.7 mM
dNTPs (1.25 mM)	8.4	210 µM
HDA-f (20 µM)	1.6	640 nM
HDA-r (20 µM)	1.6	640 nM
Platinum *Taq* Polymerase (5U/µL) (Invitrogen)	0.5	0.05 U/µL
dH_2O	30.2	-------------
Final Volume:	49	-------------

A positive and negative control was included in each reaction set consisting of 1 µL *L. iners* DNA or 1 µL dH_20, respectively, and 49 µL master mix. Eubacterial primers (HDA) were used to amplify the third variable region (V3) of the 16S rRNA gene. These primers were HDA-f-GC (5'-**CGC-CCG-GGG-CGC-GCC-CCG-GGC-GGG-GCG-GGG-GCA-CGG-GG**G-ACT-CCT-ACG-GGA-GGC-AGC-AGT-3') and HDA-r (5'-GTA-TTA-CCG-CGG-CTG-CTG-GCA-3'). The forward primer contained a GC clamp (in bold) which is used in DGGE to help retain double strands while the DNA denatures on the gel. The PCR reactions were carried out in a Mastercycler (Eppendorf) under the following annealing temperature touchdown program: 94°C for 2 min, 25 cycles of 94°C for 45 sec, 61°C-51°C over 10 cycles for 45 sec (each cycle dropping by 1°C then remaining at 51°C for last 15 cycles), 72°C for 45 sec, and a final elongation step of 72°C for 2 min. PCR products (5 µL) were mixed with 1 µL 6X Loading Dye and loaded onto a 1.5% agarose gel made of 1X TBE with 5 µL ethidium bromide. The gel underwent electrophoresis and was viewed under UV light in an AlphaImager (Alpha Innotech Corporation) to verify the presence of PCR product in each sample.

2.1.3. Denaturing Gradient Gel Electrophoresis

A denaturing gradient of 30-50% was used, with denaturant solutions prepared based on reference 0% and 100% denaturing solutions shown in Table 2.2.

Table 2.2. Composition of denaturant solutions used for DGGE.

	Denaturant Strength	
Component	**0%**	**100%**
40% acrylamide/bis-acrylamide (37.5:1)	5 mL	5 mL
50X TAE	0.5 mL	0.5 mL
Urea	0 g	10.5 g
Formamide	0 mL	10 mL
MQ-H_2O	Up to 25 mL	Up to 25 mL

The denaturing solutions were prepared in 50 mL Conical tubes (BD Biosciences) and mixed by gently rocking back and forth to avoid oxygenation. Polymerizing agents were added, including 50 µL TEMED (*N*,*N*, *N*^{n},*N*^{n}-tetramethylethylenediamine) (Sigma) and 90 µL of 10% ammonium persulphate (Bio-Rad). Next, 100 µL of D-Code dye was added to the 50% denaturant solution to visualize the concentration gradient, and 16 mL of each solution was taken up in 50 mL syringes and poured into a glass sandwich (D-Code Universal Detection System, Bio-Rad). A comb was added to the top of the gel which was then allowed to polymerize for 2 hr at room temperature. The DGGE tank was filled with Milli-Q H_2O (Millipore) and 140 mL 50X TAE, and was heated to 59°C. After the gel was assembled into the unit, 5 µL sample was mixed with 5 µL 2X loading dye (0.25 mL bromophenol blue [2%, Sigma], 0.25 mL xylene cyanol [2%, Sigma], 7 mL glycerol, 2.5 mL dH_2O) and loaded into the wells .The gel was run for approximately 4 hours at 130V or until the lower dye front reached the bottom of the gel. The gel was then removed from the unit and allowed to cool to room temperature. The gel was placed on a plastic sheet and stained for 20 min in 600 mL of 1X TAE with 40 µg/L ethidium bromide and viewed under UV light. DNA bands were excised using a scalpel and placed in separate 1.5 mL Eppendorf tubes. 20 µL of Milli-Q H_2O was used to wash each excised band, after which another 20 µL was added to the tubes, which were then left at 4°C overnight to allow for DNA to disperse into the surrounding solution.

2.1.4. DNA Re-amplification and Purification

Re-amplification of the excised DNA was performed using the same PCR protocol as described above, but with using an HDA-f primer without the GC clamp. One microlitre of solution from the DNA band/Milli-Q H_2O mixture was used as template and added to 49 µL master mix (Table 2.1). DNA products were viewed under UV light as described previously. Purification of the re-amplified DNA was performed using the QIAquick PCR Purification kit (Qiagen). One hundred microlitres of PB1 buffer was added to 20 µL PCR product and mixed, then added to a QIAquick column placed in a 2 mL collection tube and centrifuged at 6,000 rpm for 60 sec to bind the DNA to the membrane. Bound DNA was washed twice using 750 µL PE buffer and centrifuged at 6,000 rpm (first wash) and 13,000 rpm (second wash) for 60 sec each. DNA was eluted by the addition of 50 µL Milli-Q H_2O to the centre of the membrane followed by centrifugation at 13,000 rpm for 60 sec. The eluted DNA was then stored at -20°C until further use.

2.1.5. Sequencing

A mixture was prepared consisting of 10 µL purified PCR product, 0.5 µL of 20 µM forward primer (HDA-f) and 4.5 µL Milli-Q H_2O. Sequences of the PCR products were determined by dideoxy chain termination (Sequencing Facility, John P. Robarts Research Institute, London, Ontario). Analysis of the V3 region of 16S rRNA was conducted using the GenBank nucleotide database and BLAST

algorithm (Altschul *et al.*, 1997). Electropherograms were analyzed using the FinchTV chromatogram trace viewer (Geospiza).

2.2. Clinical Study of Vaginal Microbiota of Premenopausal Women

2.2.1. Ethics Statement

Details of the clinical study were reviewed and approved by the Health Sciences Research Ethics Board at the University of Western Ontario (Appendix 1). Participants were provided with a package detailing all relevant information of the study, including an in-depth explanation of the clinical procedure, and signed a consent form prior to sample collection.

2.2.2. Study Population and Recruitment

Recruitment of pre-menopausal women between the ages of 18-40 years with BV as well as healthy controls took place in London, Ontario and was based on a selective criteria designed to ensure that the vaginal samples reflected a representative microbiota of the general female pre-menopausal population. Recruitment posters (Appendix 2) were placed in the following locations in London, Ontario: The University of Western Ontario, St. Joseph's Health Care Centre, Lawson Health Research Institute, Victoria Family Medical Centre, Victoria Hospital and University Hospital. The poster emphasized a target audience of premenopausal women with a suspected history of BV. Communication between the study coordinator and those interested in the study was through phone and email correspondence. Participants were excluded if

they had reached menopause, had a urogenital infection other than BV in the past 6 months, were pregnant, had a history of gonorrhoea, chlamydia, estrogen-dependent neoplasia, abnormal renal function or pyelonephritis, were taking prednisone, immune-suppressives or antimicrobial medication, or had undiagnosed abnormal vaginal bleeding. Participants were asked to refrain from oral or vaginal intercourse and consuming probiotic supplements or foods for 48 hours prior to the clinical visit. No participants were menstruating at time of the clinical visit.

2.2.3. Sample Collection

Weekly clinics were held at the Victoria Family Medical Centre (London, Ontario) over a 6-month period. Participants were first reimbursed with $50 for attending, and they were given a letter of information and consent package (Appendix 3) and baseline questionnaire (Appendix 4) which listed the exclusion criteria. Two nurses collected vaginal samples, and the study coordinator was on-site to first go over the study details and procedure with each participant. A total of 7 samples were collected from each participant: one pHem-alert applicator (Gynex) was used to detect the vaginal pH; three CultureSwab polyester-tipped swabs (BD Biosciences) were used for bacterial isolation (for diagnosis of BV by Nugent score, for bacterial DNA and RNA collection); two ESwabs (Copan) were processed for culturing the organisms; and one cytobrush (Cooper Surgical) was processed for vaginal epithelial cell RNA (Figure 2.1). Swabs were inserted approximately 5 cm into the vaginal canal, pressed against the wall and rotated 4 times.

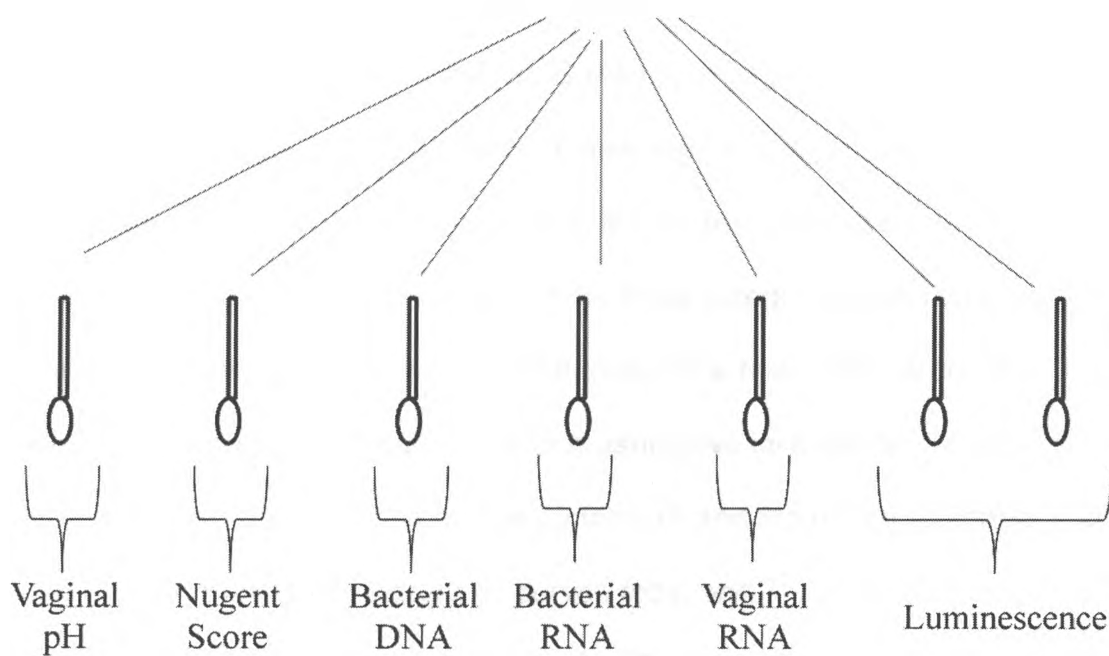

Figure 2.1. Flowchart of vaginal samples collected during the clinical study held in London, ON.

The microbial status was assessed through the Nugent scoring method (Nugent *et al.*, 1991). A CultureSwab polyester-tipped swab (BD Biosciences) was pressed against the vaginal wall and smeared onto a glass microscope slide, heat-fixed and Gram-stained. Slides were viewed under an Axio Scope light microscope (Zeiss). Each slide was given a score based on the number of 1) Gram-positive rods, 2) Gram-variable short rods and 3) Gram-negative cocci and curved rods (indicative of lactobacilli, *Gardnerella vaginalis* and *Mobiluncus* spp. respectively) (Table 2.3). Four fields of view were chosen at random at a magnification of 1,000 X, and bacterial numbers from each were recorded. The average number of bacteria in each of the three categories was recorded, and their corresponding scores were added to obtain a final score. A score of 0-3 was deemed healthy with predominance of presumptive lactobacilli, 4-6 was intermediate with fewer presumptive lactobacilli and more Gram-variable short rods and Gram-negative cocci and curves rods, and 7-10 was BV where the samples were dominated with non-lactobacilli organisms, usually in biofilms. A participant was diagnosed as having BV if the Nugent score was 7-10 and if they had a vaginal pH>4.5.

Table 2.3. Nugent score criteria for BV diagnosis.

	***Lactobacillus* spp. (Large Gram-positive rods)**	***Gardnerella* spp. (Small Gram-variable rods)**	***Mobiluncus* spp. (Curved Gram-variable rods or cocci)**
Cells per high power field (x1000)	SCORE	SCORE	SCORE
0	4	0	0
<1	3	1	1
1-4	2	2	1
5-30	1	3	2
>30	0	4	2

2.3. Bacterial DNA Preparation for Illumina Sequencing

Bacteria were collected on a CultureSwab polyester-tipped swab (BD Biosciences) which was immediately placed in 700 µL RNA*protect* (Qiagen). The tube was vortexed for 5 sec, swab discarded and the solution stored at -20°C. For DNA isolation, the solution was centrifuged at 10,000 rpm for 20 min and supernatant discarded. Next, 200 µL InstaGene Matrix (BioRad #732-6030) was added to the bacterial pellet and incubated for 30 min at 56°C. The solution was vortexed for 10 sec and incubated for 8 min at 100°C, then re-vortexed for 10 sec followed by centrifugation at 10,000 rpm for 30 sec. The supernatant was transferred to a clean microcentrifuge tube and stored at -20°C, before use as DNA template for subsequent PCR reactions.

PCR was carried out in 50 µL reactions consisting of the same components and final concentrations as was used with DGGE (Table 2.1) but with 40 µL master mix and 10 µL template (or 10 µL Milli-Q H_20 [Millipore] for negative control). Amplification was done using eubacterial primers flanking the V6 region of the 16S rRNA gene: V6-F (5'-CAACGCGARGAACCTTACC -3') and V6-R (5'-ACAACACGAGCTGACGAC -3'). Amplification was performed in a Mastercycler (Eppendorf) using the same annealing temperature touchdown program as described for amplifying DGGE DNA. Products were viewed via electrophoresis in a 1.5% agarose gel using 1X TBE, stained with ethidium bromide and viewed under UV light in an AlphaImager (Alpha Innotech Corporation). The ImageJ program was then used to quantify the brightness of each DNA band so that equal amounts of each sample were added to the

subsequent Illumina reaction. DNA samples were then sent for Illumina sequencing at The Next-Generation Sequencing Facility in The Centre for Applied Genomics at the Hospital for Sick Children in Toronto, Canada.

2.4. Luminescence Assay

Vaginal samples were collected from participants using the ESwab (Conan) swab and transport system containing 1 mL liquid Amies medium (Figure 2.2). Two ESwabs were each submerged into their respective media for approximately 2 hours at room temperature followed by a 10 sec vortex at high speed. The swabs were removed and discarded. One of the solutions was aliquoted 2 x 350 µL, and 500 µL of cryoprotectant (3% citric acid, 40% glycerol, pH 7.0) was added to each, and stored at -80°C. These samples would be available for future culturing of organisms if needed. The other ESwab solution was aliquoted 2 x 350 µL, with one stored at -80°C and the other centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to a fresh tube and stored at -80°C, which was later used for the luminescence assay.

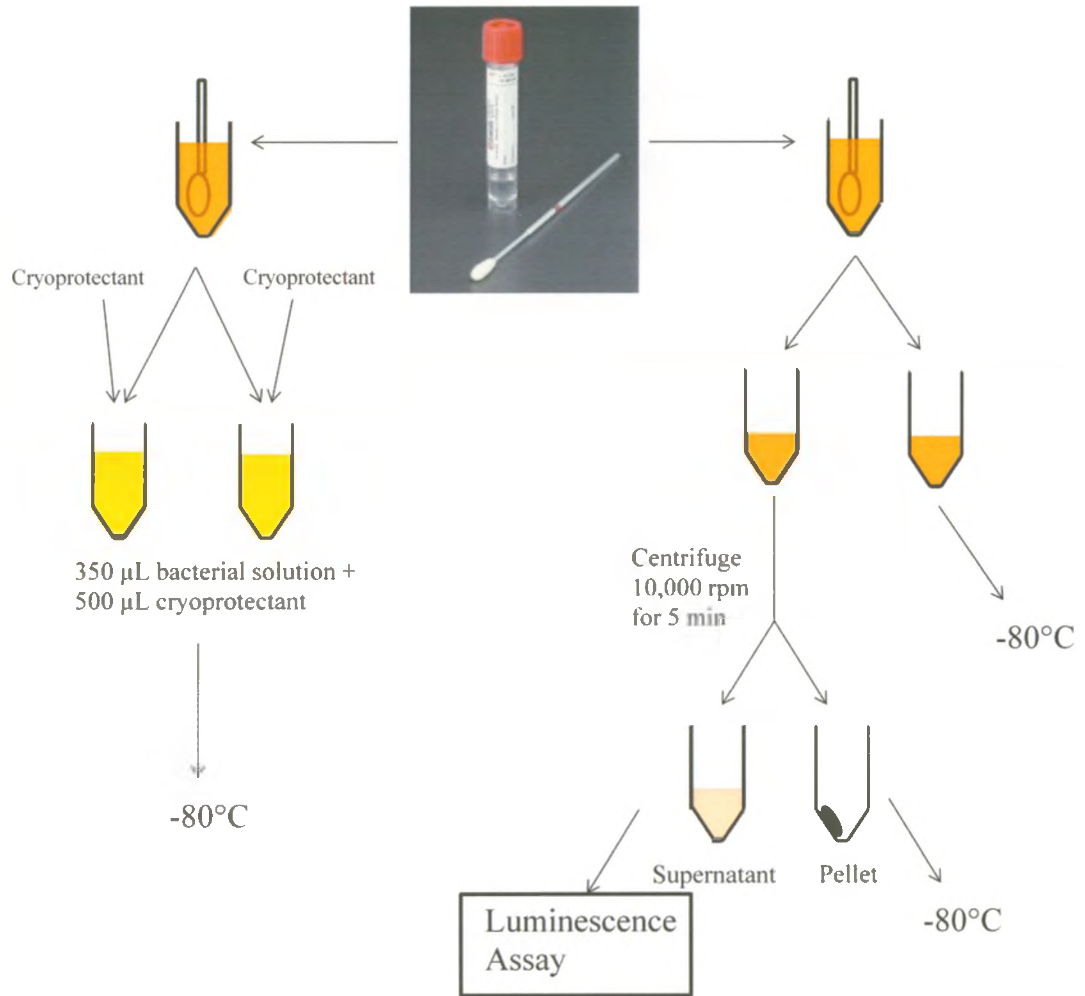

Figure 2.2. Flowchart depicting vaginal swab collection using the ESwab transport medium, including the origin of sample supernatants for the luminescence assay.

A culture of a reporter strain of *S. aureus* MN8, containing the cloning vector pAmilux with an incorporated promoter for *tst*, was grown in brain heart infusion (BHI) media with 10 µg/µL chloramphenicol overnight at 37°C with a shaking speed of 250 rpm (Mesak *et al.*, 2009; Li *et al.*, 2011). The culture was diluted to an OD_{600} of 0.02 with a) supernatant from the clinical sample, b) Amies transport medium or c) BHI, of which the latter two acted as controls. Next, 200 µL of the subculture was grown for 16 hr in a Fluoroskan Ascent FL luminometer (Thermo) where luminescence production was detected from the activated *tst* promoter every 30 min. Another 200 µL of the subculture was also grown in a Bioscreen C Reader (MTX Lab Systems) at 37°C with continuous shaking for 24 hr to monitor growth of the bacteria.

2.5. Supernatant Challenge Assay

2.5.1. Bacterial Cultures and Conditions

All bacterial strains used in the supernatant challenge assay, along with respective growth conditions, are listed in Table 2.4. The bacterial strains were divided into 3 groups for analysis: healthy, AV-associated and BV-associated bacteria (Table 2.5). *The S. aureus* MN8 strain was selected as a prototype of menstrual-TSS strains, as it has been isolated from a pre-menopausal woman suffering from the condition (Schlievert & Blomster, 1983). All aerobic bacteria were cultured in shaking conditions for 16 hr at 37°C and then sub-cultured to an OD_{600} of 0.02 using their growth media. The sub-cultures were grown for 12 hr.

The lactobacilli were grown in anaerobic conditions using the GasPak EZ system (Becton-Dickenson) for 24 hr at 37°C and sub-cultured for an additional 24 hr,

Table 2.4. Bacterial strains used for supernatant challenge assay.

Bacteria	Source	Growth Media/Conditions
Staphylococcus aureus MN8	Clinical Isolate	BHI/aerobic
Escherichia coli J96	Clinical Isolate	BHI/aerobic
Streptococcus agalactiae ATCC 13813	ATCC	BHI/aerobic
Enterococcus faecalis ATCC 33186	ATCC	BHI/aerobic
Enterococcus faecium ATCC 19434	ATCC	BHI/aerobic
Atopobium vaginae	N/A*	Vaginally-Defined Medium + 0.5% Proteose Peptone (VDMP)/anaerobic
Prevotella bivia	N/A*	VDMP/anaerobic
Lactobacillus crispatus ATCC 33820	ATCC	de Man, Rogosa and Sharpe (MRS)/anaerobic
Lactobacillus jensenii ATCC 25258	ATCC	MRS/anaerobic
Lactobacillus johnsonii DSM20553	DSM	MRS/anaerobic
Lactobacillus gasseri ATCC 33323	ATCC	MRS/anaerobic
Lactobacillus reuteri RC-14	Clinical Isolate	MRS/anaerobic
Lactobacillus rhamnosus GR-1	Clinical Isolate	MRS/anaerobic

*Not available/unknown

Table 2.5. Grouping of bacteria for supernatant challenge assay, based on associated vaginal condition.

Group		
Healthy (lactobacilli)	**AV-associated**	**BV-associated**
Lactobacillus jensenii	*Streptococcus agalactiae*	*Atopobium vaginae*
L. gasseri	*Escherichia coli*	*Prevotella bivia*
L. johnsonii	*Enterococcus faecalis*	
L. reuteri RC-14	*E. faecium*	
L. rhamnosus GR-1		

starting at an OD_{600} of 0.02. The strict anaerobes, *Atopobium vaginae* and *Prevotella bivia* were cultured and sub-cultured in a Forma anaerobic chamber (Model 1025, Thermo) for 48 hr at 37°C to allow for adequate growth. Sub-cultures underwent centrifugation at 3500 x *g* for 10 min at 4°C and the supernatants were transferred to a fresh tube. The pH of the supernatants were determined and adjusted to that of the *S. aureus* MN8 supernatant (pH 5.95), sterilized via filtration using 0.2 µm filters, transferred to a clean tube and frozen at -20°C for no longer than 1 week. Next, *S. aureus* MN8 cultures were grown for 16 hr and sub-cultured starting at an initial OD_{600} of 0.02 in half BHI and half supernatant of the challenger bacteria. The sub-cultures were grown for 16 hr at 37°C in shaking conditions and were then centrifuged at 3500x*g* for 10 min at 4°C. Sub-cultures were also grown in a Multiskan Ascent Plate Reader (Thermo-Scientific) to detect OD_{600} values to ensure similar *S. aureus* growth across the various conditions. The supernatants were subsequently used for Western Blot and real-time PCR.

2.5.2. SDS-PAGE and Western Blot

Total protein from *S. aureus* supernatant was isolated via trichloroacetic acid (TCA) precipitation. First, 300 µL of ice-cold 20% TCA was added to 1mL supernatant, which was then vortexed at high speed for 5 sec and left on ice for 30 min. The solution was centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant discarded. The pellet was washed twice with 300 µL ice-cold acetone (14,000 rpm for 5 min at 4°C), and was then air-dried and resuspended in 37.5 µL 200 mM urea and 12.5 µL 4X Laemmli buffer (40% glycerol, 240 mM

Tris/HCl [pH 6.8], 8% SDS, 0.04% bromophenol blue and 5% beta-mercaptoethanol). The protein solutions were stored at -20°C until use for gel analysis.

Acrylamide gels were made for SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and Western blot, each at a lower 12% resolving gel (pH of 8.8) and an upper 5% stacking gel (pH of 6.8) (Table 2.6). Protein solutions were thawed and heated at 100°C for 5 min, and then placed on ice for another 5 min. Next, 20 µL of the protein samples were loaded into the gels and run at 50V until the bands stacked at the interface of the stacking and resolving gel, and then at 120V through the resolving gel. The running buffer consisted of 196 mM glycine, 0.1% SDS and 25 mM Tris-HCl at pH 8.3. When the lower dye front reached the bottom of the gel, the run was stopped and gels were removed from the apparatus. One gel was stained in Coomassie Blue stain solution (50% methanol, 0.05% w/v Coomassie Brilliant Blue R-250 and 10% acetic acid) for at least 2 hr and then placed in destain solution (5% methanol and 7% acetic acid) for 2 hr. The gel was then viewed under light on a transparent surface in an AlphaImager (Alpha Innotech Corporation). The other gel was soaked in transfer buffer (25 mM Tris [pH 8.3], 192 mM glycine and 20% methanol) along with sponges, filter paper and the Hybond-ECL nitrocellulose membrane (Amersham) for protein transfer. The gel and membrane were then pressed together in between the filter pads and placed in a tank filled with transfer buffer. A current of 200 mA was run through the assembled unit for 3 hr, after which the membrane was removed and placed in Ponceau S stain solution

Table 2.6. Composition of polyacrylamide gels for SDS-PAGE and Western Blot.

Component	Stacking Gel (5%)	Resolving Gel (12%)
MQ-H_2O	5.99 mL	4.3 mL
40% acrylamide/bis-acrylamide (37.5:1)	1.0 mL	3.0 mL
1.5 M Tris (pH 8.8)	750 µL	2.5 mL
10% SDS (sodium dodecyl sulphate)	80 µL	100 µL
10% APS (ammonium persulfate)	80 µL	100 µL
TEMED (Tetramethylethylenediamine)	8 µL	4 µL

(5% acetic acid and 0.1% w/v Ponceau S) for 5 min and destained in 1X PBS for 15 min to visualize the proper transfer of proteins from the gel to the membrane. Next, the membrane was placed in blocking solution (5% skim milk and 10% horse serum in 1X PBS) overnight at 4°C and added to a solution of rabbit IgG anti-TSST primary antibody (1:1,500) (generously donated by Dr. Schlievert) in 2.5% skim milk and 5% horse serum for 1 hr at room temperature. Following this, the membrane was washed 3 times with 1X PBS and 0.1% Tween-20 for 5 min and placed in a solution of goat anti-rabbit IgG-HRP secondary antibody (1:2,500) (Invitrogen) in 2.5% skim milk and 5% horse serum for 1 hr at room temperature. The membrane was again washed 3 times with 1X PBS and 0.1% Tween-20 and exposed to a colorimetric substrate solution (0.0005% [w/v] diaminobenzidine [DAB], 1mL of 1M Tris [pH 7.9], 12 µL H_2O_2 [30%], 20 mL Milli-Q H_2O) for 3 min to visualize the TSST-1 protein bands.

2.5.3. Real-Time PCR

2.5.3.1. *S. aureus* RNA Extraction

A culture of *S. aureus* MN8 challenged with supernatants from the other bacteria (as described previously) was grown from an initial OD_{600} of 0.02 for 16 hr. Ten millilitres of RNAprotect (Qiagen) was added to 5 mL culture, and the solution was vortexed and incubated for 10 min at room temperature, then centrifuged at 6000 x *g* for 20 min at 4°C (all centrifugation steps in the RNA isolation procedure occurred at 4°C). The supernatant was then discarded and the pellet stored at -80°C. To isolate bacterial RNA, the pellet was suspended in

5 mL lysis solution (10 mM Tris-HCl, 1 mM EDTA [pH 8.0] and 50 µg/mL lysostaphin) and vortexed. The solution was incubated for 10 min at 37°C then centrifuged at 6000 x *g* for 20 min. The supernatant was discarded, and the pellet was suspended in 5 mL TRIzol (Invitrogen) and vortexed for 2 min. The sample was incubated at room temperature for 10 min, followed by the addition of 1 mL chloroform. The solution was vortexed and incubated at room temperature for 10 min, followed by centrifugation at 12,000 x *g* for 20 min. Two millilitres of the upper aqueous phase containing RNA was transferred to RNase-free microcentrifuge tubes separated into four 500 µL aliquots. Next, 55 µL of 3M sodium acetate and 550 µL isopropanol was added to each aliquot, the solution vortexed and incubated at room temperature for 10 min. The sample was centrifuged at 12,000 x *g* for 20 min, supernatant discarded and pellet washed with 500 µL of 75% ethanol. The pellet was then dried and resuspended in 20 µL RNase-free H_20 and stored at -80°C.

