GARDNERELLA VAGINALIS STRAIN DEPENDENT GROWTH AND CYTOTOXICITY IN HUMAN MONOCYTES

by

Corbett Ouellette

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Biology

Middle Tennessee State University

August 2018

Thesis Committee:

Dr. Anthony L. Farone, Chair

Dr. Mary B. Farone

Dr. Rebecca Seipelt-Thiemann

I dedicate this thesis to my parents, Dawn and Bill Ouellette, who have always believed in me no matter the situation, and bent over backwards to ensure that I received a proper education, even when it meant driving to schools on opposite sides of the city. Without their push and effort, I never would have fallen in love with science as much as I have, and for that, I will forever be grateful.

ACKNOWLEDGEMENTS

I would not have been successful without each and every person who has helped me along the way to achieve this milestone, and for that, I am eternally appreciative.

First, I would like to thank both Dr. Tony and Dr. Mary Farone, who have taught me lessons that reach far beyond the scientific realm. They have been there for me in times of need, treated me like family, and have impacted my life far beyond I had ever expected any advisor would. They have introduced me to a true love and passion in research, and left a monumental, positive impact in my life. I would also like to thank *Dr. Seipelt*-Thiemann for her help and insight on *in silico* genome analysis and developing me into a better researcher, and Dr. Matthew Elrod-Erickson for being a fantastic introduction to the intricacies of science, particularly genetics.

Second, and just as influential, is my colleague, and now lifelong friend, Dr. Eric Vick. Prior to the hundreds of hours we spent together inside and outside of lab, we attended a small talk in an MTSU auditorium and the scientists in the audience were asked to raise their hands. My hand remained down. Eric swiftly encouraged me to raise mine, and the academic confidence he instilled in me is everlasting. I will have immense respect for his knowledge, and knew that I had to work with the brightest minds to become one of the same.

I also want to thank the rest of the Farone lab, the Biology Department staff, both faculty and especially the office, and everyone else that has pushed me to be the scientist I am today. My journey is far from over, and I have every one of you to thank.

As scientists we will never be content with who we are. Just as research in the scientific community will never cease, nor will our personal novel discoveries and growth.

ABSTRACT

Bacterial vaginosis (BV) is the most prevalent vaginal infection for women of reproductive age, characterized by a lack of normal commensal bacteria and a proliferation of pathogenic species. BV infections can lead to sterility, pelvic inflammatory disease, increased chance of HIV transmission, and preterm delivery. *Gardnerella vaginalis* has been found in many cases of BV, although *G. vaginalis* has been isolated in women exhibiting no diagnostic criteria. The goal of this study was to examine the growth kinetics and cytotoxicity of different *Gardnerella vaginalis* strains, which may provide important information in identifying growth conditions of asymptomatic BV. *G. vaginalis* comparison studies utilized strains 14018, 14019, and 49145, and were grown in pure bacterial culture in brain-heart infusion (BHI) and in Roswell Park Memorial Institute (RPMI) medium, or co-cultured with undifferentiated or differentiated human monocyte cells (THP-1) in RPMI. Significant differences in growth were noted among all strains in BHI and RPMI ($p < 0.05$). Further experiments showed that there is a significant difference in the cytotoxicity of the strains when in co-culture with THP-1 cells ($p < 0.01$), and may be attributed to either different growth patterns and metabolic proteins, or possibly due to further genetic differences found among virulence factors. In silico analysis of the three strains was done to provide supporting information relevant to the growth differences. The results of this study may contribute to a better understanding of how the genetic differences found within the different strains of *Gardnerella vaginalis* correlate with differences BV.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

RESULTS

I. INTRODUCTION

Bacterial vaginosis (BV), previously described as nonspecific vaginitis, is a condition that occurs when the natural microbiota of the vaginal tract, consisting primarily of gram-positive *Lactobacillus* spp., is disrupted and replaced by other, sometimes pathogenic, bacteria (Holmes *et al.* 1981). BV can be diagnosed using Amsel criteria or Nugent scoring, both of which include the microscopic detection of vaginal clue cells, which are epithelial cells covered with or surrounded by numerous gram-positive and gram-negative bacilli and coccobacilli (Figure 1). Amsel criteria require that patients exhibit three of the four following symptoms: 1) thin, white-yellow, homogenous discharge, 2) clue cells in vaginal discharge examined microscopically, 3) vaginal pH greater than 4.5, and 4) detection of a fishy odor following mixture with 10% potassium hydroxide to vaginal discharge (Amsel *et al.* 1983). For Nugent scoring, a Gram stain of vaginal exudate is examined for the presence of clue cells (Nugent *et al.* 1991).