Contaminants were removed from the RNA samples using the RNeasy Mini Kit (Qiagen). The volume of each RNA sample was adjusted to 100 µL with RNase-free H_2O, and then 350 µL RLT buffer was added and mixed. Next, 250 µL 100% ethanol was added to the RNA and mixed with pipetting. Each sample was transferred to an RNeasy Mini spin column which was placed in a 2 mL collection tube. The sample was centrifuged at 12,000 rpm for 15 sec and flow-through discarded. Five hundred microlitres of RPE buffer was then added to the spin column and centrifuged at 12,000 rpm for 2 min. The column was placed in a new 1.5 mL Eppendorf tube, 30 µL RNase-free H_2O added to the column and

centrifuged at 12,000 rpm for 1 min to elute the RNA. Next, DNase digestion was performed to remove any DNA contaminants using the TURBO DNA-free Kit (Ambion). Three microlitres of 10X TURBO DNase buffer along with 1 µL of TURBO DNase was added to the RNA solutions and mixed. The solutions were then incubated at 37°C for 30 min, after which 3 µL DNase Inactivation Reagent was added. These solutions were left at room temperature for 5 min with occasional mixing before being centrifuged at 12,000 rpm for 1.5 min. The RNA was transferred to a fresh 1.5 mL RNase-free tube.

RNA products were viewed via electrophoresis in a 1% agarose gel using 1X TBE, stained with ethidium bromide and viewed under UV light in an AlphaImager (Alpha Innotech Corporation). The RNA concentration and quality in the samples were then determined through a biophotometer (Eppendorf) (RNA quality cut-off: 260/280 ≥ 1.8; 260/230 ≥ 1.6). Five hundred nanograms of RNA from each sample was used as a template for reverse transcription PCR. Conversion to cDNA was done using the Multiscribe Reverse Transcriptase mix (Invitrogen) with components listed in Table 2.7. The master mix was vortexed prior to the final addition of the enzyme components of the mix (RNase Inhibitor and MultiScribe Reverse Transcriptase). The reactions were carried out in a Mastercycler (Eppendorf) with the following program: initial step of 25°C for 10 min, followed by 37°C for 120 min, and a final step of 85°C for 5 min. The supernatant challenge experiment was performed once, including three technical replicates starting from the reverse transcription stage.

Table 2.7. Master Mix components for MultiScribe Reverse-Transcription.

Component	**Volume (µL)**
10X TaqMan RT buffer	1.0
$MgCl_2$ (25 mM)	2.2
dNTPs (2.5 mM)	2.0
Random Hexamers (50 µM)	0.5
RNase Inhibitor (20 U/L)	0.2
MultiScribe Reverse Transcriptase	0.25
RNase-free H_2O	3.85*
Final Volume:	10.0

*Subtract by the volume of RNA used

2.5.3.2. Quantification of *tst* Expression

The cDNA samples were used as templates for real-time PCR reactions. Primers included tst-f (5'- CTGATGCTGCCATCTGTGTT -3') and tst-r (5'- GTAAGCCCTTTGTTGCTTGC -3') for expression of the *tst* gene, and rpoB-f (5'- TCCTGTTGAACGCGCATGTAA -3') and rpoB-r (5'- GCTGGTATGGCTCGTGATGGTA-3') for expression of the *rpoB* housekeeping gene. The iQ SYBR Green Supermix (Bio-Rad) was used to carry out 20 µL reactions, which were composed of the following: 10 µL 2X iQ SYBR Green Supermix, 1 µL of each primer at 10 µM, 7 µL RNase-free H_2O and 1 µL cDNA template. Reactions were run in a Rotor –Gene 6000 thermocycler (Corbett) under the following program: an initial melting ramp from 72°C to 95°C, followed by 40 cycles of 95°C for 10 sec, 60°C for 15 sec and 72°C for 20 sec, ending with a hold at 95°C for 10 min. Data was analyzed using the Rotor-Gene 6000 Series Software 1.7 (Corbett). Expression of *tst* by *S. aureus* in each condition was compared to *tst* expression when *S. aureus* was grown in media alone. For the purpose of comparison, the control expression was set at 100%.

A series of standards were created to quantify gene expression during data analysis. A sub-culture of *S. aureus* MN8 was grown for 12 hr, and total DNA isolated using InstaGene Matrix (BioRad #732-6030). PCR was carried out in 50 µL reactions consisting of the following components: 10X PCR buffer, 1.7 mM $MgCl_2$, 210 mM dNTPs mix, 640 nM of each primer (*tst* and *rpoB* primer pairs used for real-time PCR), 5U Platinum Taq DNA Polymerase (Invitrogen) 5 µL template and Milli-Q H_20 [Millipore] to reach 50 µL. Amplification was

performed in a Mastercycler (Eppendorf) using the following program: 94°C for 1 min, followed by 30 cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 45 sec, and a final elongation step of 72°C for 2 min. Products were viewed via electrophoresis in a 1% agarose gel using 1X TBE, stained with ethidium bromide and viewed under UV light in an AlphaImager (Alpha Innotech Corporation). Concentration of DNA in each sample was determined through spectrophotometry, and a series of standards were made ranging from 5000 ng/µL to 5 x 10^{-9} ng/µL. These standards were used as templates in the real-time PCR reactions to construct standard curves with known concentrations of DNA.

Total protein from the supernatants used to challenge *S. aureus* MN8 in the real-time PCR assay were precipitated and analyzed through SDS-PAGE in order to compare protein expression among these bacteria. The procedure was identical to the protocol described previously for analyzing *S. aureus* protein expression through SDS-PAGE.

2.6. Statistical Analysis

Statistical analysis was performed on the real-time PCR data using the GraphPad Prism® 4 program. Gene expression of the various conditions was compared to the control using one-way ANOVA analysis with a Dunnett's multiple comparison test. Changes in gene expression were considered significant with p values <0.05. The standard error of the mean (SEM) for each condition tested was displayed on the bar-plots.

CHAPTER 3

RESULTS

3.1. DGGE versus 16S rRNA Sequencing by Illumina

The first step in this investigation was to identify the most effective bacterial identification technique by directly comparing DGGE to 16S rRNA sequencing by Illumina. A total of 22 vaginal samples were obtained from a clinical study held in Tanzania, Africa. From these, 6 bacterial species were detected across 6 healthy and 2 intermediate samples using the DGGE method (Figure 3.1A), as well as 6 species in 9 women with BV (Figure 3.1B). The most common organisms detected from this sample pool were *L. iners* and *G. vaginalis,* while several DNA bands from the BV subset were matched with uncultured species using the BLAST algorithm. Overall, 8 different species were detected in this HIV-positive sample pool, whereas 16S rRNA sequencing by Illumina detected 59 bacterial species in the same 22 samples (Figure 3.2). The lowest abundance of the 8 species detected by DGGE was 11%. The group of 8 species found through DGGE represented a subset from those detected by the Illumina method.

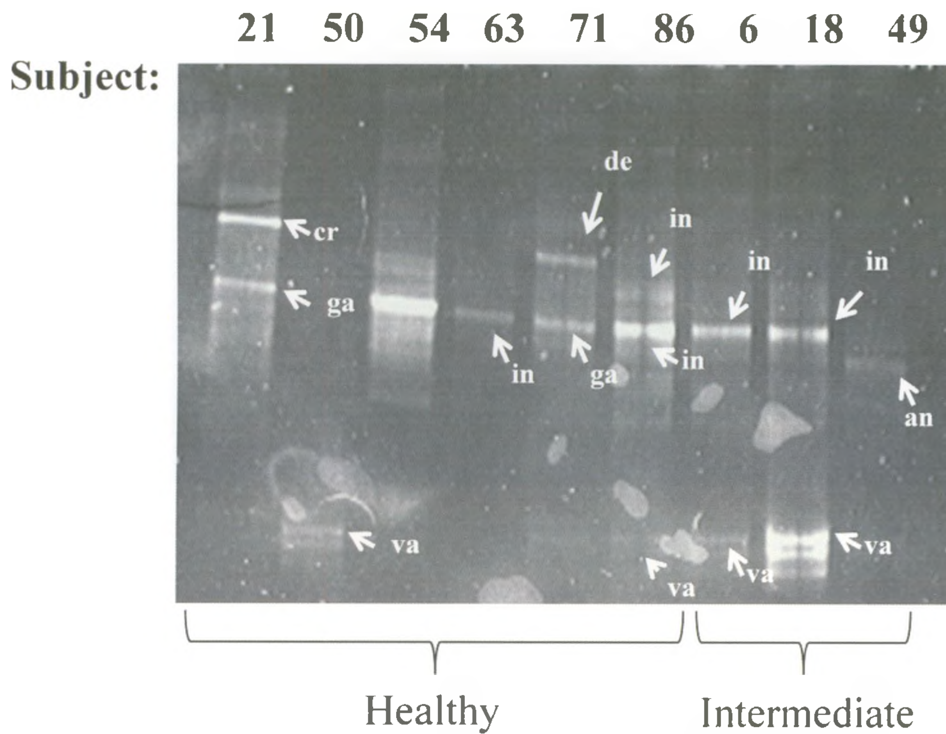

Figure 3.1A. PCR amplicons of vaginal bacterial DNA from the African study of women with healthy and intermediate vaginal status, as determined by Nugent scoring and Amsel's criteria. Extracted DNA was electrophoresed on a 30-50% denaturing gradient gel. DNA bands are labeled as follows: cr= *Lactobacillus crispatus*; ga= *L. gasseri*; va= *Gardnerella vaginalis*; in= *L. iners*; de= *L. delbrueckii* sp. *bulgaricus*; an= *Streptococcus anginosus*.

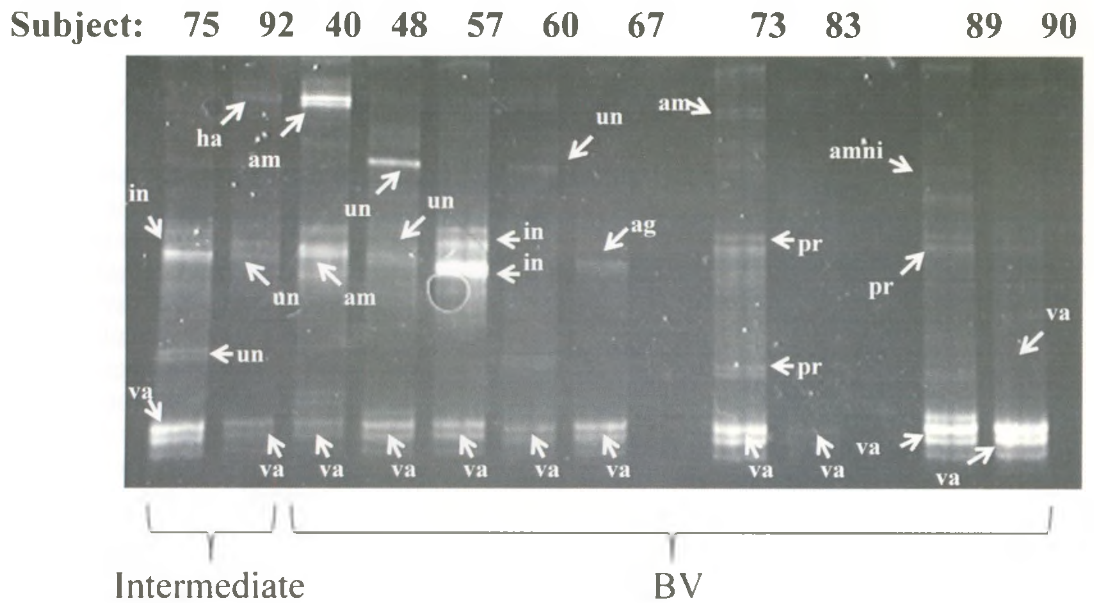

Figure 3.1B. PCR amplicons of vaginal bacterial DNA from the African study of women with intermediate and BV vaginal status, as determined by Nugent scoring and Amsel's criteria. DNA bands are labeled as follows: in= *Lactobacillus iners*; un= uncultured organism; va= *Gardnerella vaginalis*; ha= *Haemophilus influenzae*; am= *Leptotrichia amnionii*; ag= *Streptococcus agalactiae*; pr= Prevotella spp.; amni= *Prevotella amniotica.*

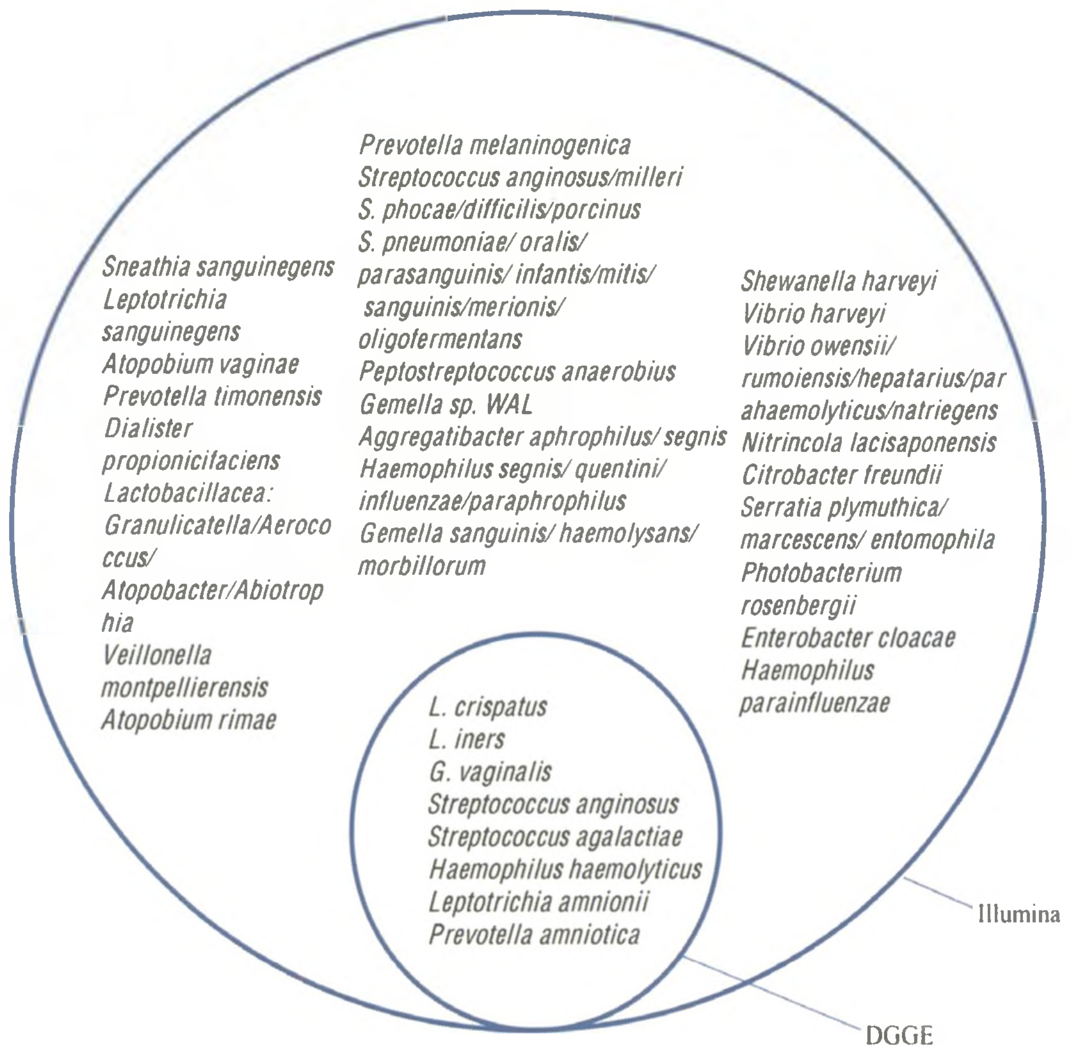

Figure 3.2. Venn diagram comparing bacterial species found through DGGE versus Illumina sequencing. A total of 8 species in 22 clinical samples were detected via DGGE, compared to 59 by the Illumina method. Figure adapted from Gloor *et al*. (2010).

3.2. Vaginal Microbiota of Pre-menopausal Women With and Without Bacterial Vaginosis in London, ON

A clinical study was undertaken to collect vaginal samples and determine the microbiomes of women with and without BV, and to look for the presence of *S. aureus* in this population. A total of 34 pre-menopausal women were recruited from London, ON. Of these, 11 were scored healthy, 10 intermediate and 13 BV based upon Nugent score and vaginal pH (Figure 3.3). The mean age of participants was 24.6 years. None of the subjects were diagnosed with AV nor had a microbiome indicative of this condition.

The vaginal microbiota of participants were determined through 16S rRNA sequencing by Illumina (Figure 3.4), and bacterial DNA from 21 participants was successfully isolated and amplified for sequencing. These included samples from subjects assigned to the healthy, intermediate and BV groups.

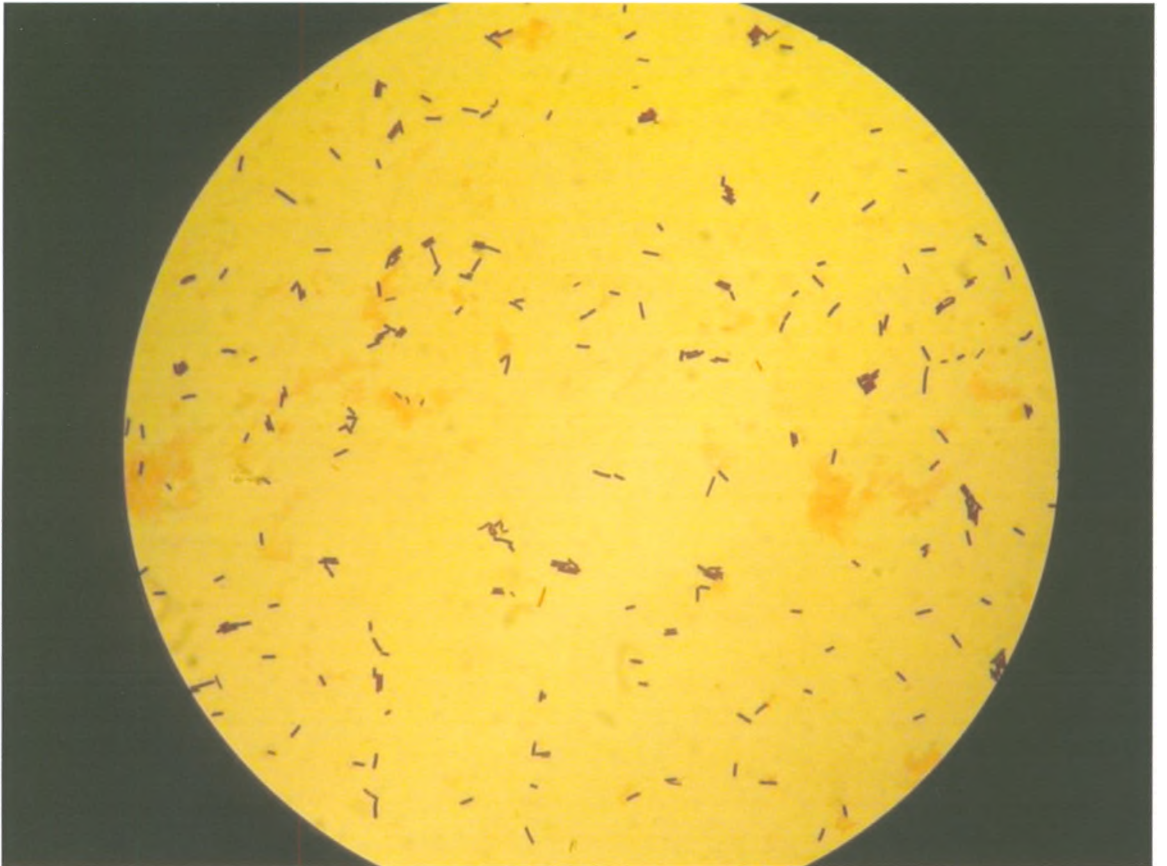

Figure 3.3A. Gram-stained vaginal smear of a subject from London, ON with a healthy microbiota (Nugent score 1-3), examined under oil immersion (x 1,000 magnification).

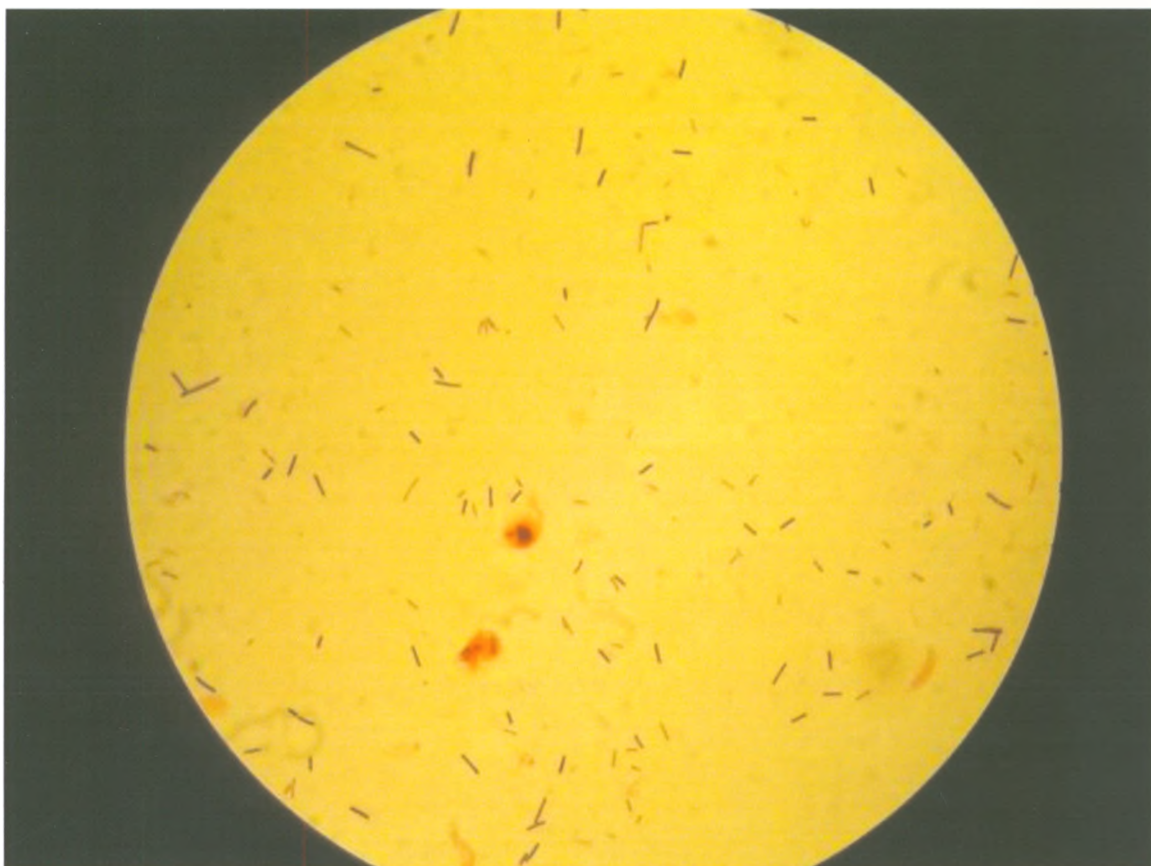

Figure 3.3B. Gram-stained vaginal smear of a subject from London, ON diagnosed with an intermediate microbiota (Nugent score 4-6), examined under oil immersion (x 1,000 magnification).

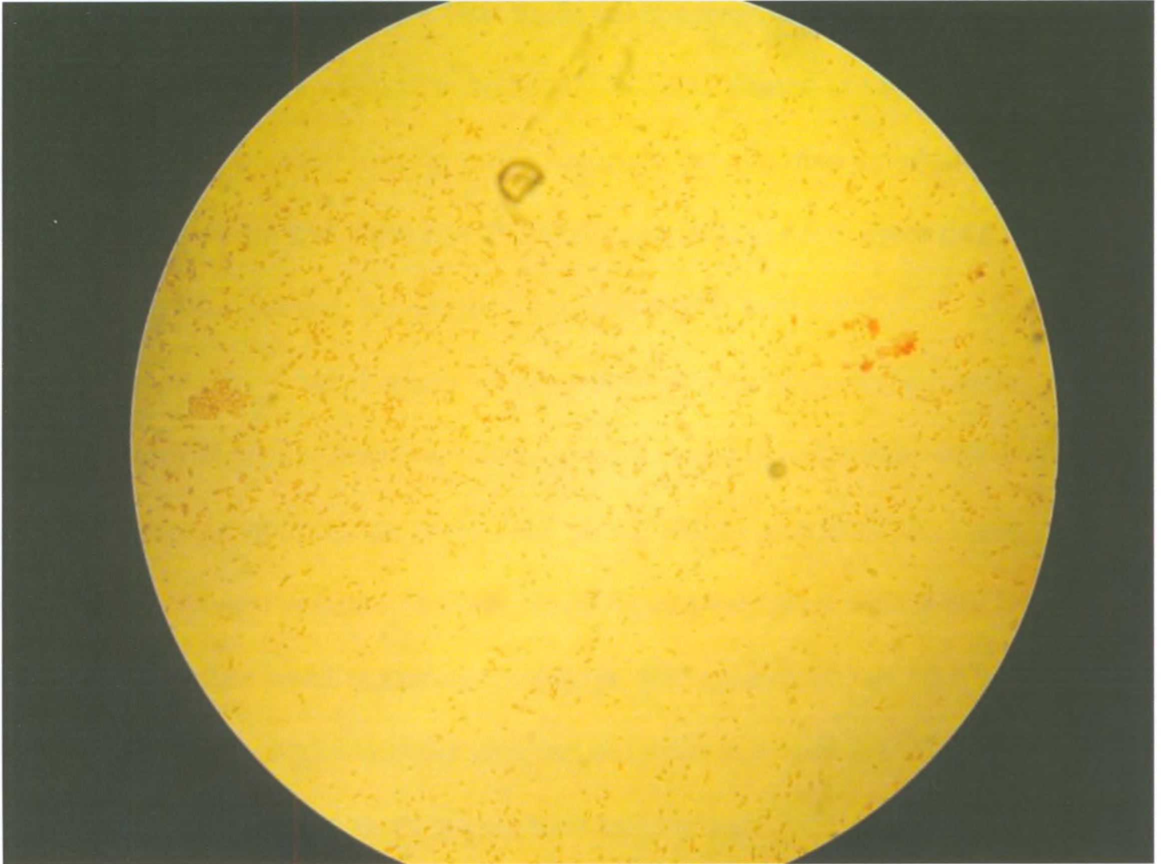

Figure 3.3C. Gram-stained vaginal smear of a subject from London, ON diagnosed with Bacterial Vaginosis (Nugent score 7-10), examined under oil immersion (x 1,000 magnification).

The vaginal microbiota of healthy versus BV participants were very distinct. The predominant species associated with healthy and intermediate samples was *L. crispatus* (50.7% and 41.6%, respectively) followed by *L. iners* (27.4% and 20.5%, respectively), with an average species abundance of 3.6 species per sample (Figure 3.4). Other bacterial species, including those associated with BV, were present in relatively low abundance in these subjects. Three outliers were present among these samples, having diversities of 11, 12 and 17 species per sample. One intermediate sample had no *L. iners* or *L. crispatus* (≥1% abundance), but instead consisted of *L. jensenii*, *L. gasseri/johnsonii* and *G. vaginalis* of nearly equal abundances. Participants assigned to the BV group, however, had a predominant *Gardnerella vaginalis* population (average abundance of 49.7%), along with *Leptotrichia amnionii* (3.9%) and *Atopobium vaginae* (3.7%). The BV samples had a higher bacterial species diversity than healthy and intermediate, with an average of 10.5 species per sample. *Staphylococcus aureus* was not detected in any of the 21 participants whose samples were sequenced by Illumina.

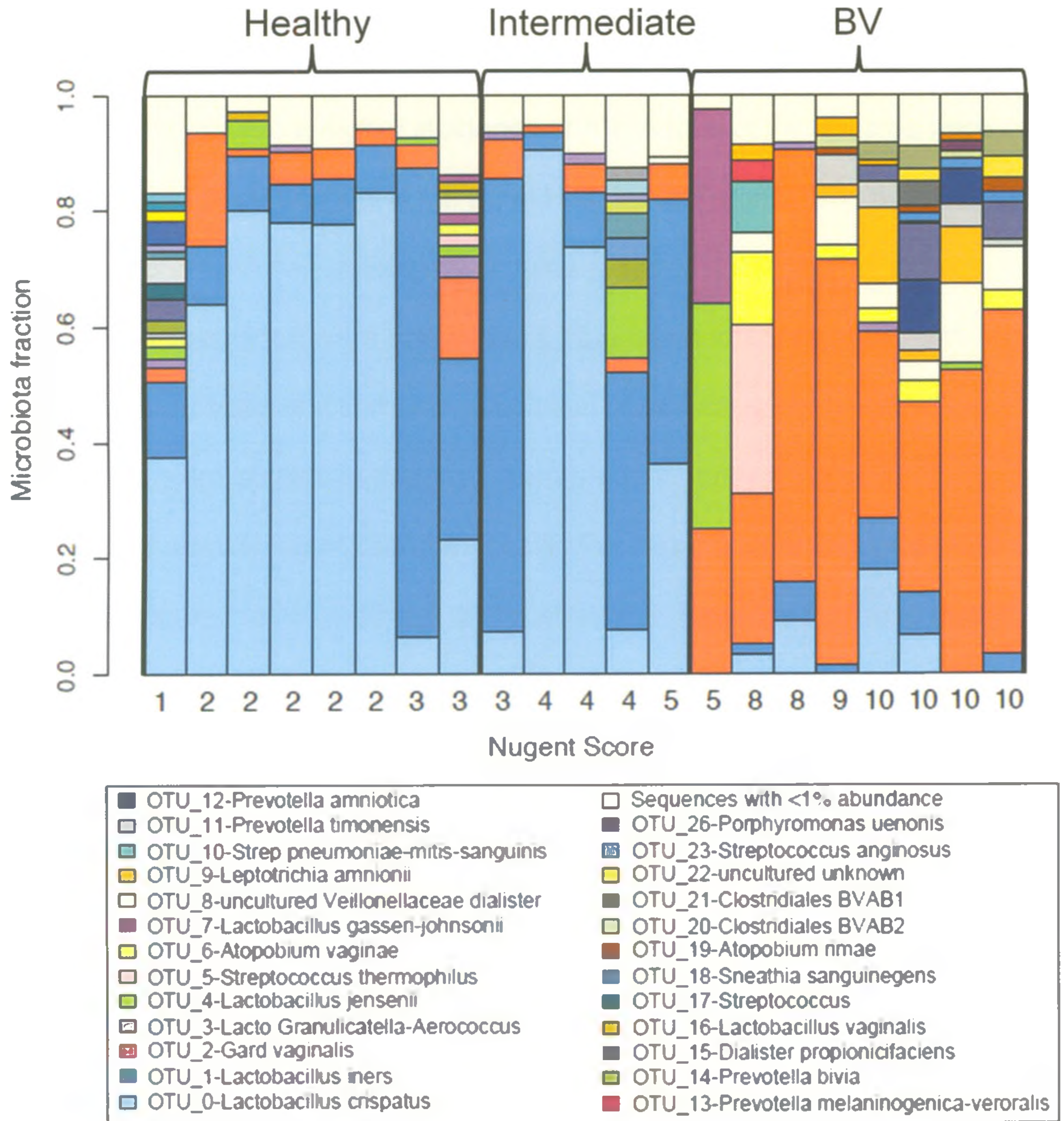

Figure 3.4. Vaginal microbiota of 21 subjects recruited from the clinical study, as determined through 16S rRNA sequencing by Illumina. Vaginal state determined via Nugent Scoring; score of 1-3 is healthy, 4-6 is intermediate, and 7-10 is BV. Each column represents a participant, with each colored bar representing a type of bacteria.

3.3. Abundance of *Staphylococcus aureus* in Pregnant Women in Toronto, ON

An additional dataset was studied from pregnant women, as that population tends to have a higher incidence of AV. A total of 74 samples were obtained from pregnant women at Mt. Sinai Hospital in Toronto, and these were analysed by 16S rRNA sequencing by Illumina (Figure 3.5A). Most of the samples in this population were dominated by *L. iners* and *L. crispatus*, with others being composed of a complex population of several species. However, some samples were outliers in that they were predominantly composed of a single bacterial species other than lactobacilli. For instance, participant 44 had a vaginal microbiome made up mostly of Mycobacteria. Reasons for these outliers are unclear but may be due to the sampling method employed. The study from which the samples were taken is double-blind and ongoing, and so the prevalence of AV in these subjects cannot be determined. *Staphylococcus* spp. were found in 13 of the 74 samples (17.6%) when applying a cut-off of ≥1% abundance (Figure 3.5B). Due to the amplification and sequencing of the sixth variable region (V6) of 16S rRNA, the *Staphylococcus* species detected were separated into a *S. aureus/haemolyticus* group and *S. epidermidis*. The *S. aureus/haemolyticus* group was detected in 11 of the 74 samples (14.9%), while *S. epidermidis* was only present in 4 samples (5.4%). The highest relative abundance of *Staphylococcus* spp. in a sample was 57.3% (Figure 3.5B, Participant 32), and the average relative abundance in all samples containing *Staphylococcus* spp. was 20.1%.

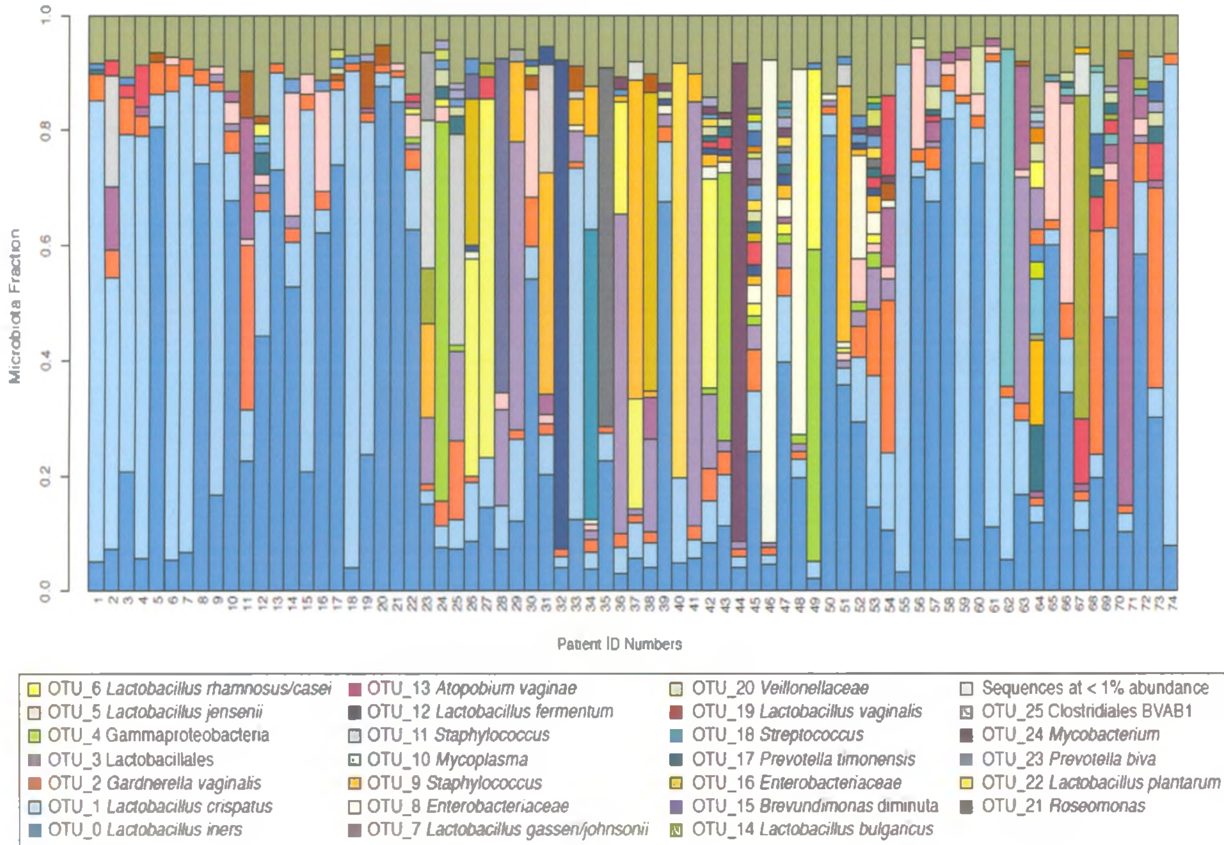

Figure 3.5A. Illumina sequencing data depicting the vaginal microbiota of 74 pregnant women recruited in Toronto. Each column represents a subject, and each colored bar within the columns represents a bacterial species.

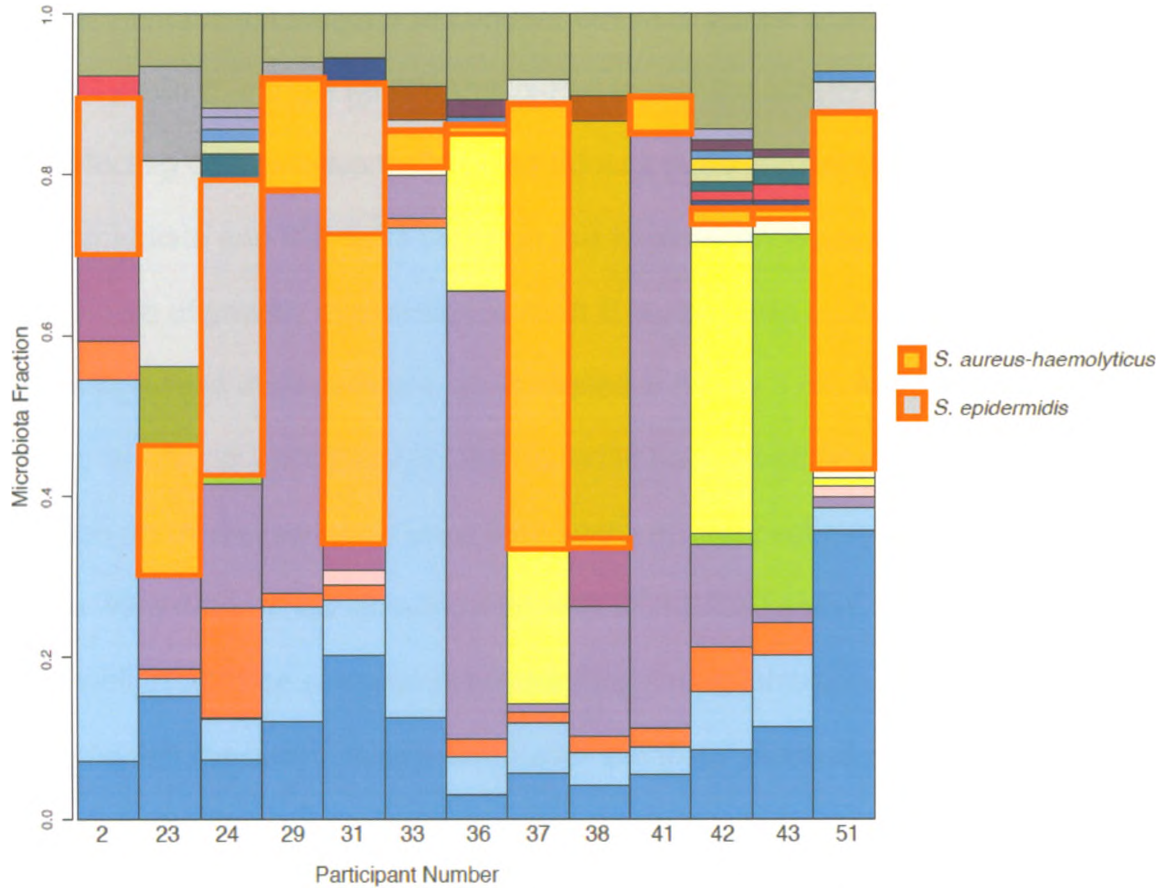

Figure 3.5B. Bacterial 16S rRNA Illumina sequencing data showing *Staphylococcus* spp. present in 13 subjects recruited from Toronto, ON. Each column represents a subject, and each colored bar within the columns represents a bacterial species as listed in Figure 3.5A. Bars encased in red depict staphylococci. Orange bars= *S. aureus/haemolyticus* group; grey bars= *S. epidermidis*.

3.4. Expression of *tst* in *S. aureus* in Women With and Without Bacterial Vaginosis

In order to investigate the ability of the different vaginal environments to influence the production of TSST-1, vaginal samples collected from non-pregnant, pre-menopausal subjects in London, ON were tested against cultures of the reporter strain *S. aureus* MN8/pAmilux-Ptst to monitor activity of the *tst* promoter (reflecting *tst* expression). Samples from all three vaginal states (healthy, intermediate and BV) were tested in this luminescence assay, and following 16 hours of growth, expression of *tst* in *S. aureus* was found to be completely suppressed in all samples, as illustrated in Figure 3.6A. In contrast, samples from two healthy participants were found to have no effect and only some inhibition on *tst* expression (Figure 3.6B and 3.6C, respectively). Total protein levels were determined via a bicinchoninic acid (BCA) assay in these two samples, as well as in three samples demonstrating strong inhibition. This was done to determine if the lack of suppression was due to an absence of protein in these 2 samples. Results showed similar protein levels in all the samples, regardless of anti-*tst* activity. The expression of *tst* occurred primarily at the post-exponential phase (approximately 10 hours) except for the luminescence seen in Figure 3.6B, in which expression began early and peaked at the mid-exponential point (6 hours). Luminescence graphs of *tst* promoter activity in response to samples from all other participants in the study are shown in Appendix 5.

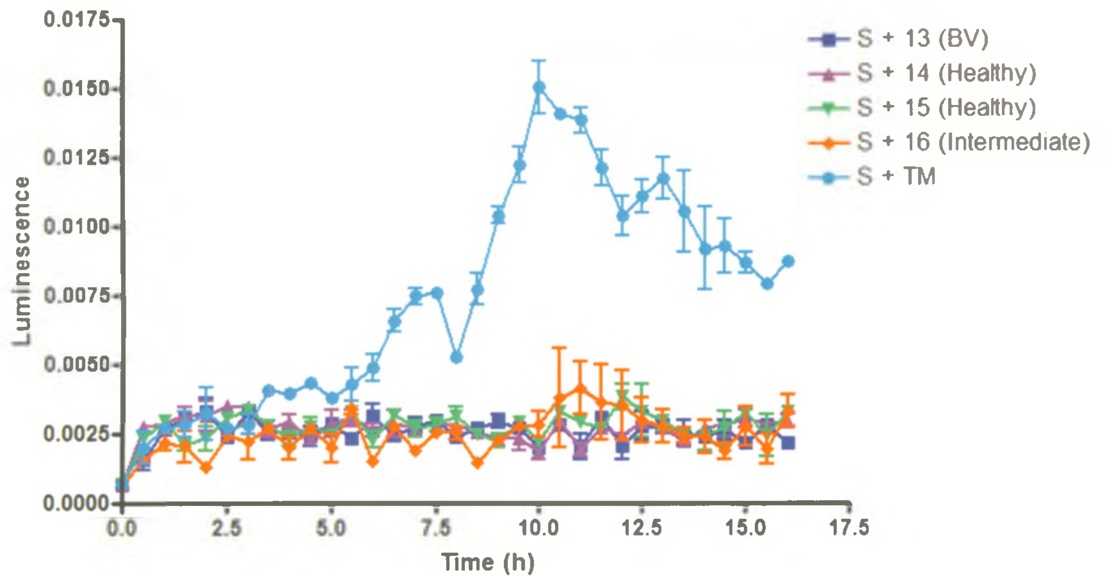

Figure 3.6A. Luminescence activity of *S. aureus* MN8 *tst* gene in response to vaginal swab contents from 4 different women of varying vaginal health (Participants 13-16) including healthy, intermediate and BV as determined through Nugent scoring. Toxin production was suppressed in response to all health groups. S=*Staphylococcus aureus* MN8; TM=Transport Medium, the medium in which the swabs were preserved.

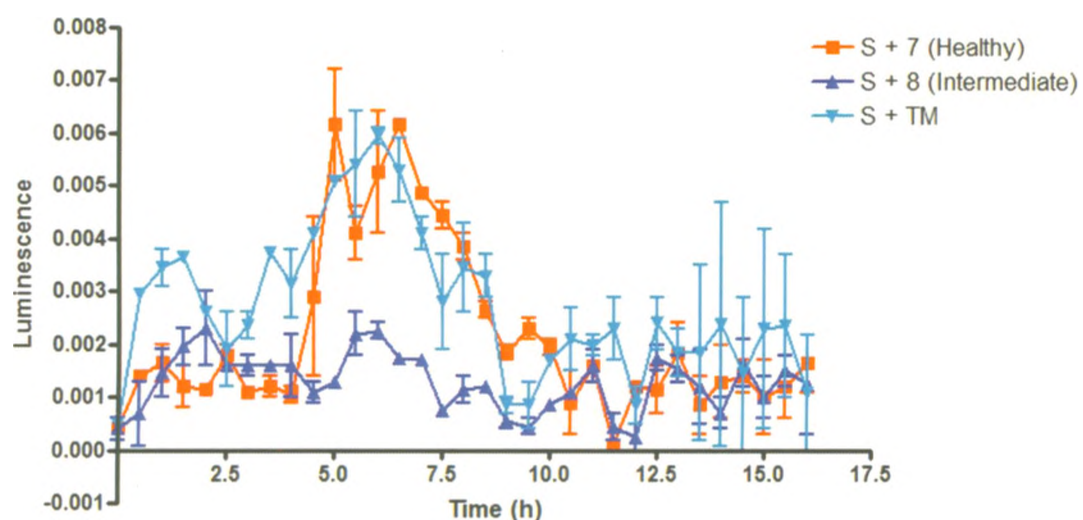

Figure 3.6B. Luminescence activity of *S. aureus* MN8 *tst* gene. Evidence of a lack of *tst* suppression from a healthy participant (Participant 7) during exponential phase (5-8hr). S=*Staphylococcus aureus* MN8; TM=Transport Medium, the medium in which the swabs were preserved.

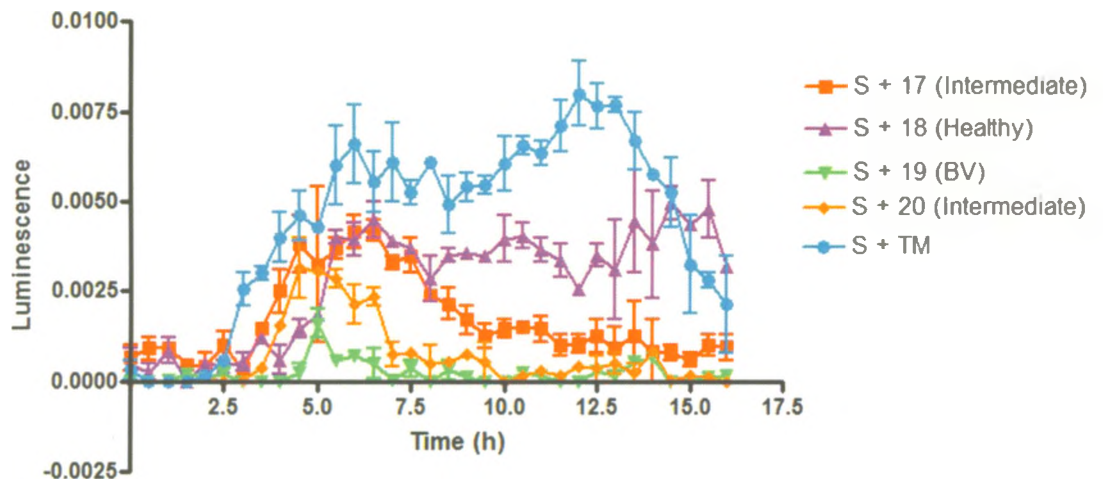

Figure 3.6C. Luminescence activity of *S. aureus* MN8 *tst* gene. Evidence of a lack of *tst* suppression from a healthy participant (Participant 18) during stationary phase (8-16hr). S=*Staphylococcus aureus* MN8; TM=Transport Medium, the medium in which the swabs were preserved.

3.5. TSST-1 Production in Response to Vaginal Bacteria Associated with Bacterial Vaginosis, Aerobic Vaginitis and a Healthy Microbiota

In order to investigate whether specific species found in the different vaginal microbiota samples have an influence on TSST-1 production, cultures of *S. aureus* MN8 were grown with supernatants of AV-associated bacteria, including *S. agalactiae*, *E. coli* and *E. faecalis*. The probiotic strain *L. reuteri* RC-14 was also included, as this strain has been shown to suppress TSST-1 production (Li *et al.*, 2011). Growth of *S. aureus* in these conditions was monitored and grew to similar OD_{600} values (Appendix 6). Proteins from these cultures were precipitated and TSST-1 detected on a nitrocellulose membrane to detect TSST-1 production semi-quantitatively in each of these conditions (Figure 3.7). The TSST-1 production in *S. aureus* MN8 varied depending on the supernatant in which it was grown. *Staphylococcus aureus*, when challenged with *S. agalactiae*, showed similar toxin levels to when it was grown with its own supernatant. The supernatant from *E. coli* suppressed TSST-1 production only slightly, while those from *E. faecalis* and *L. reuteri* RC-14 exhibited the greatest suppression of toxin production. These findings warranted further investigation into the influence of indigenous vaginal strains on TSST-1 production in *S. aureus*.

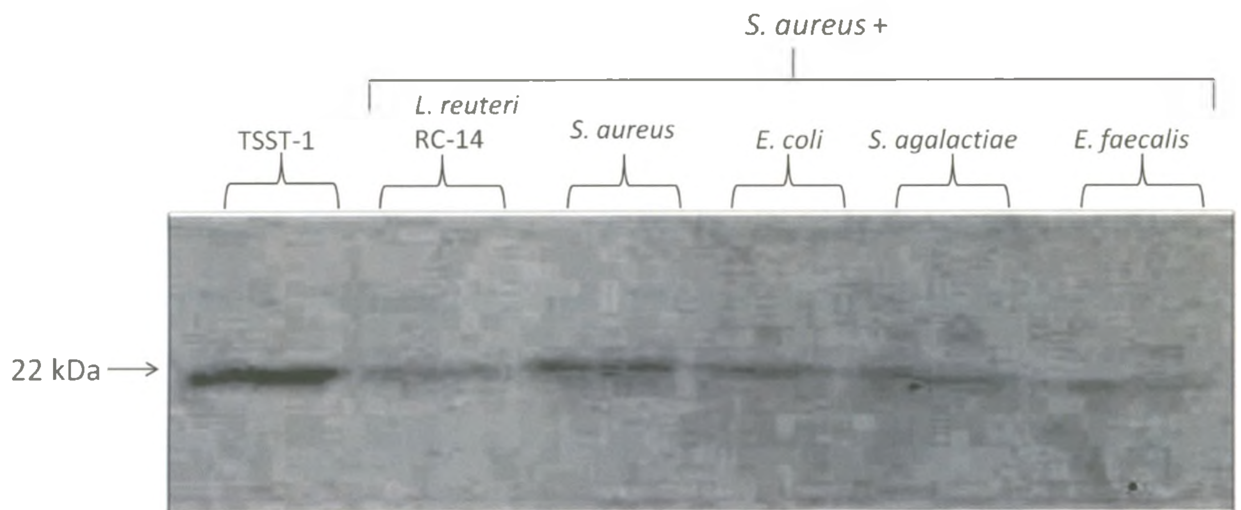

Figure 3.7. Western blot detecting TSST-1 production from S. aureus MN8 supernatants when challenged with supernatants of indicated bacteria.

The supernatant challenge experiment was repeated and *tst* expression in *S. aureus* MN8 was monitored by real-time PCR. Bacteria used to challenge *S. aureus* were separated into 3 groups for data analysis: lactobacilli, AV-associated and BV-associated (Table 2.5). Lactobacilli were found to significantly decrease *tst* expression (Figure 3.8A). *L. gasseri, L. jensenii* and *L. johnsonii* decreased *tst* expression by 76.15%, 82.67% and 67.15%, respectively ($p<0.05$). The probiotic strains *L. rhamnosus* GR-1 and *L. reuteri* RC-14 decreased expression by 78.51% and 83.78%, respectively ($p<0.05$).

In general, the AV-associated bacteria increased *tst* expression. Secreted products from *S. agalactiae* significantly increased *tst* expression by 509.15% relative to *S. aureus* grown in BHI ($p<0.05$) (Figure 3.8B). *E. faecalis* and *E. faecium* increased *tst* expression by 144.66% and 149.11%, respectively, while *E. coli* decreased expression by 36.91%. These latter changes in gene expression were not statistically significantly when compared to the control of *S. aureus* grown in BHI.

Finally, the BV-associated organisms slightly increased *tst* expression. In particular, *P. bivia* and *A. vaginae* increased expression by 190.58% and 334.06% (Figure 3.8C). This trend was not significantly different to the control of *S. aureus* grown in half BHI and half VDMP.