BV is of a concern due to its association with preterm delivery of low-birth weight infants (Hillier *et al.* 1995), as well as with an increase in human immunodeficiency virus transmission (HIV), pelvic inflammatory disease, and post-surgical infections (Sewankambo *et al.* 1997, Taylor *et al.* 2013). The National Health and Nutrition Examination Survey reported that nearly 30% of women in the United States between the ages 14 to 49 were positive for BV via Nugent scoring in 2001-2004 (Allsworth and Peipert 2007, Koumans *et al.* 2007). Risk factors for BV include numerous ethnic and economic demographics, numbers of sexual partners, douching frequency, smoking behaviors, and high body mass index (Koumans *et al.* 2007).

BV has been linked to a lack of commensal bacteria and the proliferation of potentially pathogenic bacteria. The loss of commensal *Lactobacillus* spp. results in decreased production of lactic acid and hydrogen peroxide, creating a less acidic environment. The increasing pH is optimal for growth of the bacterium *Gardnerella vaginalis* and other anaerobic bacteria associated with BV (Mijač *et al.* 2006,Saunders *et al.* 2007).

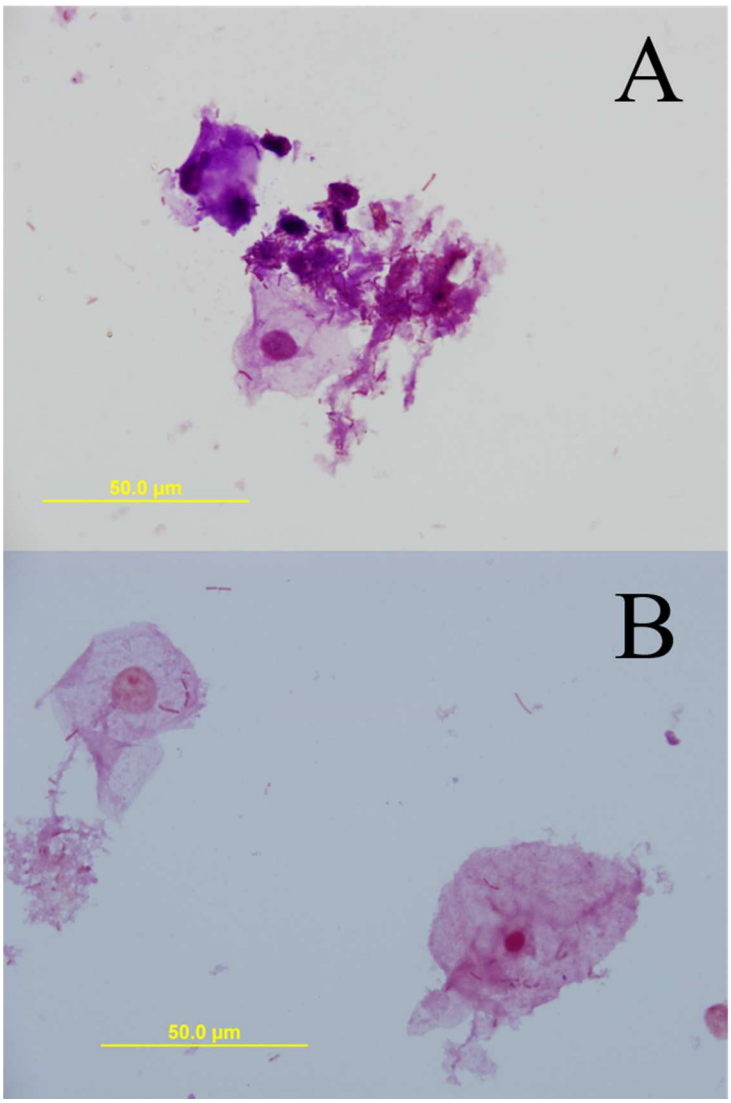

Figure 1. Clinical samples showing evidence of BV-positive and BV-negative vaginal smears. Samples were Gram stained and observed by light microscopy. (A) Cells from a patient who met Amsel criteria for a positive diagnosis of BV. The excessive coating of vaginal epithelial cells with gram-variable bacteria is a typical example of clue cell morphology. (B) Cells from a patient who did not meet Amsel criteria for a positive BV diagnosis. Epithelial cells with scattered gram-positive bacilli are visible.

BV is considered a polymicrobial infection characterized by loss of gram-positive *Lactobacillus* spp. and growth of other dysbiotic gram-positive and gram-negative anaerobic bacteria, which includes, but is not limited to the following organisms: *G. vaginalis*, *Mycoplasma hominis, Mobiluncus mulieris, Peptostreptococcus* spp., *Atrobium* spp., and *Fusobacterium nucleatum.* The spectrum of BV-associated bacteria has expanded to include *Eggerthella* spp. and *Bifidobacterium* spp. (Fredricks *et al.* 2005, Menard *et al.* 2008, Oakley *et al.* 2008). To determine the potential impact of some of these bacteria in the pathogenesis of BV, Patterson *et al.