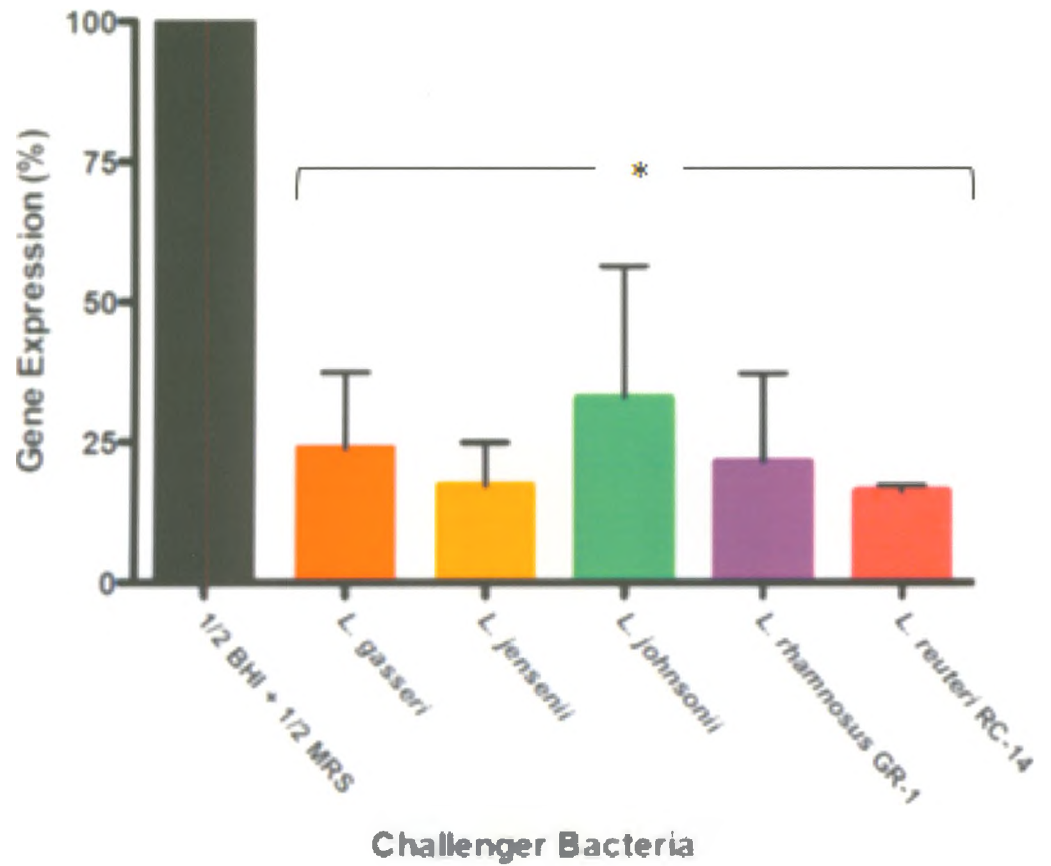

Figure 3.8A. Fold change in gene expression of *tst* in S. aureus MN8 in response to supernatants from lactobacilli, as detected by real-time PCR. ½ BHI + ½ MRS = growth media used. * indicates $p<0.05$

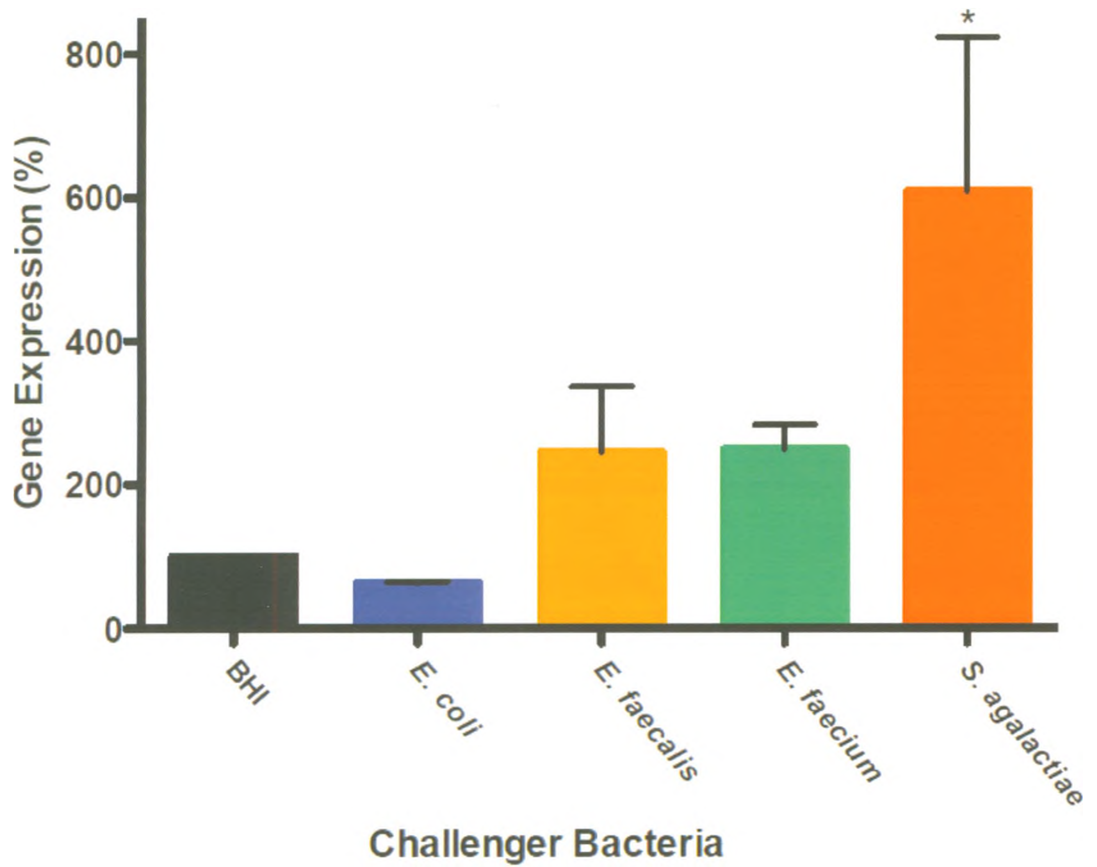

Figure 3.8B. Fold change in gene expression of *tst* in S. aureus MN8 in response to supernatants from AV-associated aerobic organisms, as detected by real-time PCR. BHI = growth media used. * indicates $p<0.05$

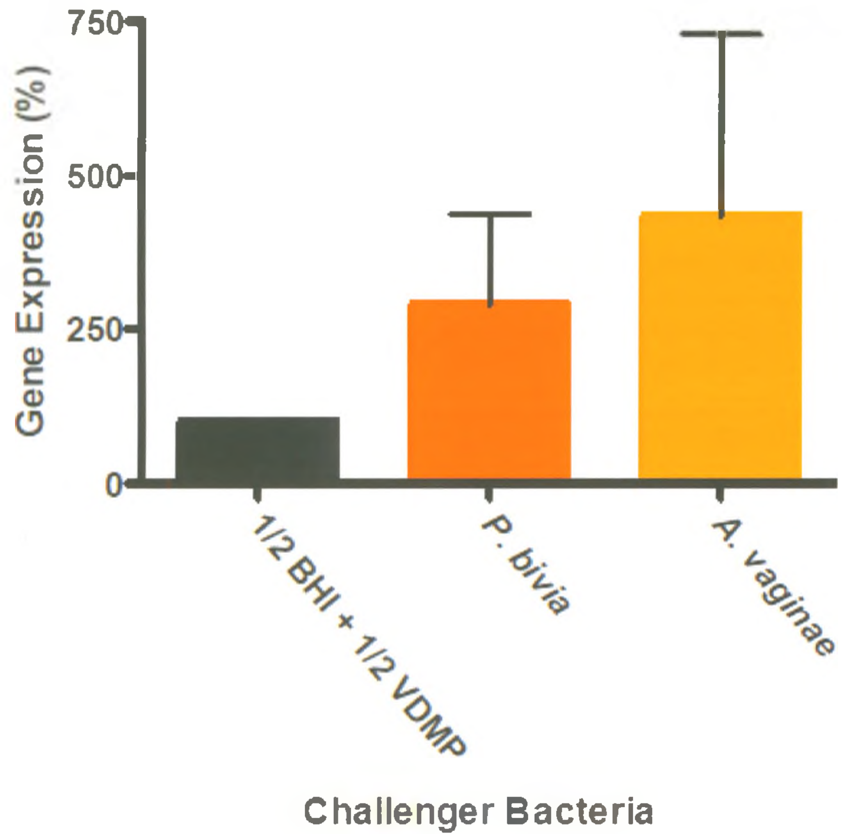

Figure 3.8C. Fold change in gene expression of *tst* in S. aureus MN8 in response to supernatants from BV-associated bacteria, as detected by real-time PCR. ½ BHI + ½ VDMP = growth media used. VDMP= Vaginally-Defined Medium + 0.5% Proteose Peptone.

Total protein expression from these challenger bacteria varied greatly in comparison to *S. aureus* as analyzed by SDS-PAGE (Figure 3.9). Among the aerobes, *E. faecium* was found to have the most complex protein secretion profile, while *E. coli* and *S. agalactiae* each showed high production of two distinct proteins (Figure 3.9A). The lactobacilli demonstrated strong production of certain proteins, seen at a mid-range size of approximately 50 kDa (Figure 3.9B). In particular, *L. crispatus* and *L. gasseri* showed a similar pattern of protein expression except for an additional protein of high production noted in *L. gasseri*.

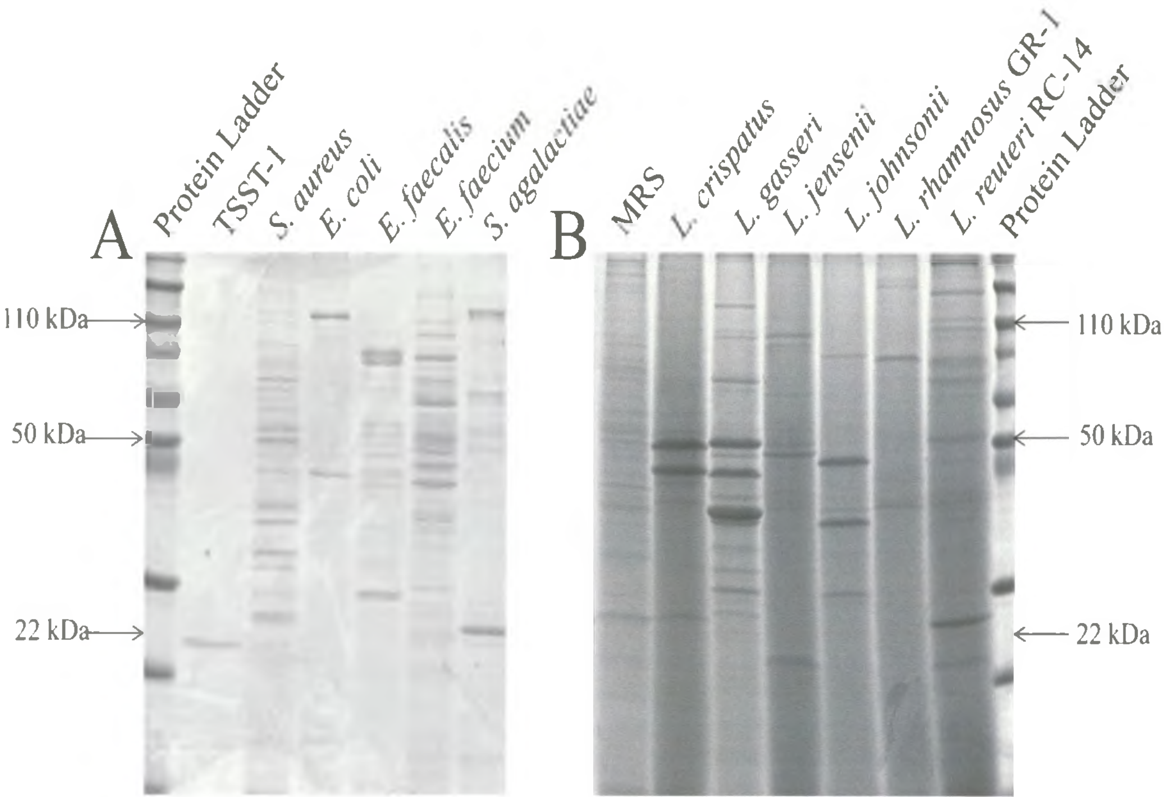

Figure 3.9. Total protein expression of bacteria used to challenge *S. aureus*, including aerobic bacteria (A) and anaerobic lactobacilli spp. (B), as determined through SDS-PAGE.

CHAPTER 4

DISCUSSION

4.1. Induction and Inhibition of TSST-1 by Vaginal Bacteria

To our knowledge, this is the first report of indigenous vaginal bacteria altering TSST-1 production in *S. aureus*. In particular, several species of *Lactobacillus* significantly suppressed *tst* expression, including *L. gasseri* and *L. jensenii* which are common vaginal residents, and *L. johnsonii* which is a gut commensal occasionally found in the vagina. We have also confirmed anti-TSST-1 activity from the vaginal probiotic strain *L. reuteri* RC-14, and showed that *L. rhamnosus* GR-1, another vaginal probiotic strain, was also capable of suppressing this toxin. Aerobic bacteria appeared to induce *tst* expression, particularly *S. agalactiae* (group B *Streptococcus*: GBS) which significantly induced expression by more than 5-fold. The BV-associated organisms tested, *P. bivia* and *A. vaginae*, showed slight up-regulation of *tst* expression. These results suggest that the vaginal microbiota can shape the virulence behavior of *S. aureus* and that a loss of lactobacilli may increase an individual's susceptibility to developing menstrual-toxic shock syndrome (TSS).

It is of interest to consider potential causative factor(s) from the bacteria responsible for altered *tst* expression. The pH of the challenger supernatants were recorded and adjusted to match the pH of the *S. aureus* culture, therefore excluding pH as a contributor to differential *tst* expression. The fact that the bacteria-free culture supernatants altered toxin expression in the challenge experiments suggests that the causative agent was a secretory compound rather than a cell surface one.

The expression of *tst* is under control of a two-component regulatory system (TCRS), and so it is possible that quorum sensing (QS) played a role in these reactions. Bacteria produce autoinducers (AIs) in the form of oligopeptides (Gram-positive bacteria) (Kleerebezem *et al.*, 1997) and acylated homoserine lactones (AHLs) (Gram-negative bacteria) (Fugua *et al.*, 2001) that are detected by the surrounding bacteria and regulate gene expression. In Gram-positive bacteria, oligopeptides are detected by a membrane-bound histidine protein kinase, which in turn activates an intracellular response regulator (Grebe & Stock, 1999). This QS system in *S. aureus* has been extensively studied for controlling the *agr* system in which *tst* expression is regulated (Cheung *et al.*, 2004).

Although most AIs are limited to intra-species communication, the AI-2 group has been identified as a family of compounds capable of inter-species signaling and is produced by both Gram-positive and Gram-negative bacteria (Schauder *et al.*, 2001). These compounds, synthesized by the LuxS enzyme from the precursor 4,5-dihydro-2,3-pentanedione (DPD), have been shown to alter virulence in *E. coli* and *S. pyogenes* (Lyon *et al.*, 2001; Sperandio *et al.*, 1999). A study by Xu *et al.* (2006) found that luxS/AI-2 signalling in *S. epidermidis* causes suppression of biofilm formation through reduced levels of exopolysaccharide adhesins, which subsequently reduced virulence in a rat model of infection. This signaling system also suppresses synthesis of capsular polysaccharide synthesis (CPS) in *S. aureus* (Zhao *et al.*, 2010). These effects of AI-2 on *Staphylococcus* spp. resemble the phenotypic changes following

activation of the *agr* QS system, in which extracellular adhesins are down-regulated and exotoxin production is increased. However, the luxS/AI-2 system does not seem to have an effect on the *agr* system in *S. aureus* (Doherty *et al.*, 2006), and so these compounds may act through alternate signaling pathways to affect toxin expression. These AI-2 compounds are thus possible causative factors for the altered *tst* expression seen in our experiments.

A recent study found that the AI-2 compounds secreted by *L reuteri* RC-14 do not have an effect on *tst* expression (Li *et al.*, 2011), which suggests that they are likely not responsible for the suppression seen in response to lactobacilli. However, AI-2 compounds secreted by the AV- and BV-associated bacteria tested in the present study should be considered, as these compounds may vary in structure depending on the producing organism and may have an influence on TSST-1 production.

Future experiments could include challenging *S. aureus* with isolated AI-2 compounds and monitor *tst* expression. However, because these compounds may vary in form depending on the producer strain and conditions, a single form may misrepresent the bacterial members being tested. The most effective approach would be to isolate the active AI-2 compounds from the challenger supernatants and test against *S. aureus* (Bassler & Surette, 2008). First, a genome survey would be conducted to determine the presence of the *luxS* gene in the challenger species. This would then be followed by a bioassay to confirm AI-2 activity from the supernatants, in which they would be added to the *Vibrio harveyi* BB170 AI-2 reporter strain to detect activation of *LuxS* (Bassler *et al.*,

1997). AI-2 activity would presumably occur as previous groups have confirmed this in cell-free supernatants of *E. coli* and *Streptococcus* spp. (Merritt *et al.*, 2003; Wang *et al.*, 2005). Mutant challenger bacterial strains lacking *luxS* could then be used to challenge *S. aureus* and observe toxin production, although the LuxS enzyme plays a role in the bacterial methyl cycle bacteria and so phenotypic changes to *S. aureus* could not definitively be attributed to reduced AI-2 levels from the challenger bacteria (De Keersmaecker *et al.*, 2006; Winzer *et al.*, 2002).

A possible explanation for the induced *tst* expression seen in response to the aerobic bacterial challengers is that these organisms secreted an array of compounds that mimicked the stationary phase of *S. aureus*. During stationary phase, the *agr* system of *S. aureus* promotes the release of exotoxins due partly to the build-up of AIs (Dunman *et al.*, 2001). Thus, since the aerobes in our study were grown into stationary phase (12 hours) prior to supernatant collection, introduction of *S. aureus* into these supernatants could have mimicked its own stationary phase to varying degrees, resulting in a range of *tst* induction by these bacteria. To determine the involvement of QS in the induction of this toxin, the real-time PCR assay could be used to monitor genes associated with TCRSs, including AgrAC, ArlRS and SrrAB. Linking these effects to QS would warrant investigation into the various oligopeptides produced from the aerobes, particularly *S. agalactiae*, *E. faecium* and *E. faecalis*.

The observation that *S. agalactiae* induces *tst* expression is particularly interesting considering the seemingly-symbiotic relationship between this species

and *S. aureus*. Multiple studies have found that *S. agalactiae* inhibits *Lactobacillus* spp. and *G. vaginalis* without inhibiting *S. aureus* (Carson *et al.*, 1997; Chaisilwattana & Monif, 1995; Monif, 1999). One study also found that *S. aureus* colonization was significantly associated with colonization by *S. agalactiae* in a population of pregnant women (Chen *et al.*, 2006). Our findings thus illustrate further ways in which GBS promotes the virulence of *S. aureus*. In the context of menstrual-TSS, women with AV who are colonized predominantly with these two species might be at higher risk of developing menstrual-TSS; elevated oxygen levels in the vagina would presumably induce TSST-1 production in *S. aureus* (Yarwood & Schlievert, 2000), and the presence of GBS may further perpetuate toxin production.

It is interesting that the *Lactobacillus* species tested (*L. jensenii*, *L. gasseri*, *L. johnsonii*) suppressed *tst* expression, considering recent findings that the vaginal probiotic strain *L. reuteri* RC-14 suppressed the toxin (Li *et al.*, 2011). This probiotic strain was included in the supernatant challenge experiment and its anti-*tst* effect was verified. Surprisingly, *L. rhamnosus* GR-1, another vaginal probiotic strain, also suppressed the toxin. A study by Laughton *et al.* (2006) found that this strain, in contrast to *L. reuteri* RC-14, was not able to suppress the RNAIII-initiating P3 promoter. This suggests that the inhibitory effect on *tst* seen by this strain was not due to inhibition of *agr* but perhaps from altered SarA activity, as this protein family has been shown to bind and activate the *tst* promoter directly and thus in an *agr*-independent pathway (Andrey *et al.*, 2010). Another regulatory system which seems to influence *tst* independently of the *agr*

system is glucose catabolite repression, in which the carbon catabolite protein A (CcpA) binds to catabolite-responsive elements (cre) in the *tst* promoter region and prevents *tst* expression (Seidl *et al.*, 2008). This represents the driving mechanism linked with glucose-mediated repression of TSST-1 production (Schlievert & Blomster, 1983). Thus, a possible cause of *tst* repression by *L. rhamnosus* GR-1, as well as by the other *Lactobacillus* strains tested, is the accumulation of glucose by the breakdown of carbohydrates in the culture media used (de Man, Rogosa and Sharpe [MRS]). To test for this, a Δ*ccpA* mutant strain of *S. aureus* MN8 could be utilized in which a partial loss of inhibition could be attributed to glucose repression.

The study by Li *et al.* (2011) isolated and identified two cyclic dipeptides, cyclo(L-Tyr-L-Pro) and cyclo(L-Phe-L-Pro), from *L. reuteri* RC-14 responsible for *tst* suppression. It would be worthwhile to screen the supernatants used in our study for the expression of these compounds. Furthermore, steps could be taken to deduce the compound(s) responsible, such as treating the supernatants with proteinase-K and trypsin to test if the compound(s) are proteins. Treatment with catalase would exclude hydrogen peroxide as the compound responsible, although another group has previously ruled out this compound for the suppression of the *agr* system (Laughton *et al.*, 2006). Separation techniques such as high-performance liquid chromatography (HPLC) could be utilized to separate the supernatants into fractions, testing them for anti-*tst* activity.

4.2. *S. aureus* TSST-1 Production in Aberrant States

We found that *S. aureus* colonization was no higher in women with BV than with healthy women. However, it is still important to determine the nature of TSST-1 production when in the vicinity of BV-associated bacteria. Mixed conditions have been noted that resemble an overlap of BV and AV, which would presumably have a higher likelihood of *S. aureus* being present (Donders *et al.*, 2002). As well, *G. vaginalis* and *P. bivia*, organisms associated with BV, have been shown to increase in numbers during menses in healthy individuals, and so it is important to predict how *S. aureus* would behave in response to these fluctuations (Eschenbach *et al.*, 2000; Srinivasan *et al.*, 2010).

The contents of vaginal swabs from the subjects recruited in London were introduced to cultures of the reporter strain *S. aureus* MN8/pAmilux-Ptst. Monitoring the luminescence produced from this strain allowed us to identify the nature of *tst* promoter activity (which reflects *tst* expression in the wild-type strain) in real time during the growth of *S. aureus*. It was discovered that samples from all three vaginal states in the sample pool (healthy, intermediate and BV) were able to significantly suppress *tst* expression, which suggests that these states have one or more compounds with anti-TSST-1 activity. The suppression seen in samples from healthy women could have been due to suppressive effects from lactobacilli, as the supernatant challenge assay of this study indicates inhibitory factors secreted by these bacteria.

The suppression of *tst* expression in response to BV samples was surprising, as these samples had greatly reduced lactobacilli numbers as well as pH's greater than 4.5, with some as high as 6.0, which is more conducive to TSST-1 production (Sarafian & Morse, 1987). However, the effect of pH would likely be minimized as the transport medium used to preserve contents of the swabs contained various buffering agents. Our results also suggest that BV-associated bacteria, as represented by *A. vaginae* and *P. bivia*, may induce TSST-1 production. Levels of oxygen and carbon dioxide would not likely contribute to suppression, as all samples were exposed to atmospheric conditions during collection and preparation. Therefore, it is possible that host derived factors were responsible for this effect, including immunological factors.

Zariffard *et al.* (2005) found that mucosal fluids from women with BV were able to induce Toll-like receptor (TLR)-2 mRNA expression as well as IL-1β and IL-6 production in peripheral-blood mononuclear cells. The TLR-2 recognizes lipoteichoic acid from the cell wall of Gram-positive bacteria, has anti-*S. aureus* activity, and responds to the bacteria through production of TNF-α, IL-1β and IL-6 (Knuefermann *et al.*, 2004; Takeuchi *et al.*, 2000). Thus, the BV samples from the current study may have had elevated cytokine levels from TLR-2 stimulation which altered *tst* promoter activity. Future experiments should utilize multiplex immunoassays to detect and correlate cytokine levels in these clinical samples with *tst* suppression.

Vaginal metabolites cannot be ruled out for the suppression effects. Future studies could reveal the complex profile of metabolites being produced from both

the microbiota and host tissue in samples from healthy women and those with BV. For instance, samples with elevated glucose levels may be better able to suppress *tst* suppression through the glucose catabolite repression system in *S. aureus*. The quantity and abundance of this sugar can be determined through commercial kits and high-throughput techniques, respectively, and correlated with anti-TSST-1 activity.

4.3. Prevalence of *S. aureus* and Other Bacteria Associated with Aberrant States

We also investigated the abundance of *S. aureus* in healthy and BV premenopausal women. To determine the most appropriate method of bacterial identification in vaginal samples, the traditional method of denaturing gradient gel electrophoresis (DGGE), which has been used by our group previously and is cost-effective, albeit laborious, was directly compared to 16S rRNA sequencing by Illumina.

Analysis by DGGE was effective at detecting *L. iners* and *G. vaginalis*. These were noted to be the two core members of the microbiota as determined through 16S Illumina sequencing (Hummelen *et al.*, 2010). However, DGGE only detected *L. crispatus* in one of the 22 samples, while sequencing by Illumina found this species in all samples. The bacterial diversity in BV samples versus healthy, as shown by 16S rRNA Illumina sequencing, was not apparent when analyzing by DGGE; the average number of species identified in healthy, intermediate and BV samples was 1.80, 1.80 and 1.89, respectively. This is in contrast to previous DGGE studies of the vaginal microbiota, in which many more

species were identified in BV samples (Heinemann & Reid, 2005; Vitali *et al.*, 2007). It is not clear why such diversity was not detected here.

Many of the sequenced DGGE amplicons contained numerous non-identifiable nucleotides which made it difficult to identify the corresponding species. This problem was likely due to the close proximity of some bands on the gel to one another, which can occur from two or more organisms with similar amplicon sequences and melting behaviour of the DNA strands. This would then lead to the excision of multiple bands and a mix of sequences.

Overall, DGGE was found to be a laborious process with limited identification power. In contrast, 16S rRNA sequencing by Illumina was much more effective at identifying the total bacterial composition. Consideration of financial expenses also favored sequencing by Illumina, as processing of multiple samples cost approximately $10/sample, while DGGE includes expenses associated with an additional set of PCR reactions as well as dideoxy chain termination sequencing. Therefore, comparison of these bacterial identification techniques warranted the use of 16S rRNA sequencing by Illumina for the remainder of the present study.

Vaginal bacterial communities from 21 premenopausal women in the London, ON area were determined through 16S rRNA sequencing by Illumina. Clinical examination determined that the vaginal condition was healthy in 9 subjects, while 7 had BV and 5 had an intermediate score based upon Nugent

scoring. Women with AV were not recruited, as this condition is less well known in the community and harder to identify as compared to BV.

Colonization by *S. aureus* was not evident in any of the women, which might be attributed to the relatively small sample size. Parsonnet *et al.* (2005) found *S. aureus* to be present vaginally in 9% of women. Based upon this finding, recruitment of 11 or more healthy women would likely be necessary to yield the detection of *S. aureus*. Interestingly, this species was also absent in the subjects with an intermediate and BV state, despite the lack of *Lactobacillus* species present in these samples and a concurrent reduction in anti-*S. aureus* activity (Charlier *et al.*, 2009; Ocana *et al.*, 1999). Thus, there was no evidence that *S. aureus* was more prevalent in women with BV versus without. The nature of these bacteria being facultative anaerobes permits some growth in anaerobic conditions, and some have been detected in samples from women with BV (Devillard *et al.*, 2005). However, bacterial growth would presumably be hindered in the strict anaerobic conditions of BV as compared to the micro-aerobic healthy environment.

This data set showed similar bacterial profiles to that seen in other studies, in that *L. iners*, *L. crispatus* and *G. vaginalis* made up the core members of the microbiota (Hummelen *et al.*, 2010; Zozaya-Hinchliffe *et al.*, 2010). However, the microbiota of pre-menopausal women varied from person to person, regardless of health status. For instance, 4 of the 5 intermediate samples consisted primarily of *L. crispatus* and *L. iners*, while the other was composed of *L. jensenii*, *L. gasseri/johnsonii* and *G. vaginalis*. As well, although healthy vaginal samples

typically contain less diverse populations compared to BV, very complex populations were seen in two healthy samples (17 and 12 species per sample) and one intermediate sample (11 species per sample) compared to the other samples (average of 3.6 species per sample). Members of these populations included *A. vaginae*, *P. bivia* and *Streptococcus* spp. Therefore, healthy women into which *S. aureus* colonize may conceivably already be colonized with a diverse population of bacteria, some of which may release compounds that induce TSST-1 production.

Another data set was analysed which included 74 pregnant women in Toronto, ON. Although the prevalence of AV in the general population is not well understood, the condition is thought to be more common in pregnant women, affecting approximately 10% of this population (Zodzika *et al.*, 2011), and so *S. aureus* was expected to be more prevalent in these subjects. In preparation for sequencing by Illumina, the sixth variable region (V6) of the 16S rRNA gene was chosen for amplification as this region is best for discriminating between different *Lactobacillus* species, which were the bacteria of primary interest at the time that this data set was initiated. As a result, some species of *Staphylococcus* could not be discriminated between, and *S. aureus* and *S. haemolyticus* were grouped together during analysis, while *S. epidermidis* was the other species identified.

Staphylococcus spp. were found in 13 of the 74 subjects (17.6%), and the *S. aureus/haemolyticus* group in particular was present in 11 of the 74 subjects (14.9%), which is higher than the reported 9% prevalence of *S. aureus* in healthy premenopausal women. This latter difference may be because of the inclusion of

S. haemolyticus which is known to be able to colonize the vagina (Iwantscheff *et al.*, 1985). Interestingly, this *Staphylococcus* group made up as much as 55% abundance of the vaginal microbiota of some of the subjects. To our knowledge, this is the first report identifying such *Staphylococcus* abundances relative to other bacterial constituents using high-throughput sequencing. It would be interesting to examine the abundance of *S. aureus* in healthy women compared to the onset of menstrual-TSS, as it might document whether an abundance threshold of *S. aureus* is a factor that predicates the condition. Such a study would be difficult given the unpredictability of TSS.

Through analysis of sequenced vaginal samples of healthy, intermediate, BV and AV-like women, *S. aureus* appeared to be more prevalent in AV-like states in pregnant women. However, larger sample sizes are needed to confirm this finding. In addition, diagnosis of AV should be established to make certain of the correlation. This would necessitate microscopic diagnosis where the following criteria are met: 1) absence of Gram-positive rods (lactobacilli) and replacement with bacteria of other morphology, 2) presence of leukocytes, 3) more than 50% of leukocytes being bloated with lysosomes (toxic leukocytes), 4) presence of Gram-positive single or chained cocci, and 5) the presence of small, rounded epithelial cells (parabasal epitheliocytes) (Donders *et al.*, 2011). In addition, a different region of the eubacterial 16S rRNA gene should be selected to discriminate *S. aureus* from other related species.

4.4. Future Directions

The present study reveals some insight into how the vaginal microbiota may affect TSST-1 production by *S. aureus*. This opens up a range of new areas of investigation into menstrual-TSS. The course of disease progression requires colonization by *S. aureus*, TSST-1 production and translocation of the toxin across the vaginal epithelium into the bloodstream. Future studies could include examining how certain conditions affect translocation of TSST-1.

Translocation of TSST-1 across the vaginal epithelium into the blood is an important step in TSS. Here, the superantigens (SAGs) elicit their pro-inflammatory effect on host immune cells. The toxin is recognized by epithelial cells and crosses the cell layer via transcytosis (Brosnahan *et al.*, 2008). An inflammatory process also plays a major contribution to TSST-1 translocation, as TSST-1 and α-toxin from *S. aureus* trigger the release of pro-inflammatory cytokines from the epithelial cells, damaging the integrity of the layer (Brosnahan *et al.*, 2009; Peterson *et al.*, 2005). It is conceivable that the vaginal microbiota can alter TSST-1 translocation, as many mucosal commensal bacteria are able to affect the integrity of the mucosal epithelial layer. Several studies have shown the ability of lactobacilli to improve mucosal epithelial integrity, through promoting translocation of occludin to the tight junctions, activation of Toll-like receptor (TLR)-2 signaling, and preventing epithelial damage produced by pro-inflammatory cytokines (Karczewski *et al.*, 2010; Resta-Lenert & Barrett, 2006; Rosenfeldt *et al.*, 2004). Therefore, a translocation experiment could be undertaken to investigate the effects of indigenous *Lactobacillus* spp. on TSST-1

translocation across the vaginal cell line VK-2, using a Transwell membrane filter system (Corning) to establish a cell monolayer. The effects of AV-associated bacteria could also be investigated, as the pro-inflammatory nature of AV suggests that these bacteria may cause epithelial layer damage and a significant increase in TSST-1 translocation. Finally, investigating the required number of TSST-1-producing colony-forming units to induce disease would allow for a more targeted approach to prophylaxis. For example, *S. aureus* that co-aggregate with lactobacilli on the epithelial cell surface may not produce much toxin, whereas in the presence of GBS it may take fewer staphylococci to trigger disease.

Staphylococcus aureus often colonize tissues in the form of a biofilm, consisting of a matrix of extracellular polymeric substance surrounding a microbial population. These *S. aureus* biofilms have been found in the fluid of menses and on the surface of used tampons (Veeh *et al.*, 2003), and *in vitro* studies have shown that these organisms attach well to tampon surfaces (Reid *et al.*, 1995). Given the number of bacterial species found in the vagina, it is likely that *S. aureus* are in biofilms with other species (Donlan, 2001). *In vitro* testing could be undertaken to assess the ability of *S. aureus* to co-aggregate with other urogenital strains and form a biofilm. If certain mixed biofilms, for example with *S. agalactiae*, provided a critical induction or stimulatory effect on *S. aureus* TSST-1 production, this might represent a risk factor for menstrual-TSS.

The influence of lactobacilli could also be tested against *S. aureus* biofilms. Our group has previously shown that the probiotic strains *L. reuteri* RC-14 and *L. rhamnosus* GR-1 are able to displace BV-associated biofilms made up

of *G. vaginalis* and *A. vaginae* (McMillan *et al.*, 2011; Saunders *et al.*, 2007), and to displace *S. aureus* from various polymer surfaces (Hawthorn & Reid, 1990; Reid & Tieszer, 1994; Velraeds *et al.*, 1998). These studies have shown that biosurfactants in the supernatants of lactobacilli and viable whole cells can reduce pathogen adhesion to surfaces and displace those that have attached. Of interest for a future study would be to investigate changes in the expression of genes necessary for biofilm formation in *S. aureus*, such as in the *lytSR* and *lrgAB* operon (Sharma-Kuinkel *et al.*, 2009) and the intercellular adhesion (*ica*) locus (Cramton *et al.*, 1999), which could all be monitored through real-time PCR. Understanding the nature of *S. aureus* biofilms in the vagina would give a better indication as to when an individual is at a higher risk of disease, as well as help develop new methods to control toxin production.

4.5. Conclusions

Our findings collectively suggest that women with AV who use tampons may be most susceptible to developing menstrual-TSS (Figure 4.1). This study showed that bacteria associated with AV (*Streptococcus agalactiae, Enterococcus faecalis* and *E. faecium*) directly increase TSST-1 production in *S. aureus*, and that a depletion of lactobacilli could remove otherwise suppressive effects on *S. aureus*. These microbial influences on TSST-1 production could conceivably augment the risk of toxin production from elevated oxygen levels and a neutral pH in the vagina of women with AV. Also, *S. aureus* would not only attach to the surface epithelial cells and ECM, but also colonize the tampon

fibers. Thus, *in vitro* testing is required to determine the virulence of *S. aureus* on tampon fibers when exposed to these various AV-like conditions.

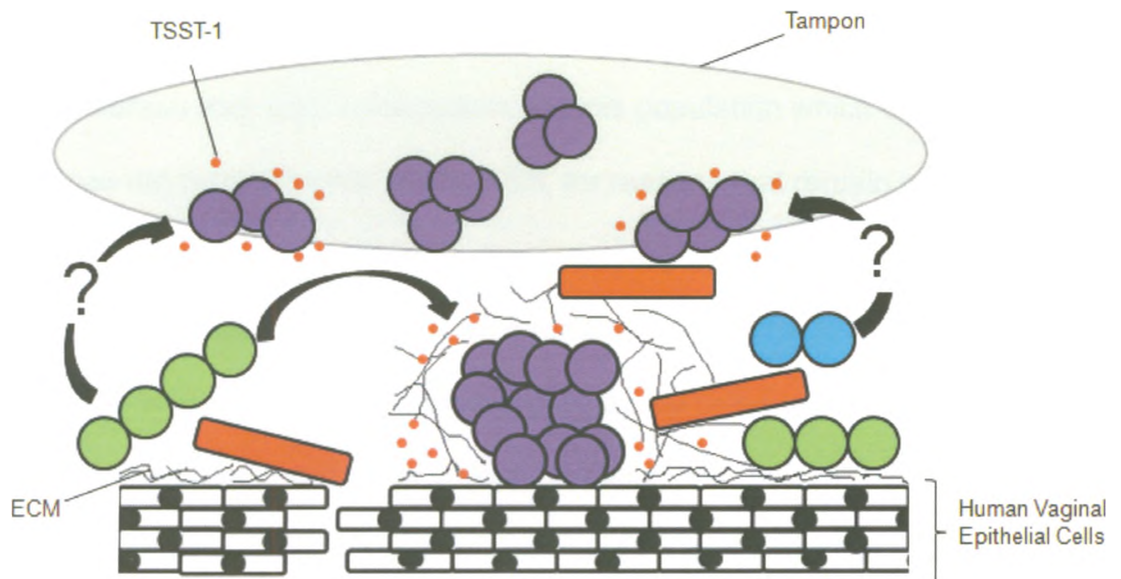

Figure 4.1. Schematic representation of *S. aureus* (purple cocci) colonization in an AV-environment, binding to the vaginal epithelial cells, ECM and tampon fibers. These bacteria are co-colonized with aerobic organisms including *E. coli* (red rods), *S. agalactiae* (green cocci) and *Enterococcus* spp. (blue cocci), of which the latter two secrete currently unidentified compound(s) that induce TSST-1 production. The combination of these secreted compounds, elevated oxygen levels, a neutral pH and a leaky vaginal epithelial layer induced by local inflammation may provide the necessary conditions for menstrual-TSS to occur in pre-menopausal women.

The BV vaginal environment seems to suppress *tst* expression despite the induction of this gene by *Atopobium vaginae* and *Prevotella bivia*. This suggests that some women with BV may have an aberrant yet somehow protective microbiota against TSS, but further research is required to explain this.

Aerobic vaginal environments are more often found in pregnant women, including *S. aureus* and GBS colonization, yet this population which does not use tampons does not have a higher rate of TSS, for reasons that remain unclear. Post-partum women who use tampons, however, may be most susceptible to menstrual-TSS, but reasons other than the tampon seem to be involved. For instance, these women appear to have a higher partial pressure of oxygen (Hill *et al.*, 2005), perhaps due to increased oxygen diffusion into the expanded vagina or post-partum haemorrhaging which occurs in 2-11% of deliveries (Gilbert *et al.*, 1987; Tamizian & Arulkumaran, 2001). Also, this group has a higher colonization rate by *S. aureus*, as suggested in a study which found 17% of post-partum women colonized versus 9% of all other premenopausal women (Linnemann *et al.*, 1982). Therefore, the use of probiotics to counter AV post-partum, especially those colonized with GBS, may be an effective approach to further reduce the rate of menstrual-TSS in our society.

CHAPTER 5

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5.0 References

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Appendix 1- UWO HSREB Full Board Submission Form

FORM 2F002 - UWO HSREB DELEGATED REVIEW (OCT 2008) *CONFIDENTIAL* PAGE 1

SECTION 1 PROTOCOL SUBMISSION REGISTRATION

Circle level of delegated review requested:
LEVEL 1 — Original + 1 copies
LEVEL 2 (default) — Original + 4 copies

1.1	Project Title
⇒ The role of vaginal bacteria in toxic shock syndrome.	

1.2	**Anticipated Project dates**	**Start Date**	June 2009
Ongoing databases/registries/banks will automatically be assigned a 5 year end date. A request for an end date extension can be submitted as the End Date approaches		**End Date**	June 2010

1.3	**Principal or Lead Investigator at this site.** (PI must be a faculty or staff member at UWO or affiliated institutions. Supervisor for student or resident projects must be a faculty or clinical advisor.)
Name	Dr. Gregor Reid
Title & Position	Assistant Director (International), Lawson Health Research Institute Professor, Microbiology & Immunology, and Surgery, UWO Director, Canadian Research and Development Centre for Probiotics
Degrees	Ph.D., M.B.A.
Departmental Affiliation	Microbiology and Immunology
Mailing Address — Building & Street Address	F2-116 Lawson Health Research Institute 268 Grosvenor St
Mailing Address — City, Province	London, ON
Mailing Address — Postal Code	N6A 4V2
Telephone	[redacted] Fax [redacted]
Email (required) For security purposes please provide your hospital, institute or UWO email address whenever possible.	[redacted]

1.4	-- Date: ____________ **Signature of Local Principal Investigator attesting that:**

a) all co-investigators have reviewed the protocol contents and are in agreement with the protocol as submitted;
b) all investigators have read the Tri-Council Policy Statement: Ethical Conduct in Research Involving Humans and the UWO Guidelines on Health Sciences Research Involving Human Subjects and agree to abide by the guidelines therein;
c) the investigator(s) will adhere to the Protocol and Consent Form as approved by the REB; and
d) the Principal Investigator will notify the REB of any changes or adverse events/experiences in a timely manner;
e) the researcher will not send nor collect data/specimens until contracts and/or data or material transfer agreements have been approved by the appropriate institutional officials;
f) if external regulatory approval is required, the investigators will not start the project or database/registry/bank until all approvals are in place

1.5 List all **local** co-investigators and collaborators. Include research personnel only if they have a significant role in the conduct of the research or database/registry/bank. Expand chart as required.

Name	Title/Position	Degrees	Role
Dr. John McCormick	Associate Professor	B.Sc., Ph.D.	Protocol development and analysis of samples
Dr Wayne Miller	Associate Scientist	B.A., B.Sc., Ph.D.	Protocol development and analysis of samples
Mr. Roderick MacPhee	Graduate Student	B.Sc.H.	Study Coordinator, Analysis of samples
Dr Jo-Anne Hammond	Assistant Professor Family Medicine UWO	MD	Recruitment of subjects
Ms Sarah Cribby	Lab Technician	B.Sc.	Analysis of samples
Mr. Ruben Hummelen	Graduate Student	B.Sc., M.Sc.	Clinical Assistance

1.6a	To whom should REB notices and correspondence regarding this protocol be sent – the PI or an Administrative Contact? *Note that this must be a local person within the institution. The Local Principal Investigator is ultimately responsible for all aspects of the project and is required to sign-off on all requests for changes and modifications to the protocol. In some instances the REB may override the naming of an administrative contact but will notify Principal Investigator of this determination and that materials will be sent directly to him/her instead.*	PI (default option)	
		Admin Contact	X
1.6b	If Administrative Contact selected provide name and contact information below.		

Contact Name	Roderick MacPhee
Title & Position	Graduate Student, Study Coordinator
Department	LHRI, Rm F3-127
Building & Street Address	SJHC, 268 Grosvenor St
City, Province	London, ON
Postal Code	N6A 4V2
Telephone	[redacted] Fax

Email (required) For security purposes please provide your hospital, institute or UWO email address whenever possible.	[redacted]

1.7	Will the research utilize patients (or their records), resources or staff at any of these sites? If YES to any item, please note that the UWO Office of Research Ethics will routinely share information as to the ethics approval status and other ethics-related or conduct-of-research issues of this submission with the LHRI Grants & Contracts Office. In some instances it may be necessary to also inform the LHSC/SJHC Privacy Office.	NONE of the following sites are involved with this research **(check all that apply)**	
		LHSC – Victoria Hospital - YES	
		LHSC – University Hospital - YES	
		LHSC – South Street Hospital - YES	
		LHSC - London Regional Cancer Program - YES	
		Children's Hospital of Western Ontario - YES	
		UWO Fowler Kennedy Clinic - YES	
		St. Joseph's Health Care London- YES	X
		Parkwood Hospital - YES	
		Regional Mental Health Care (London) - YES	
		Regional Mental Health Care (St Thomas) - YES	
		Byron Family Medical Centre - YES	X
		Victoria Family Medical Centre - YES	X
		St. Joseph's Family Medical Centre - YES	
1.8	Type of research activity Select all that apply	Chart or record review / data collection	
		Biological specimen collection and / or use	X
		Survey, interview, questionnaire, focus group	X
		Physiological or psychological testing	
		Registering a data base/registry/specimen bank	
		Other (specify)	X- Middlesex-London Health Unit

1.9a	Is this a student or resident project? i.e. Is completion of this research a requirement for a course, degree or training/placement program?	YES	X
		NO	
1.9b	If YES, please describe the course, degree or program (e.g. name of course, Honours BA paper, Masters or Ph.D. theses etc) and the student's role in the research (e.g. questionnaire design, data collection, interviews, data analyses etc).		

⇒ This study will be part of the thesis project for a Masters program in Microbiology and Immunology. The student, Roderick MacPhee, is acting as Study Coordinator and will play a role in various aspects of the study, including questionnaire design, participant recruitment and data analysis.

1.10a	Is this initiative funded?	YES	X
		NO	

1.10b	If YES, identify source of funds and name of person to whom the funds have been awarded.
Kimberly Clark Corporation, granted to Gregor Reid and John McCormick.	

1.11a	Was this research and/or database/registry/bank initiated from outside UWO or the hospital or research institute? e.g. a commercial sponsor; other health care centre, other researchers etc.	YES	
		NO	X
1.11b	If YES, identify the external parties and describe the relationship with the local researchers. Note; that research and databases/registries/banks sponsored and/or maintained by commercial sponsors maybe required to pay an ethics review fee. If the Office of Research Ethics thinks this is necessary you will be contacted for billing information.		

1.12a	Is there collaboration with a commercial sponsor or the possibility of commercialization?	YES	X
		NO	
1.12b	If YES, describe		
Study has been funded by Kimberly Clark Corporation, Neenah, WI. This clinical part is not likely to lead to anything commercial.			

1.13a	**REB Administration Fee** The University requires an administration fee for **industry funded** protocols submitted for ethical review. Please select billing option A) or B). *Note: It is ultimately the responsibility of the local investigator to ensure the fee is paid. In the event the Office of Research Ethics is not able to recover the fee in a timely manner from the party named below, an invoice will be sent to the Local Principal Investigator for payment. Failure on the part of the Sponsor or the Investigator to pay the fee in a timely manner may result in the withholding or withdrawal of ethics approval until such time as the matter is resolved.*		
A)	**The funds for this study will be administered by Lawson Health Research Institute (LHRI) The fee will be paid from a LHRI research account.** If NO, please provide complete billing information below in part "B".	**YES**	**X**
		NO	
B)	The funds for this study will not be administered by LHRI. Send invoice to:		

BILLING INFORMATION – COMPLETE ALL SECTIONS			
Company or Institution			
Contact Person			
Street Address			
City		Province / State	
Country		Postal Code	
Telephone Number		Fax	
CONTRACT &/OR PROTOCOL REFERENCE NUMBER REQUIRED To ensure the sponsor is able to match the invoice for the ethics administration fee with the contract you must provide the contract and/or protocol reference number.			

If you feel that due to extenuating circumstances the REB fee should be waived or adjusted, provide a brief written explanation to the Office of Research Ethics *prior* to the submission of this protocol. Include the following:

- Indicate how the funding will be used (budget)
- Identify who will own the data or any intellectual property arising from the agreement
- Indicate if there are any restrictions (e.g. publication delays) imposed upon the investigator by the sponsor and if so, what they are.

Do not assume that prior waivers or discounts will also apply to this submission (Email tfletch6@uwo.ca or dgrafton@uwo.ca or write to Office of Research Ethics Room SSB 4180 UWO)

1.13b | Conditions for Industry Funded Research

Investigators are reminded of the following requirements :

- all agreements and contracts must be approved by the appropriate research administration office for their institution prior to starting the study (e.g. LHRI Clinical Research Office of Grants and Contracts; UWO Offices of Research Development Services or Industry Liaison etc.);
- contracts and agreements must not put undue limitations on an investigator's right to publish;
- contracts and agreements must not prohibit a study investigator from informing research participants of any risks that may arise during a study;
- investigators and their staff are not permitted to accept 'finders fees' for subject recruitment, nor accept compensation for services rendered that is significantly greater than their normal wages or fees for time spent; and,
- research related expenses should be covered by the project Sponsor or other research funds not by OHIP, the participant's health insurance or the institution's operating budget.

1.14	**Conflict of Interest - General** This section to be completed for <u>all</u> submissions not just those funded by industry. Note also that this declaration applies to <u>all</u> co-investigators as well as the Principal Investigator.		
	Do any of the investigators or their immediate families have any proprietary interests in the product under study or the outcome of the research including patents, trademarks, copyrights and licensing agreements?	**YES**	
		NO	X

1.15	**Conflict of Interest - Industry Sponsored Protocols Only** Note also that this declaration applies to <u>all</u> co-investigators as well as the Principal Investigator.	**NOT APPLICABLE**	
		YES	**NO**
1.15a	Are any of the investigators or their immediate families receiving any personal remuneration (including investigator payments and recruitment incentives) from industry sponsors for taking part in this investigation?		X
1.15b	Is there any compensation for this study that is affected by the study outcome?		X
1.15c	Do any of the investigators or their immediate families have equity interest in the sponsoring company? (this does not include Mutual Funds)		X
1.15d	Do any of the investigators or their immediate families receive payments of other sorts from this sponsor (e.g. grants, compensation in the form of equipment or supplies, retainers for ongoing consultation and honoraria)?		X
1.15e	Are any of the investigators or their immediate families members of the sponsor s Board of Directors (or comparable body)?		X

1.16	If YES to any of the above in 1.14 or 1.15 please describe the arrangement and discuss the implications of a potential conflict of interest. If the conflict of interest cannot be eliminated, what the conflict is and how that conflict is being managed should be disclosed in the Letter of Information. The document should explain what additional protections have been put in place to protect the study subject.
⇒	

1.17	Does this submission involve the **creation or approval** of formal and ongoing databases, registries or specimen banks? This does not include one-time data collection projects or the use of existing data bases, registries or specimen banks.	YES	
		NO	X

IF NO, GO TO SECTION 2

1.18	Describe what type of approval you are seeking with this application (*check all that are*	Collection of **local** data/specimens for ***locally maintained*** database/registry/bank	
		Collection of **local** data/specimens for ***off site*** database/registry/bank	

	applicable) **"Locally maintained" or "local database"** refers to databases etc located at UWO or its affiliated hospitals or research institutes.	Establishment of **local** database/registry/bank that will contain ***local data only***	
		Establishment of a London-based multi-site database/registry/bank that will contain data/specimens from multiple sites (local and/or non-local). Non-local sites must obtain REB approval from their own institution prior to submitting data	
		Other (explain)	

1.19	Indicate which of the options best describe this database/registry/bank	NEW Clinical database/registry that is also to be used for research	
		PRE-EXISTING Clinical database/registry that will now also be used for research	
		NEW Research only database/registry/bank	
		PRE-EXISTING Research only database/registry/bank	
		Other (specify)	

1.20	Who owns the database/ registry/ bank? Who is responsible for ongoing data or specimen stewardship? E.g. Is there a Management or Steering Committee.? If so describe the membership, role and responsibilities of the committee.

⇒

1.21a	Will data or specimens be made available to other researchers in the future?	YES	
		NO	
1.21b	If YES, what is the process for determining who may access data/specimens for **future** research?		
⇒			
1.21c	If YES, will researchers who are granted permission to access the data or specimens in the future have access to personal identifiable information?	YES	
		NO	
If YES, it must be made very clear in the Letter of Information/Consent form that researchers in the future may have access to identifiable data. If the database is maintained locally, the UWO HSREB must approve subsequent uses of the data when identifiable data is included.			

SECTION 2 PROJECT DESCRIPTION

Complete each section where indicated. Do not direct the reviewer to 'SEE ATTACHED'. Your protocol will be RETURNED UNREVIEWED if the project description information is incomplete, illegible or improperly filled out.

2.1a	Provide a brief one or two sentence overview of the proposed research or database/registry/bank describing the population and purpose. *e.g. Charts of patients less than 3 months of age presenting to the CHWO Emergency Department with a rash will be reviewed to examine possible seasonal variations. e.g. - Clinical data from breast cancer patients will be amalgamated into a provincial database called BRCAData to facilitate assessment of clinical trial outcomes in breast cancer patients. This ongoing database is located at Princess Margaret Hospital in Toronto and is owned by OCRN*

⇒ **Seven vaginal swabs will be collected from 20 premenopausal women: 10 healthy women and 10 with bacterial vaginosis. These swabs will collectively be used for assessing the effects of the vaginal microbiota on toxic shock syndrome toxin (TSST-1) regulation in *Staphylococcus aureus* MN8.**

2.1b	Provide **KEYWORDS** about the research. (Max 5)	**Bacterial vaginosis, toxic shock , microbiota**

2.2	**Background, Justification & Objectives and Hypotheses:** Provide a clear statement of the purpose and objectives of the research or database/registry/bank. (i.e. Why is the research being done or the database established?) (1 page maximum – adhere to page limitations) References may be appended to this section and need not heed the 1 page maximum

⇒ Menstrual-associated toxic shock syndrome (TSS) became prominent in the early 1980s when a significant number of TSS cases occurred in otherwise healthy young women using high absorbency tampons (1). The staphylococcal superantigen (SAg) TSST-1 is believed to be responsible for essentially all menstrual-associated TSS cases (2). This is likely related to the apparent unique ability of this particular SAg to cross mucosal barriers (3). Although there are key host and environmental factors known to influence the development of staphylococcal TSS, including elevated protein, CO_2 and O_2, and neutral pH (4), there are essentially no mechanistic studies relating to how the vaginal microbiota influences the development of this potentially fatal disease.

In this study we will examine the microbiota of human vaginal samples in order to correlate regulation of TSST-1 production by *S. aureus* and presence of particular bacterial species.

Objective: To examine to role of resident vaginal microorganisms in TSST-1 expression by *S. aureus*. Vaginal samples will be obtained from 10 healthy, pre-menopausal women and 10 with bacterial vaginosis as determined by Nugent scoring. Contents of the swabs will be identified and examined for their ability to affect the expression of TSST-1 toxin by *S. aureus* MN8.

Hypothesis: A "healthy" vaginal microecology will inhibit expression of TSST-1 by *S. aureus*, whereas an imbalance in the vaginal microecology will promote increased expression of TSST-1.

2.3	**Methodology** - Describe the study design or data collection process and describe in detail what will be done to the participants and/or their data or specimens Investigators are encouraged to use flow charts or diagrams in their descriptions. (2 page maximum – adhere to page limitations)

⇒ In this study, samples from 20 premenopausal women will be included in data analysis; 10 with bacterial vaginosis and a vaginal pH greater than 4.5, and 10 healthy women with a vaginal pH less than 4.5. It is expected that as many as three women will need to be screened for every woman that satisfies the above criteria (expected total of 60 participants). Seven vaginal swabs will be collected from the posterior fornix of the vaginal tract of each participant through the use of a speculum. These swabs will be used (1) for the diagnosis of BV through Nugent scoring, (2) identification of bacterial species present in the vagina, (3) co-culturing with *Staphylococcus aureus* MN8 to assess effects on toxic shock syndrome toxin (TSST-1) regulation, (4) isolation of lactobacilli to study effects of these species on TSST-1 transport across vaginal epithelial cells, isolation of (5) bacterial and (6) vaginal epithelial cell mRNA for gene expression analysis, and (7) for measurement of vaginal acidity. Swabs will be stored on ice for transport to LHRI.

The vaginal microbiota of each participant will be determined as follows. DNA will be isolated from one swab from each participant by suspending the swab in PBS, followed by DNA extraction using a commercial DNA extraction kit. Eubacterial primers will be used for PCR amplification of 16S rRNA genes and the PCR products will be separated by Denaturing Gradient Gel Electrophoresis (DGGE)(5). After staining and visualization of the gels, DGGE fragments will be excised from the gel, the DNA contained within the fragments will be re-amplified by PCR, and the resultant PCR products will be purified and sequenced for identification of bacterial species represented by each fragment.

Microorganisms will be isolated from a swab from each participant and added to *S. aureus* MN8 cultures. Expression of TSST-1 in each culture will be determined by Western immunoblotting using a TSST-1-specific antibody, and by quantitative RT-PCR analysis of transcriptional levels of *tst* (encoding TSST-1).

Literature Cited

1. **McCormick, J. K., J. M. Yarwood, and P. M. Schlievert.** 2001. Toxic shock syndrome and bacterial superantigens: an update. Annu. Rev. Microbiol. **55**:77-104.
2. **Bergdoll, M. S., and P. M. Schlievert.** 1984. Toxic-shock syndrome toxin. Lancet **ii**:691.
3. **Schlievert, P. M., L. M. Jablonski, M. Roggiani, I. Sadler, S. Callantine, D. T. Mitchell, D. H. Ohlendorf, and G. A. Bohach.** 2000. Pyrogenic toxin superantigen site specificity in toxic shock syndrome and food poisoning in animals. Infect. Immun. **68**:3630-4.
4. **McCormick, J. K., J. M. Yarwood, and P. M. Schlievert.** 2001. Toxic shock syndrome and bacterial superantigens: an update. Annu Rev Microbiol **55**:77-104.
5. **Burton, J. P., and G. Reid.** 2002. Evaluation of the bacterial vaginal flora of 20 postmenopausal women by direct (Nugent score) and molecular (polymerase chain reaction and denaturing gradient gel electrophoresis) techniques. J. Infect. Dis. **186**:1770-80.