* (2010) compared virulence traits of *G. vaginalis* to seven other bacteria recovered from vaginal tracts of women with BV. They compared adherence to cultured vaginal epithelial cells, biofilm formation, and cytotoxicity for vaginal epithelial cells. Of the bacteria examined, only *G. vaginalis* exhibited all of the virulence characteristics, suggesting that *G. vaginalis* may exhibit an increased contribution to etiology. Although *G. vaginalis* has been detected in up to 76% of BV cases, *G. vaginalis* has also been detected in women without symptoms of BV, indicating that the detection of *G. vaginalis* is not necessarily indicative of disease due to strain differences (Smayevsky *et al.* 2001).

G. vaginalis was initially described by Leopold in 1953 and further characterized by Gardner and Dukes in 1955 (Dunkelberg 1991). First classified as *Haemophilus vaginalis* due to its isolation on blood bilayer medium used for *Haemophilus* spp., it was later placed into the new genus *Gardnerella* (Gardner and Dukes, 1955). This genus was originally described as including gram-negative and gram-variable bacteria that do not produce catalase or oxidase enzymes and are facultative anaerobes, synthesizing acetic

acid as the primary product of fermentation (Gardner and Dukes 1955, Greenwood and Pickett 1980). *G. vaginalis* is currently the only species in the genus.

G. vaginalis typically stains as a gram-variable coccobacillus, exhibiting both gram-positive and gram-negative cells. Electron microscopy indicated that the bacterium has a thin layer of peptidoglycan, typical of gram-negative bacteria, but does not possess the outer cell envelope of gram-negative bacteria. Chemical analyses established that the bacteria do not produce gram-negative lipopolysaccharide, but rather the lipoteichoic acid of gram-positive bacteria, confirming *G. vaginalis* is a gram-positive bacterium that can also stain as gram-negative due to its thin cell wall (Sadhu *et al.* 1989).

The isolation of *G. vaginalis* involves multiple selective and differential media, due to the polymicrobial nature of the vaginal microbiota. *G. vaginalis* can be differentiated by beta-hemolysis on human blood bilayer Tween 80 agar, a gram-variable reaction in Gram stains, and a negative catalase test (Piot *et al.* 1982). Other diagnostic tests for *G. vaginalis* include pulsed-field gel electrophoresis and PCR (Aroutcheva *et al.* 2001). Screens for resistance to antibiotics, such as to amphotericin B, gentamicin, and nalidixic acid, have also been incorporated into diagnostic testing (Catlin 1992).

The limited biochemical tests available for identifying *G. vaginalis* are indicative of the phenotypic differences between isolates. These strain, or biotype, differences between *G. vaginalis* isolates were recognized during early taxonomic studies to distinguish *Haemophilus vaginalis* as a new genus using biochemical and DNA hybridization assays (Greenwood and Pickett 1980, Piot *et al.* 1980). Among the strains first characterized were those submitted to the American Type Culture Collection (ATCC), ATCC 14018 and 14019, both of which were isolated from women with clinical

signs of BV (Gardner and Dukes 1955). ATCC strain 49145 is also a clinical isolate (ATCC 2018). ATCC strains 14018, 14019, and 49145 are the only *G. vaginalis* isolates that have been acquired, authenticated, produced, preserved, and distributed by the ATCC to date.

Strains of *G. vaginalis* have been differentiated using both phenotypic and genotypic approaches (Piot *et al.* 1984, Benito *et al.* 1986, Ingianni *et al.* 1997). Several studies have shown a relationship between these *G. vaginalis* strains and symptoms or severity of BV (Atroucheva *et al.* 2001, Tosun *et al.* 2007, Santiago *et al.* 2011, Pleckaityte *et al.* 2012, Schellenberg *et al.* 2016). Some of the observed associations are related to the reported virulence properties of the bacterium, which include production of the enzymes vaginolysin and sialidase, as well as biofilm formation (Patterson *et al.* 2010, Castro *et al.* 2015,).

Vaginolysin is a cholesterol-dependent cytolysin that is specific for human cells (Rottini *et al.* 1990, Cauci *et al.* 1993, Gelber *et al.* 2008). Vaginolysin lyses human erythrocytes and also induces membrane blebbing on the surfaces of vaginal epithelial cells (Randis *et al.* 2013). Vaginolysin binds to CD59 on human erythrocytes and vaginal epithelial cells, resulting in pore formation and membrane disruption (Cauci *et al.* 1993, Gelber *et al.* 2008). Increased expression of vaginolysin has been detected in strains isolated from women with BV when compared to strains isolated from women without symptomatic BV; however, no relationship between *in vivo* vaginolysin and BV have been confirmed (Castro *et al.* 2015).

Sialidases cleave sialic acid residues from the surfaces of cells and are used by bacteria to obtain substrates for energy prodction and to expose binding sites of cell

surfaces (Kim *et al.* 2011). Sialidase activity has been detected in women with BV, and sialidase activity is also associated with destruction of the vaginal mucus layer (Briseldin *et al.* 1992, Lewis *et al.* 2013, Moncla *et al.* 2016). The exposure of glycans on cellular surfaces and mucus destruction by sialidases could increase binding sites for *G. vaginalis* adhesion to the vaginal epithelium. A putative gene for a sialidase has been identified in some strains of *G. vaginalis,* and is present in 75% of strains associated with BV (Hardy *et al.* 2017, Schellenberg *et al.* 2017). High numbers, ≥ 106 genome equivalents per ml, of the putative sialidase gene in vaginal fluid, as detected by qPCR, were also strongly associated with increased biofilm formation in BV cases (Hardy *et al.* 2017).

Biofilm formation in *G. vaginalis*-associated BV is linked to both antibiotic resistance and high rates of BV reoccurrence (Swindinski *et al.* 2005, Munzy and Schwebke 2015). Biofilms might also allow *G. vaginalis* to counter the natural resistance mechanisms of the vaginal environment by providing protection from hydrogen peroxide and lactic acid (Patterson *et al.* 2007). Recent transcriptomic analyses of *G. vaginalis* growth in planktonic vs. biofilm environments detected increased expression of a gene encoding for a putative protein that may be involved in masking the bacterium from the host immune defenses, the Rib protein (Waldemarrson *et al.* 2006, Yeoman *et al.* 2010, Castro *et al.* 2017).