2.4a	Will any testing using an xray, CT, MRI or ultrasound be done?	YES	
		NO	X

2.4b	If YES provide a complete description of the testing and describe the exposure and give an assessment of risk. • If an MRI is being used UWO HSREB wording MUST be included in the Informed Consent documentation. - see HSREB Guidelines 2-G-0004 (formerly Appendix 3) • **http://www.uwo.ca/research/ethics/med/hsreb-guidelines.htm** • If radiological testing or therapy (including Xray, CT, MRI, ultrasound etc) is being used, researchers may find the following website a useful resource in determining the appropriate language for Informed Consent documentation to explain these procedures and their risks to participants **http://www.radiologyinfo.org/**

⇒

2.5a	**Number of local subjects or records** For databases/registries indicate anticipated number of subjects **from this centre** or give approximate numbers to be included each year.	60
	Total number of subjects or records. For databases/registries indicate anticipated number of subjects in **entire database/registry/bank** or give approximate numbers to be included each year	60
	Number of sites or centres participating.	
2.5b	**Justify the sample size and/or provide sample size calculation.** If a *formal sample size calculation was not used* give a rationale for the proposed number of subjects; charts or records to be reviewed; or specimens collected.	

⇒ Due to the specificity of pH values included in the inclusion criteria, it is predicted that vaginal swabs from only 1 in every 3 participants will be eligible for further analysis. Therefore, recruitment of 60 participants should fulfill the study of vaginal microbiota from 20 women.

SECTION 3 RESEARCH PARTICIPANTS

3.1a	Will the study, database/registry/bank involve data or samples from males and females?	MALES	
		FEMALES	X
3.1b	If NO, explain why only one gender is being selected. (e.g. condition under study is gender specific)		

⇒ Only females will be studied because we are looking at differences of the vaginal flora

3.2	What is the age range of the participants who will participate in the research or from whom the data or specimens are obtained?	LOWER AGE LIMIT	18
		UPPER AGE LIMIT	40

3.3	Participant **Inclusion** Criteria: Premenopausal females aged 18-40.

⇒

3 4	Participant **Exclusion** Criteria (if applicable).

⇒ Pregnant; sexually transmitted infection (specifically, gonorrhoea or chlamydia); abnormal renal function or pyelonephritis; are receiving prednisone or immunosuppressive drugs, are receiving treatment for any urogenital infection or are taking any antimicrobial therapy: have a personal history of known or suspected estrogen-dependent neoplasia such as breast or endometrial cancer; have undiagnosed abnormal vaginal bleeding; had sexual intercourse within the past 48 hours; have taken any 'acidophilus' or probiotic health food supplements in the past 48 hours; are menstruating at time of clinical visit; had any urogenital infections in the past 6 months.

3 5a	Will the participants be current or ongoing hospital or clinic patients or clients?	YES	
		NO	X
3 5b	If YES, list all procedures, tests, drugs etc. utilized for the purpose of this study which are ***not part of ordinarily accepted care*** of the patient. If additional or extended hospitalization or outpatient visits are required include the number of days or visits.		

⇒

3.6	**Delays or withholding of standard care**	NOT APPLICABLE STUDY DOES NOT INVOLVE PATIENTS	
3 6a	Are any standard therapies or diagnostic procedures to be withheld during the course of the study?	YES	
		NO	X
3.6b	Will management or treatment of the participant's condition be prolonged or delayed because of the research? Will a placebo be used in lieu of standard care?	YES	
		NO	X
3.6c	If YES to any of the above, discuss the potential risks and benefits to the participants and provide a rationale why standard care must be withheld or delayed		
⇒			

3.7	**Deception or partial disclosure** This section refers to instances of deliberate deception or the withholding of key information that may influence a participant's performance or responses.		
3.7a	Do any of the procedures in this study include the use of this type of deception or partial disclosure of information to participants?	YES	
		NO	X
3.7b	If YES, provide a rationale for the planned deception or partial disclosure.		
⇒			
3.7c	If YES, describe the procedures for a) debriefing the participants and b) giving them a second opportunity to consent to participate after debriefing. If debriefing and reconsent are not viable options please explain.		
⇒			

3 8a	Will the participants be compensated or reimbursed for their time, expenses	YES	X

	and/or contribution to the research?	NO	
3.8b	If YES, provide details. Specify the amount, what the compensation or reimbursement is for, and how payment will be determined for participants who do not complete the study. This information must be included in the Information/Consent documentation. At a minimum, researchers are strongly urged to cover parking and other incidental costs if participants are required to go to the hospital outside of normal appointments.		

⇒ Participants will receive $50 for parking and travel to the clinic.

SECTION 4 BIOLOGICAL SPECIMENS (e.g. blood, tissue, muscle biopsies or tumor samples

4.1a	Are biological specimens (e.g. blood, tissue, muscle biopsies or tumor samples) to be taken or analyzed for the purposes of **this** research protocol?	YES	X
		NO	
4.1b	Are biological specimens being collected for **future** genetic testing or other unspecified testing or studies?	YES	
		NO	X
If YES to either 4.1a or 4.1b complete the balance of this section. If NO to both 4.1a and 4.1b go Section 5.			

4.2a	Describe facilities and procedures to protect the ***physical*** comfort and safety of the participants from whom samples will be taken. If specimens have been collected previously and stored, indicate "Not Applicable". In the case of invasive sampling e.g. taking blood, biopsies indicate who will take the sample and give their qualifications to do so.		
⇒The family doctor or a nurse will collect the samples in the confines and privacy of a treatment room		Not applicable	

4.3a	Describe what specimens will be taken and what they will be used for. In the case of blood samples also provide the total amount of blood that will be taken
⇒ **Vaginal swabs.** Swabs will first be analyzed for diagnosis of BV or a healthy vaginal microbiota using Nugent scoring. Another swab will be used to determine pH of vaginal fluid. Contents of the swabs will be applied in a blinded fashion to *S. aureus* MN8 cultures, and both protein and transcriptional levels of this toxic shock toxin will be examined in *S. aureus*. The microbiota of the swabs will be identified by 16s rRNA denaturing gradient gel electrophoresis (DGGE). In this way, we will determine any correlation between an aberrant aerobic or anaerobic microbiota and increased expression of TSST-1, as well as whether a healthy vaginal sample with predominant lactobacilli content, is able to inhibit TSST-1. One swab will also be used for the collection of lactobacilli species to study the effect of these bacteria on the transport of the TSST-1 toxin across vaginal epithelial cells. Two other swabs will be used for the isolation of bacterial and vaginal epithelial cell mRNA for analysis of gene expression. Data collected from these swabs will thus expand upon the activity of these bacteria and vaginal epithelial cells, and how this activity may influence the regulation of TSST-1 production by *Staphylococcus aureus* MN8.	
4.3b	Explain who will control or own the specimens?

⇒ Dr. Reid will control the specimens until the experiments have been performed.

4.3c	Explain how and where the specimens will be stored.

⇒ The specimens will only be stored in the fridge for a day until processing. Bacterial DNA will be used for gel electrophoresis study.

4.3d	Describe how long the specimens will be retained and how they will be destroyed.

⇒ The swabs will be destroyed within one week of collection as per standard waste procedures in the hospital.

4.4a	What was the **original** purpose or use of the tissue or specimens?	Collected specifically for research purposes	X
		Originally collected for diagnostic purposes	
		No purpose or use - unwanted or discarded tissue or biomaterials	
4.4b	The subsequent use of tissue or biomaterials (except blood) **originally collected for diagnostic purposes** must, be approved by the Department of Pathology Tissue Use Committee prior to submission to the HSREB and a copy of their approval appended to this form. If the Tissue Committee approval is not available at the time of submission to the HSREB, ethics approval will be withheld until a copy of Tissue Committee approval/registration is received.		
	Tissue Use Committee approval	Not applicable	X
		Pending	
		Approval attached	

SECTION 5 DATA COLLECTION

5.1	Are any data, other than biological specimens, to be collected? e.g. test results, surveys, questionnaires, hospital chart information etc.	YES	X
		NO	

If YES complete the balance of this section - If NO go to Section 6

5.2	What is the source of that data?	Patient HOSPITAL chart or clinical record	
		Other HOSPITAL departmental records e.g. Pharmacy, Physiotherapy, Laboratory	
		Directly from research participant or other informant	X
		NON-HOSPITAL clinician records e.g. Family Physician office	
		NON-HOSPITAL laboratories or treatment sites e.g. physiotherapy clinic, laboratory	
		Departmental or clinic (clinical) database	
		Other (specify)	

5.3	List all data collection instruments and forms to be used in this research. e.g. Chart abstraction sheets, questionnaires, surveys, interview outlines etc. If there are no forms, you must provide a comprehensive list of data to be collected or topics to be covered. Do not insert questionnaires etc into the body of this applications; append a copy of all forms at the end this application.
⇒ Questionnaire- Appendix C	

SECTION 6 RISKS AND BENEFITS OF THE RESEARCH

6.1	**RISKS, HARMS & DISCOMFORTS:** Discuss the risks of the proposed research and/or database/registry/bank. Even though patients may not be involved directly as in the case of records review or previously collected specimens, it is important, in addition to physical harms, to consider possible psychological, emotional, social and economic harms and stressors and intrusions on privacy.
⇒ There will be no risk or harm to participants, although they may experience some discomfort associated with collection of vaginal swabs. Any such discomfort will be minimal and for a short period of time.	

6.2	**BENEFITS:** Discuss any possible direct benefits to the research participants as a result of their participation in the study. Please note that monetary compensation is not considered a benefit. If no direct benefits are apparent, discuss possible benefits to society at large or to the patient/participant population being studied.
⇒ Toxic shock syndrome still kills and afflicts a considerable number of people, mainly women, each year. The subjects will have the satisfaction of knowing that their provision of samples could help identify new methods to control and prevent this condition.	

6.3	The TCPS requires that researchers propose a continuing review process appropriate for the risk of this submission. The higher the risk the more frequent the review. **Please indicate your recommendation as to the frequency of the REB's continuing review.** At a minimum, all protocols continuing past 12 months will require the completion of the REB's Surveillance Report Form annually.	ANNUAL (default)	X
		EVERY 6 MONTHS	
		EVERY 3 MONTHS	
		EVERY MONTH	

SECTION 7 PRIVACY & CONFIDENTIALITY ISSUES

LHSC and SJHC require that all persons accessing patient information complete the hospital Privacy & Confidentiality Education Program. Contact the LHSC-SJHC Privacy Office for more information.

If research data are lost, stolen or accessed inappropriately, it must be reported to the HSREB immediately. If the data relate to hospital patients or records, a report must also be made to the hospital's Privacy Office.

7.1a	Indicate if any of the following **personal identifiers** will be ***collected*** for research purposes during the course of the research. (Excluding the consent form which will contain the participant's name.) If any are to be collected, indicate which will be ***retained*** **(upon study completion)** with the research data set or biological specimen once data or biological specimen collection is complete? Indicate "Retained" if there will be a Master list kept after data collection is complete that links participant identifiers to de-identified data.	Researchers may find it helpful to consult the CIHR Best Practices for Protecting Privacy in Health Research. http://www.cihr-irsc.gc.ca/e/29138.html	**Collected**	**Retained**
		No personal identifiers	X	X
		Full or Partial Name or Initials		
		Location or Contact info: address, phone, postal code etc		
		Full or Partial Date of Birth or Death		
		Personal Numbers: e.g. OHIP Health Card, SIN		
		Institutional / Hospital Chart or Record #		
		Facilities and service providers		
		Other personal identifiers(specify)		
7.1b	IF ANY OF THE ABOVE IDENTIFIERS WILL BE **COLLECTED** give the level of detail to be collected. E.g. full name or initials only; full date of birth or year only; full postal address or 3 digit postal code; names of service providers or type of institution only etc.			

⇒ Samples will be number coded by pharmacy at St. Joseph's Health Centre and no investigator will be privy to the information. The nurse or physician collecting the swabs will place the swabs in pre-marked vials (1, 2, 3, 4 …). The receiving technician will not know the name of the subject nor if she is healthy or has BV. Of note, it is possible that the receiving technician will have a good idea which sample is from a BV subject (based upon experimentation), but this cannot influence the ultimate results as these consist of simply documenting which bacteria are present, and how *S. aureus* reacts to the flora.

7.1c	IF ANY OF THE ABOVE IDENTIFIERS WILL BE **COLLECTED** provide a comprehensive rationale explaining why it is necessary to collect this information. IF ANY OF THE ABOVE IDENTIFIERS WILL BE **RETAINED** once data collection is complete provide a comprehensive rationale explaining why it is necessary to retain this information. (Including the retention of master lists that link participant identifiers with de-identified data.) Acceptable reasons will generally be limited to the following purpose and investigators will need to defend their reasons for collecting and/or retaining identifiers and how the identifiers will be used to achieve the stated purpose. • Contact or linkage for follow up or ongoing data collection • Provide data for clinical monitoring of the participant • Enable data to be withdrawn from data set if participant withdraws consent • Return individual results to participant • Conduct a data linkage with a high degree of accuracy Do not just copy one of the above reasons into the box below. Investigators must, in their own words, explain fully and defend their reasons for collecting and/or retaining identifiers and explain how the identifiers will be used to achieve the stated purpose.
⇒	

7.2	In addition to the UWO HSREB, identify all agencies or individuals other than the local research team who, for **monitoring or auditing purposes**, may require access to identifiable or confidential data collected for this research or database/registry/bank, now or in the future. e.g. the Sponsor(s),CRO's, regulatory agencies such as Health Canada or the FDA etc. Include the following sentence into all recruitment informed consent materials where the participant's identity is known and access to the records or follow up by the HSREB is possible. "Representatives of The University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of the research."
⇒ None	

7.3	Describe the procedures to be used for preserving the confidentiality of data or specimens both during the data or specimen collection and in the release of the findings. e.g. all identifiers removed once data collected, data coded by unique identifiers with master list held separate from data etc. If a device has a serial or code number that will allow the sponsor to identify individual patients this should be noted in the Letter of Information.
⇒	

7.4a	Will anyone other than employees, clinical staff or students of the institution where the patients' original records or samples are located, approach participants or have direct access to a subject or their records for purposes of collecting data or conducting this research?	YES	
		NO	X
7.4b	If YES indicate who these people are, what their role is, why they need access and what safeguards have been instituted to ensure they adhere to acceptable security practices and maintain confidentiality.		
⇒			

7.5a	Indicate which of the following security measures will be/has been undertaken to protect the data and records	**(check all that apply)**	
		All hospital and research staff accessing patient information have completed the hospital Privacy & Confidentiality Education Program	
		Data will be encrypted	
		Data will be password protected	X
		Data will be stored on a hospital or other institutional network drive that has firewalls and security measures in place	X
		Hard copy records will be stored in a locked cabinet in a secure location	
		Access to records and data limited to authorized persons	X
		All identifiers to be removed once data collected/verified	
		Master list linking data with identifiers stored separately from data.	
		Consultation with hospital Privacy Office or LHRI Research Office re privacy and security issues	
7.5b	Describe these measures in more detail.		
⇒	No subject names will be used in any samples. Thus, there will be no confidentiality or privacy issues. All electronic data will be kept on a password-protected computer in a locked laboratory and all paper files will be kept in a locked filing cabinet in a locked office.		

7.6	Indicate how long the specimens and/or data will be retained and if not being kept indefinitely, describe the method of disposal or destruction
⇒ The specimens will be destroyed upon processing.	

7.7a	Are participant data or biological specimens being sent or taken **off-site** to a sponsor, co-investigator or central data collection site or registry? NB a formal contract or data or material transfer agreement must be in place between the local institution and the recipient before data or specimens are sent.	YES	X
		NO	
7.7b	Will data be taken off site for analyses? E.g. In the case of patient information is there plans for the researcher to conduct analyses away from the site? E.g. at home?	YES	
		NO	X

If YES to either 7.7a or 7.7b complete the balance of this section.
If NO go to Section 8.

7 8a	Will personal identifiers be included with the data or specimens sent or taken **off-site**? Note Data and specimens that leave the site should not include the patient's name or other identifiers unless there is a compelling reason. Data that includes identifiable personal health information MUST be encrypted before being sent or taken off site or utilized via secure remote access. Master lists must be stored separately from the data. E g data files and the master list should not be stored on the same portable device.	YES NO	 X
7 8b	If YES, indicate which, if any, of these participant identifiers will be included with the data or specimens sent off-site?	Full or Partial Name or Initials	
		Contact info: address, phone, postal code etc	
		Date of Birth or Death	
		Personal Numbers e.g. OHIP Health Card, SIN	
		Institutional / Hospital Chart or Record #	

7 8c	IF ANY OF THE ABOVE IDENTIFIERS WILL BE **SENT OR TAKEN OFF-SITE** provide a comprehensive rationale explaining why it is necessary for this information to go off-site. Acceptable reasons will generally be limited to the following purpose and investigators will need to defend their reasons for sending identifiers off-site and how they will be used to achieve the purpose stated • Contact or linkage for follow up or ongoing data collection • Provide data for clinical monitoring of the participant • Enable data to be withdrawn from data set if participant withdraws consent • Return individual results to participant • Conduct a data linkage with a high degree of accuracy • Data analyses Do not just copy one of the above reasons into the box below Investigators must defend their reasons for collecting and/or retaining identifiers and explain how the identifiers will be used to achieve the stated purpose.
⇒	

7.9d	If NO, will there be a code or identifiers that allow linkage of the data and/or specimens back to the study and/or the research participant?	No personal identifiers sent/taken off-site but data and/or specimens are coded and linkage is possible.	X
		Data and/or specimens completely de-identified and no linkage is maintained.	
7.9e	If a LINKAGE IS POSSIBLE describe how the data and/or specimens are to be coded to allow the linkage and who will retain a master list linking participants and their data. Note that in most instances the master list should remain in a secure location at the local site.		

⇒ Each participant will be assigned a participant ID number which will be placed on the consent form of each participant. These consent forms will act as the only link between participant name and ID, and will be stored securely with the administrative assistant of the local site These files will not be accessible to the local or off-site investigators. All samples, including those sent off-site, will only contain the ID number.

7 9f	Indicate which of the following security measures will be/has been undertaken to protect the data and records	(check all that apply)	
		Data will be encrypted	
		Data will be password protected	
		Data will be de-identified	X
		Data will be shipped by courier or other bonded shipping method	
		Data will be personally delivered by researcher or research staff or picked up by co-investigator or sponsor	X
		All identifiers to be removed prior to shipping	
		Master list linking data with identifiers retained at local site.	
		Data sent by fax to a secure location	

7 10	If YES to either 7 7a or 7 7b, indicate **where** data, records or specimens are sent or taken Be as specific as possible.
⇒ A vaginal swab from each participant will be smeared on a glass slide, which will then be delivered to the Microbiology Lab of the London Laboratory Services Group, located at Victoria Hospital of the London Health Sciences Centre, for the purpose of BV diagnosis.	

7 11	If YES, describe **how** data or specimens are sent or taken **off-site** or **accessed from off-site**? (E.g. hard copy, fax, electronic transmission email/web site, portable media devices, secure remote access, courier etc.) and describe transmission safeguards and security measures i.e. Are data encrypted prior to transmission? If yes describe level of encryption? Physical security (private faxes, shipping of disks etc)? Note that data containing identifiable personal health information MUST be encrypted to an appropriate level.
⇒ The glass slides smeared with a vaginal swab will be delivered personally by the research investigator to Victoria Hospital.	

7.12	If YES, Describe the **off-site** procedures for securing and storing written records, videotapes, computer discs, recordings and questionnaires, data and specimens
⇒ The Microbiology laboratory at Victoria Hospital will receive only the glass slides with participant ID, and these slides will be stored only until analysis, after which they will be disposed. Staff at this site will follow their local procedures for safe storage and disposal of specimen.	

7 13	If YES, indicate how long the data or specimens will be retained **off-site** and describe the method of disposal if data or specimens are not returned to the local site or not retained indefinitely
⇒The glass slides will be retained at the Microbiology laboratory only until analysis is performed. The glass slides will then be disposed of under proper disposal procedures established at this laboratory	

SECTION 8 PARTICIPANT RECRUITMENT & CONSENT PROCESS

Disclaimer: The Review Board does not assess the legal validity of the consent form nor does it provide any other legal advice.

8 1a	Will potential participants, or people associated with them, be contacted directly to collect data from them or recruit them to the study?	YES	X
		NO	
8.1b	If YES, identify who will be contacting the potential participants (and the others) to recruit them and how it will be done. *If you answer "yes" to this section*, ensure that you submit a letter of information with your study. In the case of patients, initial contact must be made by a member of the patient's health care team, circle of care, or someone the patient would expect to have relevant information about them and their medical problems. In the case where others such as teachers or family members are asked to give information about the study participant, the participant's permission is usually required for the other person to release the information.		

⇒ Dr. Jo-Anne Hammond will recruit subjects from her patient pool who fit the criteria. In addition, recruitment posters will be posted at the following sites: Victoria Family Medical Centre, Lawson Health Research Institute, St. Joseph's Hospital, Victoria Hospital, University Hospital, University of Western Ontario, Fanshawe College and Brescia University College. These posters will contain the contact information of Roderick MacPhee (study coordinator) so that potential participants may contact us directly. As well, the recruitment poster will be posted on the Lawson Health Research Institute website of clinical trials. A message will also be placed in the LHSC E-Cast online newsletter which is available to hospital staff. Lastly, staff from the following clinics will be asked to recruit eligible subjects from their patient pool: Middlesex-London Health Unit, Byron Family Medical Centre and St. Joseph's Family Medical Centre.

8.2a	What type of consent will be or was obtained from participants?	No consent to be obtained - Researcher requests waiver from REB	
		Explicit written consent – use of formal consent documentation	X
		Explicit verbal consent e.g. telephone surveys	
		Explicit consent - other e.g. completion of questionnaire, survey evidence of consent	
		Passive consent e.g. notices posted with option to opt out	
		Prior consent e.g. prior research consent (append example copy)	
		Other (please describe)	

8.3	**If a waiver to obtain prospective consent** is being requested you must explain why. As per the Ontario Personal Health Information Protection Act (PHIPA) requests to waive consent are generally limited to the following reasons. Investigators **must** indicate which are relevant and explain why.	

8.3a	**Inappropriateness of consent requirement:** There may be potential harms to individual from direct contact e.g. Psychological, social or other harm or distress could be caused by contacting individuals or families.	
⇒n/a		
8.3b	**Inappropriateness of consent requirement:** Contact with individuals not permitted under a previous agreement, law or policy.	
⇒n/a		
8.3c	**Impracticability** of seeking consent due to size and nature of population being studied.	
⇒n/a		
8.3d	**Impracticability** of seeking consent due to proportion of prospective participants likely to have relocated or died since original data or specimen collection	
⇒n/a		
8.3e	**Impracticability** of seeking consent due to lack of existing or continuing relationship between participants and data holder.	
⇒n/a		

8.4	**If a waiver to obtain consent is being requested**, what is the probability of harm related to retaining the ability to identify data or specimens and the adequacy of security measures? If there are risks, potential benefits to the public and individuals must significantly outweigh potential harms to research participants.			
	PROBABILITY OF RISK TO PARTICIPANT AS A RESULT OF ACCESSING, USING OR DISCLOSING THEIR DATA OR SPECIMENS	**High**	**Moderate**	**Minimal or None**
8.4a	Physical injury			
8.4b	Emotional or psychological harm			
8.4c	Social harm (stigmatization, insurability, employability)			
8.4d	Financial harm			
8.4e	Intrusion on privacy			
8.4f	Loss of trust (e.g. in institution or care givers etc.)			
8.4g	Other (specify)			

8.5a	In the case of previously collected or discarded biomaterials or specimens did the donor consent to use for general research purposes? If specimens are to be collected prospectively indicate "Not Applicable"	YES	
		NO	
		DON'T KNOW	
		Not applicable	X
8.5b	If YES, prior explicit or implicit consent has already been obtained (e.g. for use of discarded or left over specimens) please describe the nature of the consent (e.g. usual hospital surgical consent) and how it was obtained and documented. If possible attach a sample of the document.		
⇒			

IF YOU ARE ASKING FOR A WAIVER TO OBTAIN PROSPECTIVE CONSENT AND/OR HAVE PRIOR CONSENT YOU HAVE NOW COMPLETED THE FORM.

IF YOU WILL BE OBTAINING CONSENT OR NOTIFYING POTENTIAL PARTICIPANTS PLEASE COMPLETE THE REST OF THE FORM.

8.6a	Will posters, advertisements, public notices or telephone solicitation be used to recruit or notify participants?	**YES**	X
		NO	
8.6 b	If YES attach copies of all posters, handouts, notices or advertisements etc. In the case of audio announcements or telephone recruitment provide a written copy of the script. If video or electronic media are used e.g. video tape or CD's provide only one (1) copy of tape or CD. This will be retained by the Office of Research Ethics and will not be returned to the Investigator. Advert attached as appendix A.		