Immune defenses that protect against the formation of biofilms and the onset of disease in the female lower genital tract include innate barrier of multiple layers of non-keratinized epithelial cells, which protect the underlying tissue from damage during intercourse and act as a mechanical barrier against invading pathogens (Eschenbach *et al.* 2001). A layer of mucus acts as a physical barrier against pathogens and is rich in

antimicrobial peptides which help clear invading microbes (Ming *et al.* 2007). The lamina propria of the vaginal tract includes T lymphocytes as well as phagocytic cells including monocytes, macrophages, neutrophils, and dendritic cells (Lee *et al.* 2015) Although BV is not typically associated with clinical indicators of inflammation, including infiltration of leukocytes, swelling, and pain, both cytokines and antibodies to *G. vaginalis* have been detected in women with BV (Cauci *et al.* 2002, Mitchell and Marrazzo 2014). Additionally, in a study by Vick *et al.* (2014), exposure of differentiated THP-1 human monocytes with *G. vaginalis* (ATCC 14018) *in vitro* resulted in significant increases in the inflammatory cytokines IL-1β, IL-18, and TNF-α. After 24 h of exposure, *G. vaginalis* had also caused significant cell death when compared to the unexposed controls, although cell viability was 66%. Cleavage of caspase-1 in *G. vaginalis*-treated THP-1 cells confirmed the cell death as pyroptosis in response to the inflammatory stimulation. Vick (2014) subsequently compared inflammatory responses of THP-1 cells to different *G. vaginalis* strains. Strain ATCC 14019 did not induce an inflammatory response equivalent to the other two strains ATCC 14018 and ATCC 49145.

Differences in inflammation and cell death in response to co-culture of THP-1 cells with different strains of *G. vaginalis* suggests that different *G.* vaginalis strains have different phenotypic and/or genotypic characteristics that activate inflammation and initiate cell death at various levels. Since the amount and types of bacteria in the vaginal tract have been linked to cytokine responses (Kremleva and Sgibnev 2016). Strain growth characteristics are of great interest and led to three overarching goals to this study.

Project Aims:

1) To compare the growth of *G. vaginalis* strains ATCC 14018, 14019, and 49145 in various media, a) brain heart infusion medium, b) RPMI 1640 complete medium used for culture of THP-1 cells;
2) To compare growth of strains with undifferentiated and differentiated THP-1 cells; and
3) To compare the sequenced genomes of these strains genetic variation that could potentially affect the growth of these bacteria.

II. MATERIALS AND METHODS

Monocyte culture conditions

THP-1 human monocyte cells (ATCC TIB-202) were grown in complete RPMI 1640 medium (RPMI; Sigma-Aldrich), which contained 10% fetal bovine serum (Atlanta Biologicals), 100 IU penicillin, and 100 µg/mL streptomycin (both from Sigma-Aldrich). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were grown for 3-5 days and then passaged at a 1:5 dilution into new, complete RPMI. Alternatively, when using cells for experiments, cells were split at a 1:2 dilution with new, complete RPMI to maintain a high cell count in the flasks for infections.

Bacterial culture conditions

G. vaginalis strains were purchased from the ATCC and included strains 14018 (ATCC 14018), 14019 (ATCC 14019), and 49145 (ATCC 49145). Bacteria were grown at 37°C with 5% CO_2 on brain heart infusion agar (BHI; Becton Dickinson). Freshly streaked plates were incubated for 3-5 days to ensure purity of the cultures before subculture to broth. *G. vaginalis* strains were grown in BHI broth (Becton Dickinson) at 37°C in a humidified atmosphere with 5% CO_2 for 48 h before harvesting for experiments.

Growth curves for **Gardnerella vaginalis**

G. vaginalis strains were harvested from a 48 h starter BHI broth culture. The optical densities of cultures were measured at 600 nm (OD_{600}) by spectrophotometer

(Thermo-Scientific Spectronic 20D+) to determine cell density utilizing a coefficient determined from previous experiments:

[(Starter culture OD_{600}) x 6.55 x 10^7 cells/mL] / 0.1 OD_{600}

A volume representing 1 x 10^7 cells was removed from each of the strain starter cultures and placed into microcentrifuge tubes in triplicate and pelleted at 12,000 *x g* for 10 min at 4°C in a microcentrifuge (Hermle Labnet, Z33MK). Supernatant was discarded and the cell pellet was resuspended in 500 µL of either BHI or complete RPMI without antibiotics and then added to 4.5 mL of the same medium. The OD_{600} was measured and this was designated as the time = 0 point. Incubation of the cultures was continued at 37°C in a humidified atmosphere with 5% CO_2.