8.7a	Will minors or persons not able to consent for themselves be included in the study?	YES	
		NO	X
8.7b	If YES, describe the consent process and indicate who will be asked to consent on their behalf and discuss what safeguards will be employed to ensure the rights of the research participant are protected. Whether or not a separate assent form is used, investigators and parents or guardians should discuss the study with the person (when appropriate) and explain exactly what will happen and what the person's rights are. In certain circumstances, the REB may find it acceptable for mature or emancipated minors to give consent without also requiring consent from parents or guardians.		
⇒			

8.8	Describe opportunities (if any) available to participants to consent to future, as yet unknown, research on their data or specimens. In the case of de-identified data this is not an option, but researchers need to think carefully about the logistics and likelihood of being able to contact participants in the future if they promise contact for future consent.	Not Applicable	X
⇒			

8.9	Describe what opportunities (if any) will be available to participants to withdraw their data or specimens in the future. Please note that it may be necessary to deny participants the right to withdraw data or specimens to protect the integrity of research already using the data. If this is the case, this limitation on their ability to withdraw must be made clear in the Letter of Information. In the case of de-identified data withdrawal is not an option.	Not Applicable	X
⇒			

8 10	If YES, Attach a copy of all documentation that will be (or has been) used to inform and obtain consent from the potential participants about the research. Separate Information/consent documents or a combined Information/Consent document may be used. Wording regarding the participant's consent must comply with the UWO guidelines, be relevant to the Canadian and Ontario scene and participants must be given a copy of the Letter of Information or combined Information/consent document to keep for reference. The letter of information and consent documentation included in this submission should be on institutional letterhead and be presented as though it were the actual letter being given to participants. If announcements, posters or advertisements will be used to inform potential participants that this research is underway, attach a copy of all hardcopy advertisements /announcements/verbal scripts etc that will be used THE CHECKLIST ON NEXT PAGE IS DESIGNED TO ASSIST YOU IN THE PREPARATION OF THE INFORMED CONSENT DOCUMENTATION WHEN USED IN CONJUNCTION WITH THE GUIDELINES FOR PREPARATION OF INFORMED CONSENT DOCUMENTATION IT WILL ENSURE YOUR MATERIALS MEET THE REB's MINIMUM REQUIREMENTS. CHECK THE DOCUMENTS CAREFULLY FOR COMPLETENESS IT IS THE INVESTIGATOR'S RESPONSIBILITY TO CORRECT ALL SPELLING OR GRAMMATICAL ERRORS AND ENSURE THE DOCUMENTS MEET UWO HSREB SPECIFICATIONS **BEFORE** SUBMITTING THE PROTOCOL TO THE HSREB. INCOMPLETE OR POORLY PREPARED CONSENT DOCUMENTATION IS A MAJOR REASON WHY ETHICS APPROVAL IS DELAYED. **See Guideline 2-G-005 (formerly Appendix 4) on the following website. http://www.uwo.ca/research/ethics/med/hsreb-guidelines.htm**

These are the contact persons for participants who have questions regarding their rights and the conduct of the research. The correct person must be inserted into this sentence and this phrase included in the Letter of Information. **"If you have any questions about your rights as a research participant or the conduct of the study you may contact..."**	
If participants are recruited from within the LHSC or SJHC system or research is taking place at LHSC or SJHC sites	Dr. David Hill, Scientific Director, Lawson Health Research Institute at [redacted]
If participants recruited from sites other than LHSC or SJHC and research not taking place at LHSC or SJHC sites.	The Office of Research Ethics at (519) 661-3036 or by email at ethics@uwo.ca

CHECKLIST – INFORMATION & CONSENT DOCUMENTATION *To be used in conjunction with the Guidelines for preparation of Information & Consent documentation.* **See 2-G-005 on the following website. http://www.uwo.ca/research/ethics/med/hsreb-guidelines.htm**		
HAVE YOU INCLUDED OR ADDRESSED THE FOLLOWING ISSUES?		
X	*Required*	Title of the research
X	*Required*	Identity of researchers & affiliation
	When appropriate	Identity of sponsors if industry funded
X	*Required*	Invitation to participate in research & complete description of what is involved, including the time commitment and location
X	*Required*	Provide summary explanation of research
X	*When appropriate*	Indicate number of participants – total & local
X	*Required*	Describe Risks / Harms / Benefits – even if there are none
X	*Required*	Voluntary participation, can refuse to participate, withdrawal of data etc
X	*Required*	Discuss anonymity &/or confidentiality of information
X	*When appropriate*	Explanation of compensation
	When data going off-site	Where data going, security measures, what will happen to it etc
X	*Required*	Local contact person(s) for participants re questions about database/registry/bank and/or treatment and care
X	*Required*	Local contact person for participants re subject rights
	When appropriate	Access to records or follow up by HSREB to monitor the study
X	*When appropriate*	No waiver of rights
X	*Required*	No indication of institutional approval
X	*Required*	Statement that participant will receive copy of letter of information to keep
X	*Required*	Non identification in publication of results without specific consent
X	*Required*	Consent Statement as per UWO standard
	When appropriate	Consent options
X	*Required*	Signatures of appropriate persons
X	*Required*	Information/consent documents addressed to research participant
X	*Required*	Language Level - lay language, grade 8 level for general population
X	*Required*	No spelling or grammatical errors
X	*Required*	Appropriately Formatted – on letterhead, pages numbered, appropriate type size, page layout, header/footer, headings etc
X	*Required (When appropriate)*	**Consent Form Wording:** I have read the Letter of Information, (have had the nature of the study explained to me) and I agree to participate. All questions have been answered to my satisfaction.

Appendix 2- Recruitment Poster

Western

LAWSON
HEALTH RESEARCH INSTITUTE

Volunteers needed for study to help prevent Toxic Shock Syndrome

Females aged 18-40 with a history of bacterial vaginosis are needed to provide samples at the Victoria Family Medical Centre from which we hope to identify factors involved in the development of toxic shock syndrome.

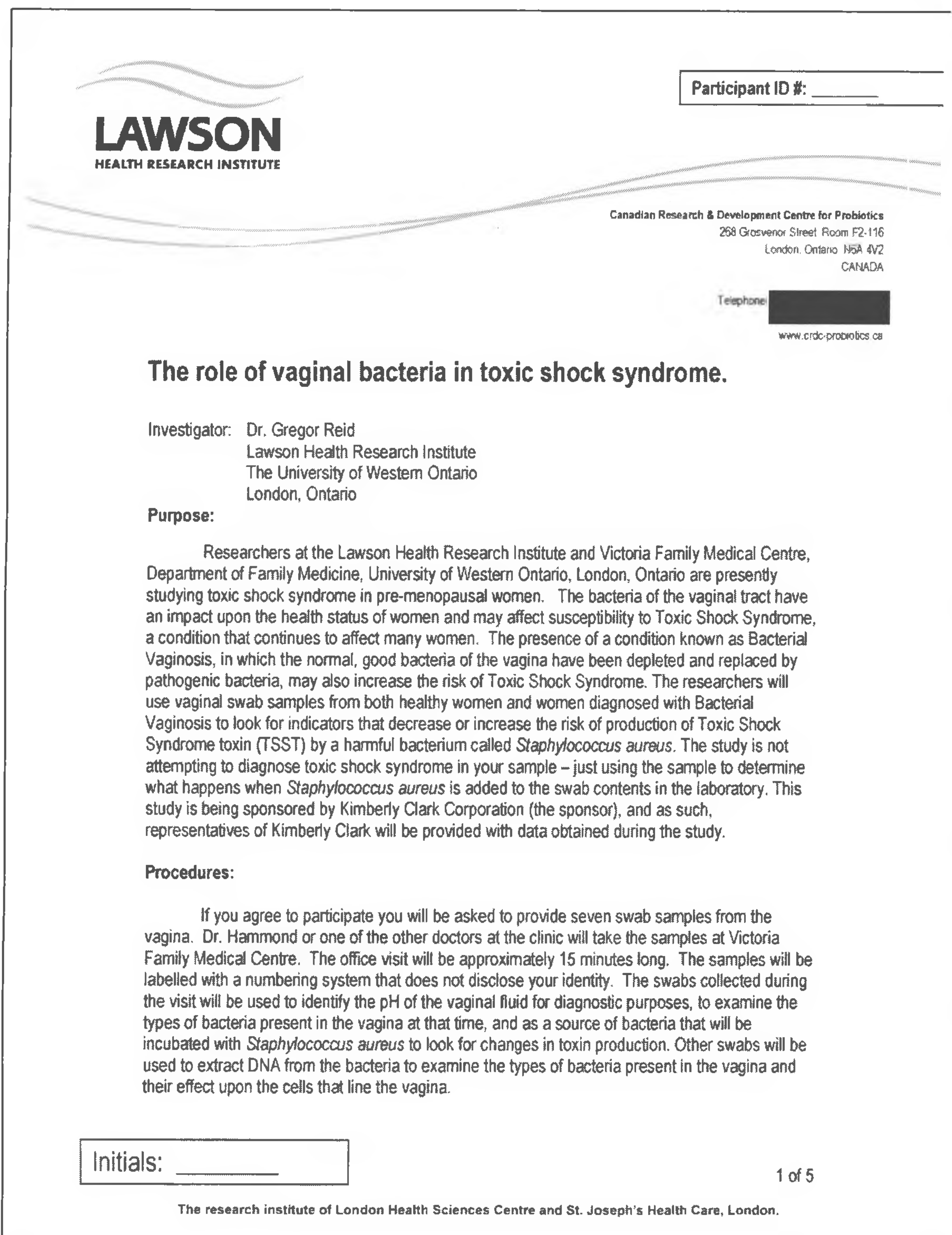
If interested, or for more information, please contact:

Roderick MacPhee
Study Coordinator
Tel:
Email:

You will be reimbursed for your time and travel expenses.

Roderick MacPhee Tel: Email:	Roderick MacPhee Tel: Email:	Roderick MacPhee Tel: Email:	Roderick MacPhee Tel: Email:	Roderick MacPhee Tel: Email:	Roderick MacPhee Tel: Email:	Roderick MacPhee Tel: Email:	Roderick MacPhee Tel: Email:

Appendix 3- Letter of Information and Consent



Participant ID #: ______

LAWSON
HEALTH RESEARCH INSTITUTE

Canadian Research & Development Centre for Probiotics
268 Grosvenor Street Room F2-116
London, Ontario N6A 4V2
CANADA

Telephone:

www.crdc-probiotics.ca

The role of vaginal bacteria in toxic shock syndrome.

Investigator: Dr. Gregor Reid
Lawson Health Research Institute
The University of Western Ontario
London, Ontario

Purpose:

Researchers at the Lawson Health Research Institute and Victoria Family Medical Centre, Department of Family Medicine, University of Western Ontario, London, Ontario are presently studying toxic shock syndrome in pre-menopausal women. The bacteria of the vaginal tract have an impact upon the health status of women and may affect susceptibility to Toxic Shock Syndrome, a condition that continues to affect many women. The presence of a condition known as Bacterial Vaginosis, in which the normal, good bacteria of the vagina have been depleted and replaced by pathogenic bacteria, may also increase the risk of Toxic Shock Syndrome. The researchers will use vaginal swab samples from both healthy women and women diagnosed with Bacterial Vaginosis to look for indicators that decrease or increase the risk of production of Toxic Shock Syndrome toxin (TSST) by a harmful bacterium called *Staphylococcus aureus*. The study is not attempting to diagnose toxic shock syndrome in your sample – just using the sample to determine what happens when *Staphylococcus aureus* is added to the swab contents in the laboratory. This study is being sponsored by Kimberly Clark Corporation (the sponsor), and as such, representatives of Kimberly Clark will be provided with data obtained during the study.

Procedures:

If you agree to participate you will be asked to provide seven swab samples from the vagina. Dr. Hammond or one of the other doctors at the clinic will take the samples at Victoria Family Medical Centre. The office visit will be approximately 15 minutes long. The samples will be labelled with a numbering system that does not disclose your identity. The swabs collected during the visit will be used to identify the pH of the vaginal fluid for diagnostic purposes, to examine the types of bacteria present in the vagina at that time, and as a source of bacteria that will be incubated with *Staphylococcus aureus* to look for changes in toxin production. Other swabs will be used to extract DNA from the bacteria to examine the types of bacteria present in the vagina and their effect upon the cells that line the vagina.

Initials: ________

If you suffer any reactions (pain, discomfort, irritation or other signs or symptoms) that concern you and that you feel are due to the study, you should report these to Dr. Hammond. Upon enrolment in the study, you will be given the opportunity to provide written consent for researchers to notify you via your referring physician or family doctor should any clinical abnormality be detected from the sample analysis.

Exclusion Criteria:

You should not participate in this study if you have any of the following: are pregnant; recurrent sexually transmitted disease; abnormal renal function or pyelonephritis; are receiving prednisone or immunosuppressive drugs; are receiving treatment for any urogenital infection or are taking any antimicrobial therapy; have had a urogenital infection in the past 6 months; have a personal history of known or suspected estrogen-dependent neoplasia such as breast or endometrial cancer; have undiagnosed abnormal vaginal bleeding; have had a history of gonorrhoea or chlamydia; have had sexual intercourse 48 hours before the clinical visit; have taken any 'acidophilus' or probiotic health food supplements 48 hours before the clinical visit; are menstruating at time of clinical visit.

Contacts:

Participation in this study will not affect the treatment of any medical problems you experience. Should you have questions you should feel free to contact us:

Dr. J. Hammond
Referring Physician
Victoria Family Medical Centre

Or

Mr. Roderick MacPhee
Study Coordinator

If you have any questions about the conduct of this study or your rights as a research subject, you may contact the Dr. David Hill, Scientific Director, Lawson Health Research Institute,

Initials: ________

Risks:

We do not expect that you will experience any risk by participating in this study. You may experience some mild discomfort when the vaginal samples are collected, but this should not result in any increased risk.

Voluntary Participation:

Participation in this study is voluntary. You may refuse to participate, or you may withdraw from the study at any time with no effect on your future care.

Confidentiality:

All information collected for the study will be kept confidential and will only be made available to members of the research team for the purposes of data analysis. Participants will be issued an identification number for sample submission. Your identity will only be known to your referring physician and/or Dr. Hammond and will not be released to members of the research team or sponsor. Data will be stored indefinitely on a password-protected computer in a locked laboratory and all paper files will be kept in a locked filing cabinet in a locked office. All data will be filed with sample identification numbers only—no names will be stored with the data. A copy of the final data will be supplied to the sponsor. If the results of the study are published, your name will not be used and no information that discloses your identity will be released or published. Representatives of The University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of the research.

Benefits:

This study may help us better understand why some women develop toxic shock syndrome and others do not. The long-term goal is to reduce the risk of this condition through interventions such as creams, probiotics, nutrients, or pads.

Consent:

By agreeing to participate in this study, you give your consent for the information given by you and obtained from your samples can be compiled and analyzed with others participating in this study. If you are participating in another study, please let us know so that we can determine if you should participate in this study.

Initials: ________

Your Rights:

You do not waive any legal rights by signing the consent form.

Compensation:

Each participant will be compensated $50 for participation in the study to cover the cost of parking and travel expenses to the centre.

You will be provided with a photocopy of this letter.

Initials: ________

Participant ID #: ______

The role of vaginal bacteria in toxic shock syndrome.

Consent

I, ______________________________, have read the Letter of Information, have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

I ____________ (DO or DO NOT) give my permission to contact my family physician should any abnormality be identified during data analysis. If permission is granted, please supply name of physician:

____
Name of Physician

______________________________ ________________
Signature of Participant **Date**

____
Name of Participant (Please Print)

______________________________ ________________
Signature of person obtaining Informed Consent Date

____
Name (Please Print)

Note: the participant must initial each page of this letter and agreement.

Initials: ________

Appendix 4- Study Questionnaire

Baseline Questionnaire

The role of vaginal bacteria in toxic shock syndrome.

Participant ID: ______________ Date: __________

Instructions: The following questions are designed to assess the eligibility of the participant based on the recruitment criteria for this study. Please answer as honestly and clearly as possible.

Feel free to ask the study coordinator about any questions that may be unclear.

1. Are you a female between the age of 18-40 years old?
   - Yes
     - Please Specify: __________
   - No

2. Have you reached menopause?
   - ☐ Yes
   - ☐ No

3. Have you had any urogenital infections in the past 6 months? (This includes, but is not limited to, sexually transmitted infections, bladder infections, yeast infections)
   - ☐ Yes Please Specify: __________________ Date: __________
     __________________ Date: __________
     __________________ Date: __________
   - ☐ No

4. Are you pregnant?
   - ☐ Yes
   - ☐ No

5. Have you had sex (oral or vaginal) within the past 48 hours?
   - ☐ Yes
   - ☐ No

6. Have you been diagnosed with gonorrhoea or chlamydia in the past?
   - ☐ Yes
   - ☐ No

7. Have you been diagnosed with abnormal renal function (including abnormal function of kidneys, ureters, bladder or urethra) or pyelonephritis (an ascending urinary tract infection)?
   - ☐ Yes Please Specify: ____________ Date:_________
     ____________ Date:_________
   - ☐ No

8. Are you currently receiving prednisone, immunosuppressive drugs or antimicrobial medication?
   - ☐ Yes
   - ☐ No

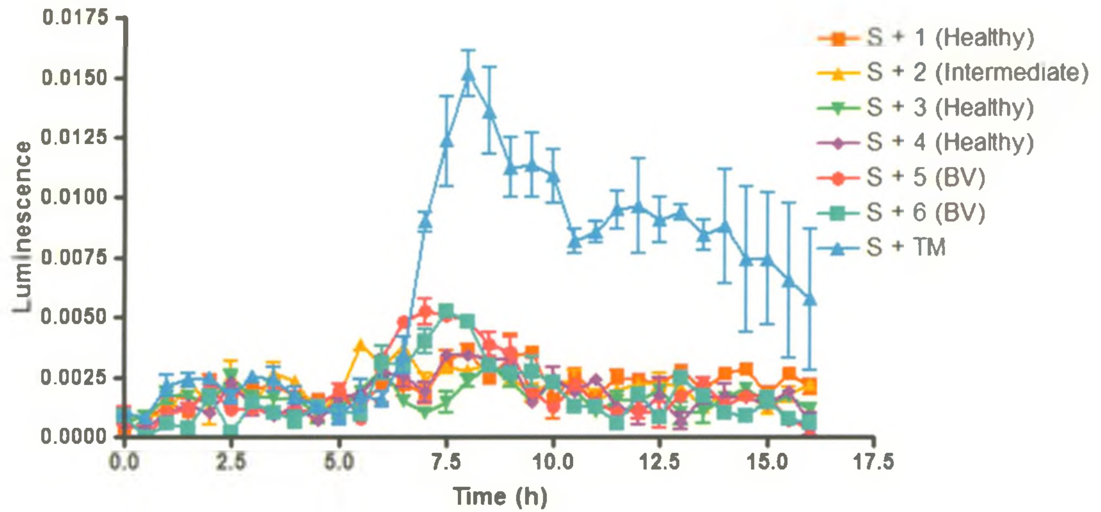
9. Do you have a history of estrogen-dependent neoplasia such as breast or endometrial cancer?
   - ☐ Yes
   - ☐ No

10. Do you have any abnormal vaginal bleeding?
    - ☐ Yes
    - ☐ No

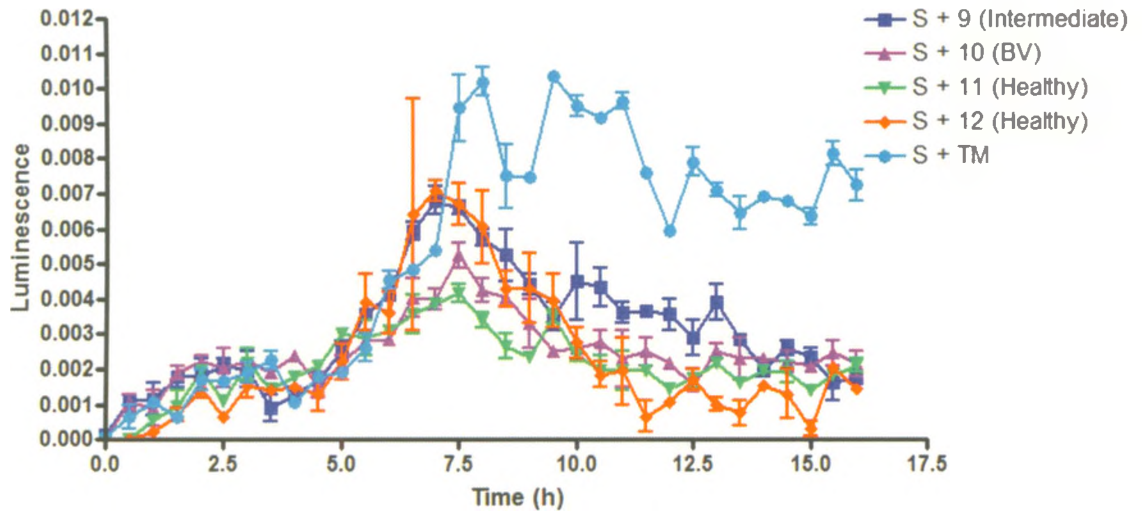
11. Have you taken probiotic supplements or foods in the past 48 hours?
    - ☐ Yes
    - ☐ No

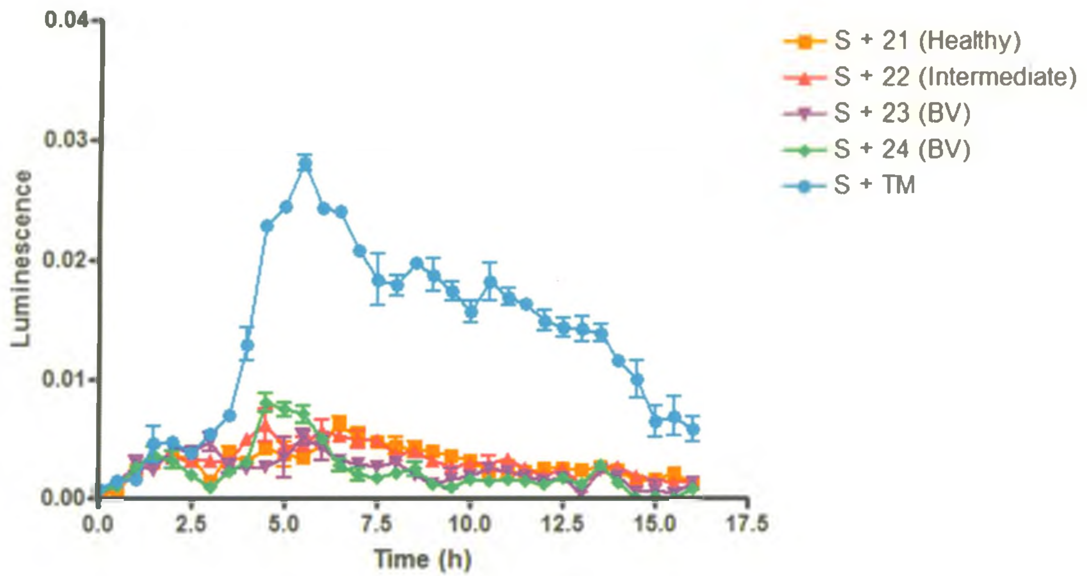
12. Are you currently menstruating at time of clinical visit?
    - ☐ Yes
    - ☐ No

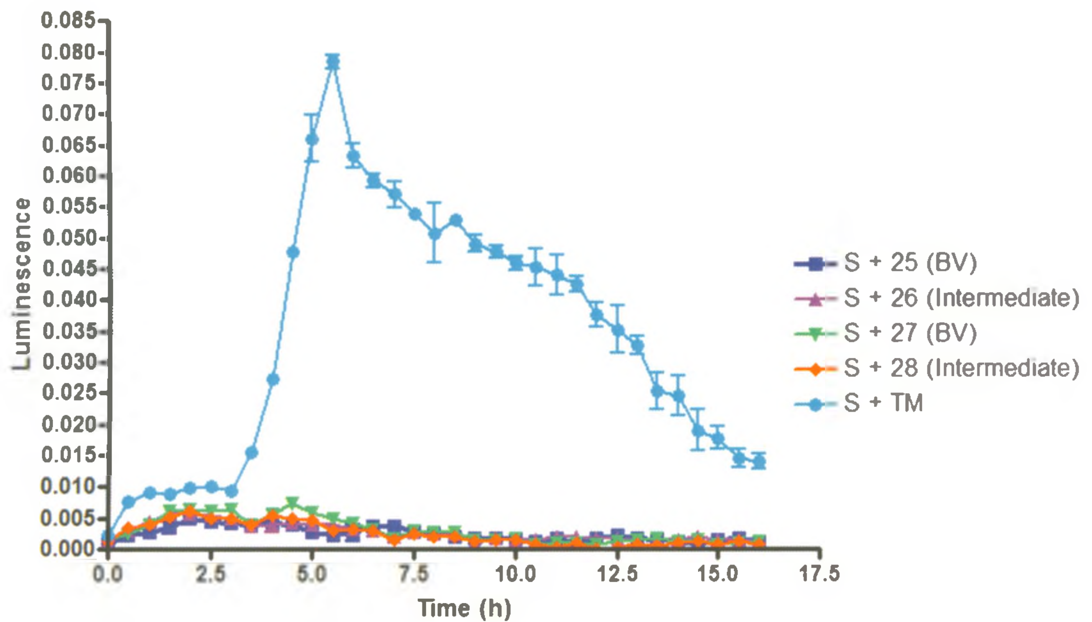
Appendix 5A- Luminescence Data

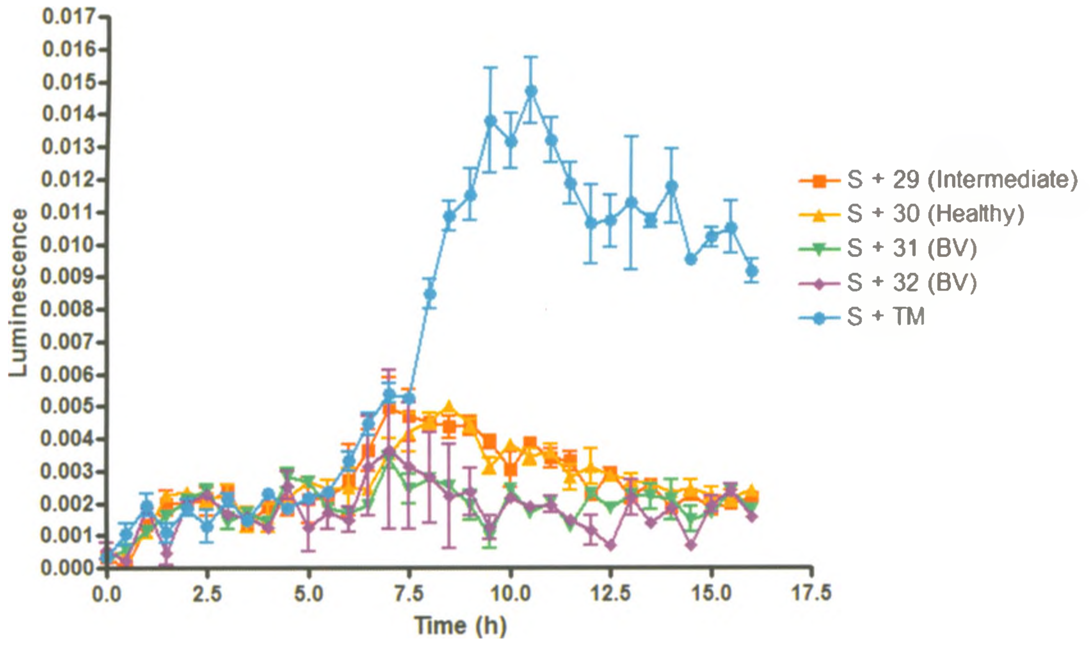


S=*S. aureus* MN8; TM=Transport Medium.

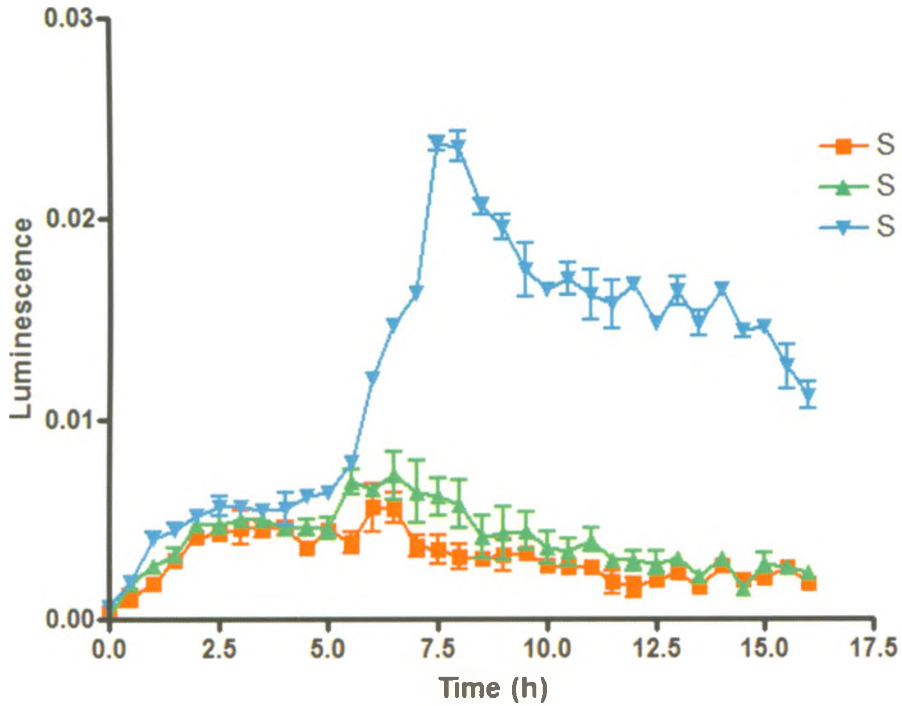



0.04
0.03
0.02
0.01
0.00
Luminescence
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Time (h)
S + 21 (Healthy)
S + 22 (Intermediate)
S + 23 (BV)
S + 24 (BV)
S + TM


0.085
0.080
0.075
0.070
0.065
0.060
0.055
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Luminescence
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Time (h)
S + 25 (BV)
S + 26 (Intermediate)
S + 27 (BV)
S + 28 (Intermediate)
S + TM

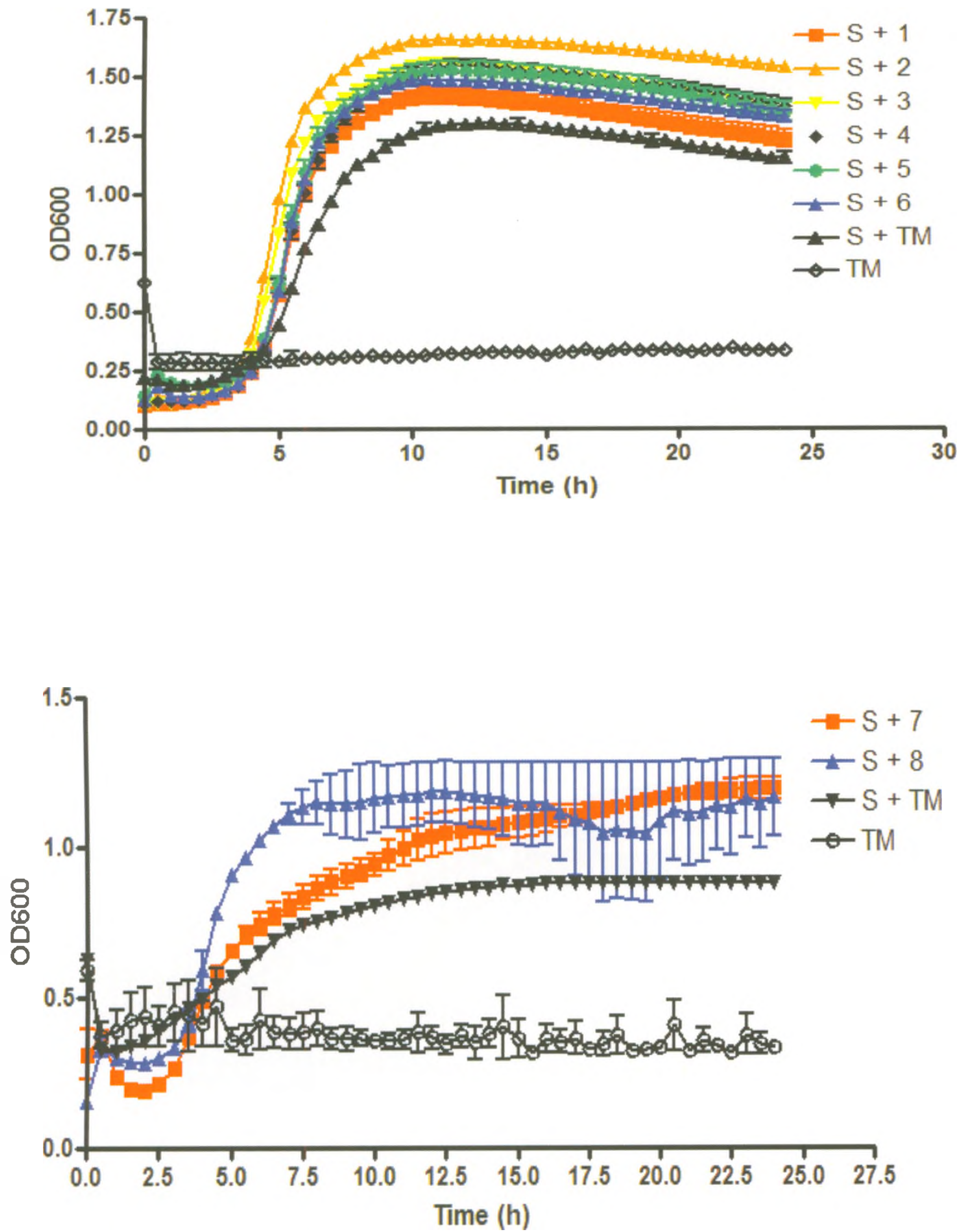


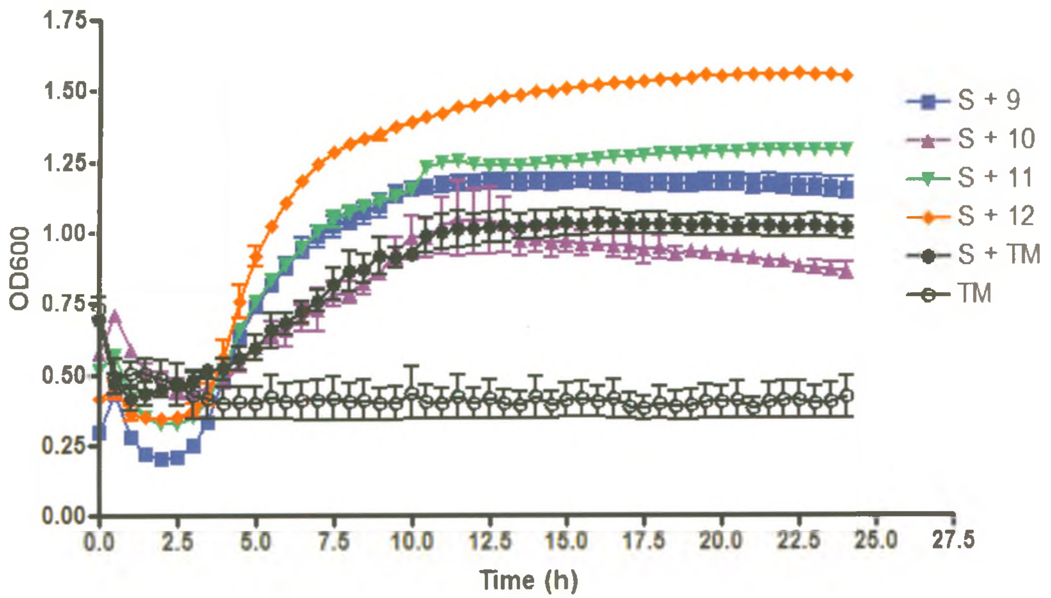
0.017
0.016
0.015
0.014
0.013
0.012
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
Luminescence
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Time (h)
S + 29 (Intermediate)
S + 30 (Healthy)
S + 31 (BV)
S + 32 (BV)
S + TM

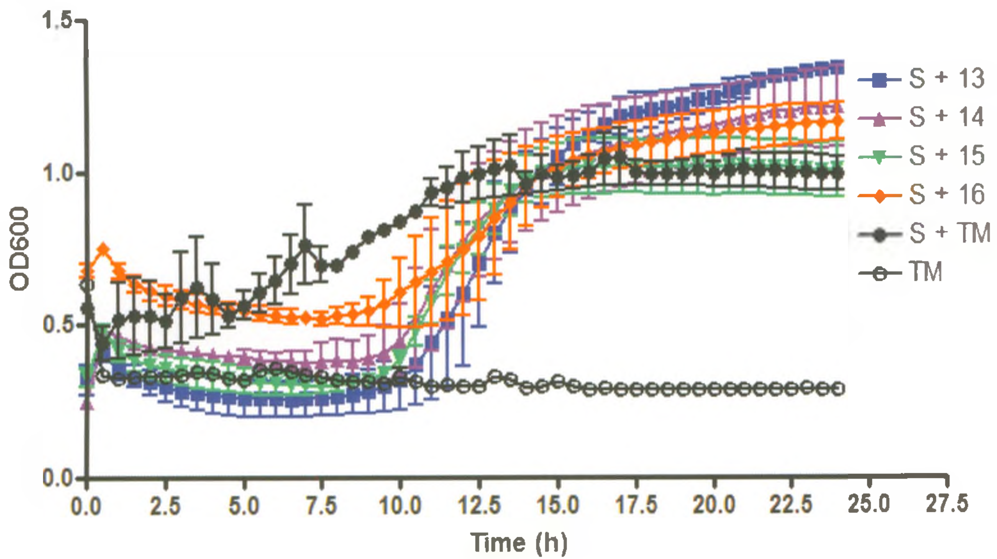


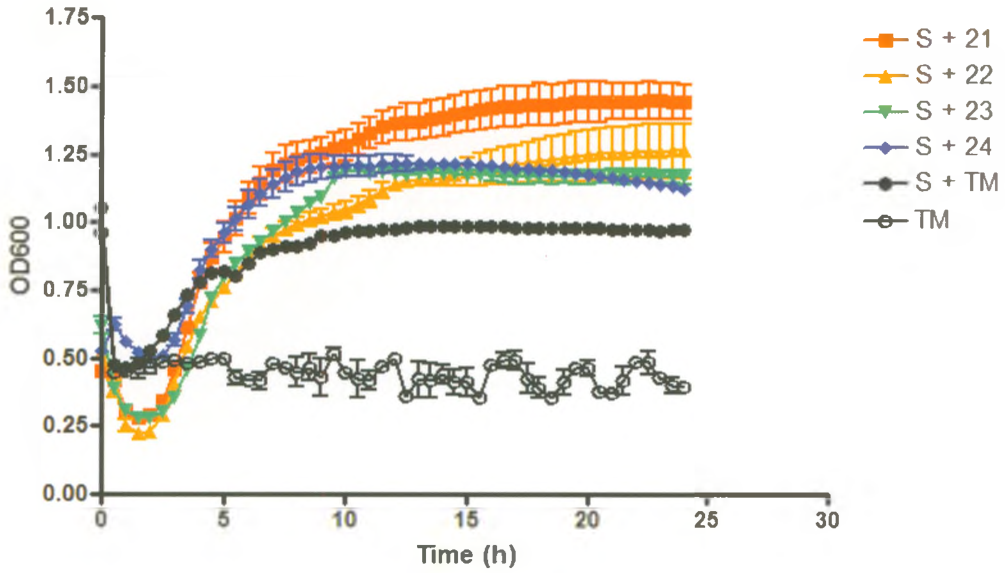
0.03
0.02
0.01
0.00
Luminescence
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Time (h)
S + 33 (BV)
S + 34 (BV)
S + TM

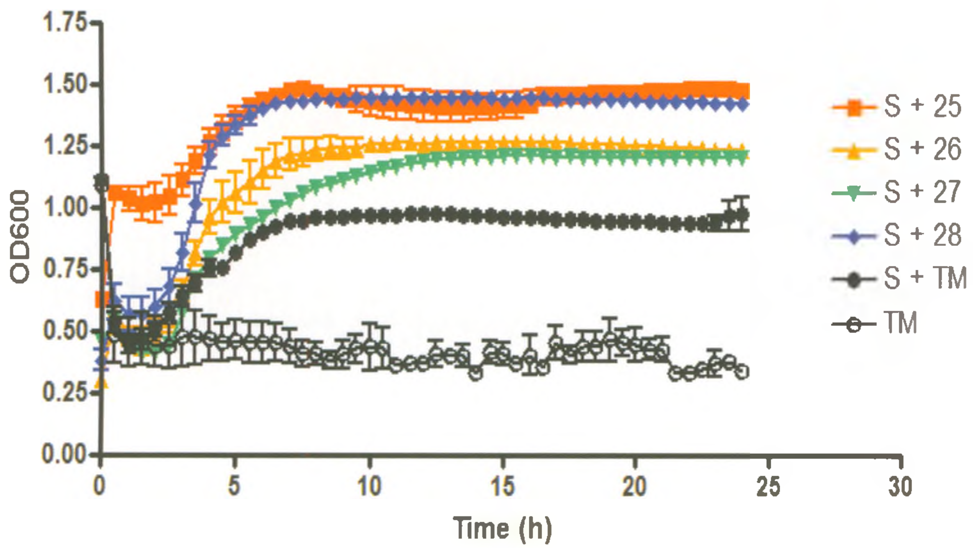
Appendix 5B- Growth Curves for Luminescence Assay

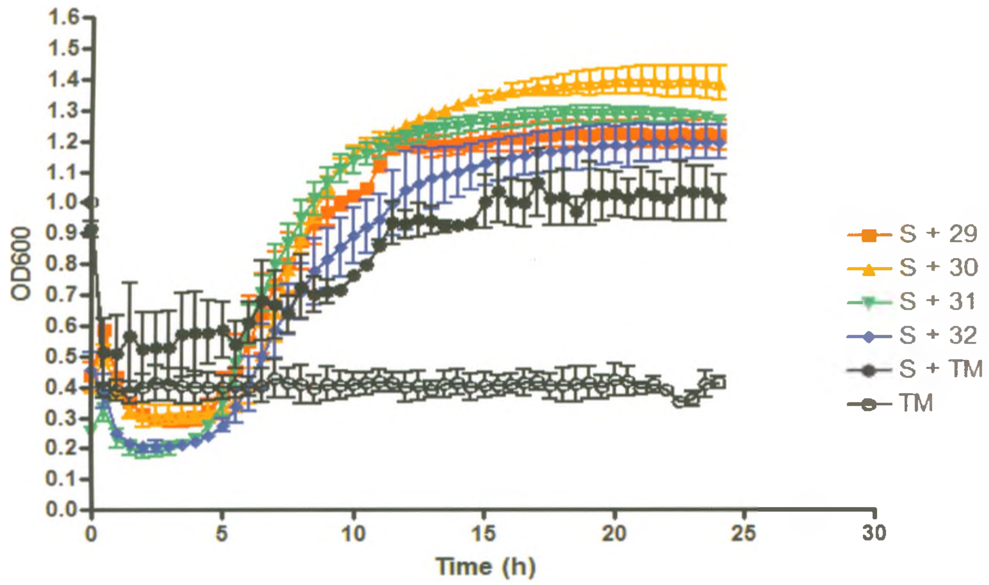


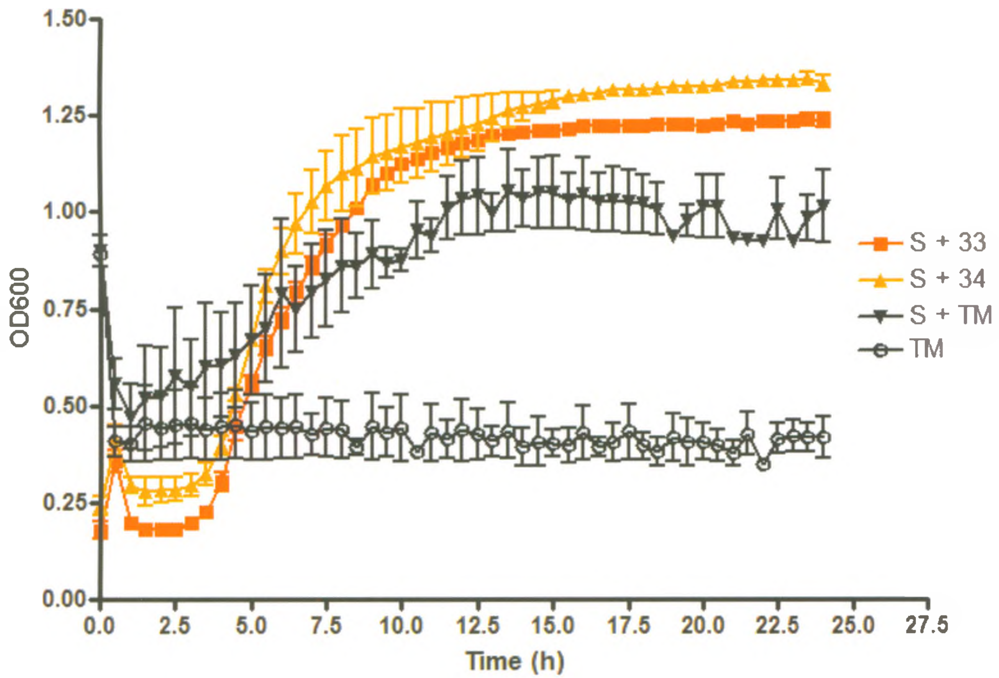

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
OD600
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
Time (h)
S + 9
S + 10
S + 11
S + 12
S + TM
TM


1.5
1.0
0.5
0.0
OD600
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
Time (h)
S + 13
S + 14
S + 15
S + 16
S + TM
TM

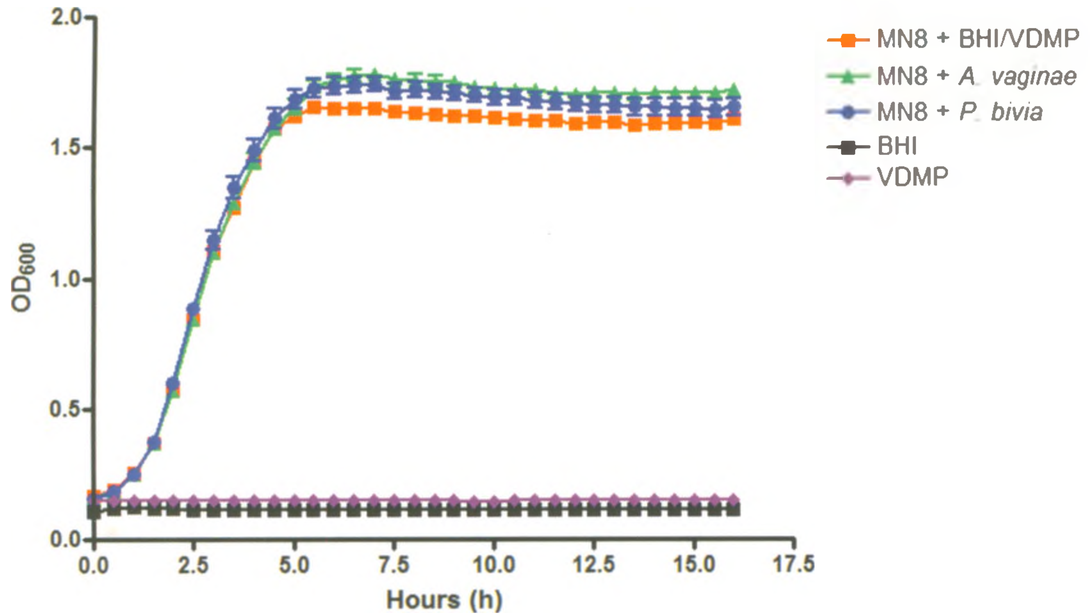

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
OD600
0
5
10
15
20
25
30
Time (h)
S + 21
S + 22
S + 23
S + 24
S + TM
TM


1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
OD600
0
5
10
15
20
25
30
Time (h)
S + 25
S + 26
S + 27
S + 28
S + TM
TM

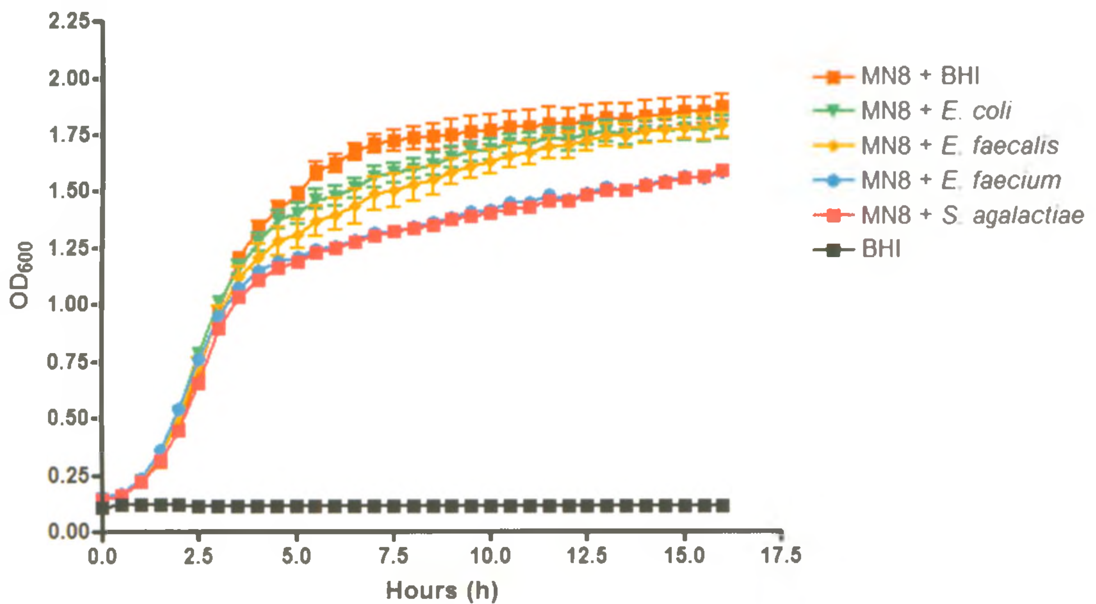

OD600
Time (h)
S + 29
S + 30
S + 31
S + 32
S + TM
TM

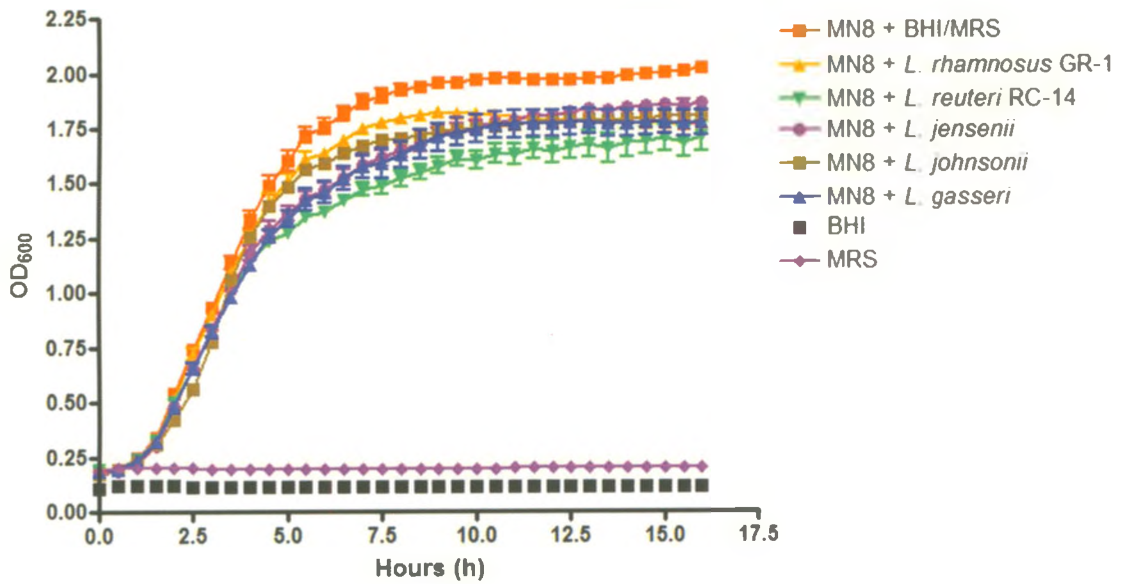

OD600
Time (h)
S + 33
S + 34
S + TM
TM

Appendix 6- Growth Curves of *S. aureus* MN8 in Response to Supernatant Challenge



MN8= *S. aureus* MN8; BHI=Brain Heart Infusion Media; VDMP= Vaginally-Defined Medium + 0.5% Proteose Peptone.





MRS= de Man, Rogosa and Sharpe media

Appendix 7 – Publications During Thesis

7.1. Review of the most recent clinical trials studying the effectiveness of probiotics for the treatment of bacterial vaginosis

MacPhee RA, Hummelen R, Bisanz JE, Miller WL, Reid G. Probiotic strategies for the treatment and prevention of bacterial vaginosis. Expert Opin Pharmacother 2010; 11(18): 2985-95.

Review

Expert Opinion

1. The vaginal microbiota
2. Bacterial vaginosis
3. The selection of urogenital probiotic strains
4. Probiotics for the treatment of bacterial vaginosis
5. Secondary prevention of bacterial vaginosis
6. Expert opinion

Probiotic strategies for the treatment and prevention of bacterial vaginosis

Roderick A MacPhee, Ruben Hummelen, Jordan E Bisanz, Wayne L Miller & Gregor Reid[†]
[†]*The University of Western Ontario, Canadian Research & Development Centre for Probiotics, Lawson Health Research Institute, London, Ontario, Canada*

Importance of the field: Urogenital infections are on average the number one reason for women to visit the doctor. Yet, treatment and preventive strategies have gone unchanged for close to 50 years. With prevalence rates for bacterial vaginosis at more than 29%, depending on the population, and similarly high incidences of vulvo-vaginal candidiasis and urinary tract infections, plus HIV, new therapies are urgently needed to improve the health of women around the world.
Areas covered in the review: This review discusses the vaginal microbiota, our improved understanding of its composition, and its role in health and disease. It also discusses the progress made in the past 10 years or so, with the development and testing of probiotic lactobacilli to improve vaginal health and better manage urogenital infection recurrences.
What the reader will gain: The reader will have an understanding of the clinical data obtained so far, and the potential mechanisms of action of probiotics. Despite the need for more clinical studies, the review illustrates a case for inclusion of probiotics as part of the approach to disease prevention, and as an adjunct to antimicrobial treatment. Challenges remain in optimizing clinical benefits, selecting new strains, preparing new products and having them tested in humans then approved with informative claims, and making products readily accessible to women in the developed and developing world.
Take home message: The vaginal microbiota is a complex structure that can change quickly and dramatically, and significantly impact a woman's health. New health-maintenance and disease-treatment approaches are badly needed, and probiotics should be considered.

Keywords: bacterial vaginosis, *Lactobacillus*, probiotics, vaginal microbiota

Expert Opin. Pharmacother. (2010) 11(18):2985-2995

1. The vaginal microbiota

The vagina is a dynamic environment colonized by a wide range of microorganisms that are collectively referred to as the vaginal microbiota. The most important constituent of the microbiota in healthy women are members of the genus *Lactobacillus*. A decade ago, *L. crispatus* and *L. jensenii* were viewed as the predominant vaginal species [1]; however, with recent advances in culture-independent community profiling, the nutritionally fastidious *L. iners* is emerging as a dominant organism, present in both healthy and lactobacilli-deficient aberrant vaginal environments [2,3]. Bacterial migration from the rectum, across the perineum to the vagina occurs naturally in women and is a source of both pathogens as well as lactobacilli. The latter are believed to offer protective effects against pathogens through displacement, modulating immunity, production of antimicrobial substances and lowering vaginal pH [4,5].

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7.2. Paper documenting the application of 16S rRNA sequencing by Illumina for studying vaginal samples

Gloor GB, Hummelen R, Macklaim JM, Dickson RJ, Fernandes AD, **MacPhee R**, Reid G. Microbiome profiling by illumina sequencing of combinatorial sequence-tagged PCR products. PLoS One 2010; 5(10): e15406.

OPEN ACCESS Freely available online PLoS one

Microbiome Profiling by Illumina Sequencing of Combinatorial Sequence-Tagged PCR Products

Gregory B. Gloor[1*], Ruben Hummelen[2,3], Jean M. Macklaim[1,2], Russell J. Dickson[1], Andrew D. Fernandes[1,4], Roderick MacPhee[2,5], Gregor Reid[1,2,5,6]

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Abstract

We developed a low-cost, high-throughput microbiome profiling method that uses combinatorial sequence tags attached to PCR primers that amplify the rRNA V6 region. Amplified PCR products are sequenced using an Illumina paired-end protocol to generate millions of overlapping reads. Combinatorial sequence tagging can be used to examine hundreds of samples with far fewer primers than is required when sequence tags are incorporated at only a single end. The number of reads generated permitted saturating or near-saturating analysis of samples of the vaginal microbiome. The large number of reads allowed an in-depth analysis of errors, and we found that PCR-induced errors composed the vast majority of non-organism derived species variants, an observation that has significant implications for sequence clustering of similar high-throughput data. We show that the short reads are sufficient to assign organisms to the genus or species level in most cases. We suggest that this method will be useful for the deep sequencing of any short nucleotide region that is taxonomically informative; these include the V3, V5 regions of the bacterial 16S rRNA genes and the eukaryotic V9 region that is gaining popularity for sampling protist diversity.

Citation: Gloor GB, Hummelen R, Macklaim JM, Dickson RJ, Fernandes AD, et al. (2010) Microbiome Profiling by Illumina Sequencing of Combinatorial Sequence-Tagged PCR Products. PLoS ONE 5(10): e15406. doi:10.1371/journal.pone.0015406

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Introduction

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