For each time point, culture tubes were removed from the incubator and shaken for 30 seconds individually by vortexing to disrupt biofilm formation, and evenly resuspend bacteria. The OD_{600} was measured every 4 h until the beginning of log phase, after which the OD_{600} was measured every hour to ensure proper determination of growth patterns until stationary phase. Optical density measurements were continued to 48 h. The statistical analysis of data was processed via two tail, heteroscedatic t-test, and analyzed via Prism7 (GraphPad Software) with statistical significance at $p < 0.05$.

Co-culture of* Gardnerella vaginalis *and THP-1 cells

Undifferentiated THP-1 cells were transferred from tissue culture flasks to 15 mL conical centrifuge tubes (Fisher Scientific) and centrifuged at 400 *x g* for 5 min at 4°C (Thermo-Scientific, Sorvall Legend XTR). The supernatant was removed and cells were

reconstituted and washed with 5 mL of phosphate-buffered saline (PBS; Sigma-Aldrich) to remove residual antibiotics by centrifugation again at 400 *x g* for 5 min at 4°C, repeating the wash process twice. Supernatant was removed and the cell pellet was resuspended with complete RPMI without antibiotics. Alternatively, experiments requiring differentiated THP-1 cells had 5 x 10^5 cells/mL seeded into wells of a 24-well plate following the washing protocol described above. Cells were differentiated into macrophages by the addition of 100 nM phorbol myristate acetate per well (PMA; Sigma-Aldrich) and incubation for 48 h prior to bacterial treatment. Cell concentration was determined by hemocytometer count and THP-1 cell viability was assessed via trypan blue exclusion. Briefly, desired dilutions of cells were mixed at a 1:1 ratio with 0.4% trypan blue in PBS (Sigma-Aldrich) and incubated at room temperature for 10 min. A 10 µl sample of the mixture was then pipetted into the well on each side of a hemocytometer with a coverslip (Fisher Scientific). Cells on the hemocytometer were observed at 20X magnification on an Olympus CX31 microscope. Cells that did not stain blue were counted and considered viable. A total cell count was taken, including both clear and blue-stained cells. Percent viability was determined by the following calculation:

(# of viable cells) / (# of total cells) x 100

Cell concentrations were adjusted to 5 x 10^5 viable cells/mL with complete RPMI without antibiotics and 500 µL of the suspension was pipetted into individual wells of a 24-well plate (Corning Costar).

For bacterial treatment of THP-1 cells, the OD_{600} was measured via spectrophotometer (Amersham Biosciences, GeneQuant Pro) for *G. vaginalis* strains grown for 48 h in BHI broth. Population density of the starter culture was determined as described above and the appropriate volumes of *G. vaginalis* culture was transferred to microcentrifuge tubes and centrifuged at 12,000 *x g* for 10 min to yield a multiplicity of infection (MOI) of 5. Following centrifugations, supernatants were discarded. For treatment of undifferentiated THP-1 cells, triplicate pellets were each resuspended with 500 µL of 2.5 x 10^5 THP-1 cells in complete RPMI without antibiotics, followed by addition of each triplicate sample to an individual, empty well of a 24-well plate. For *G. vaginalis* treatment of differentiated THP-1 cells, bacterial cell pellets were resuspended with 500 µL of complete RPMI without antibiotics. Before addition of the resuspended bacteria, spent medium was removed from the previously differentiated THP-1 wells, and new medium with *G. vaginalis* was added. Co-cultures were incubated at 37°C in a humidified incubator with 5% CO_2. All co-culture infections were conducted in triplicate for each time point, strain, and cell type.

Gardnerella vaginalis *quantification from co-cultures*

At appropriate time points, cells and media in co-culture wells were triturated and bottoms of wells scraped gently with a pipette to ensure that THP-1 cells were dislodged from the well. After cell removal, wells of the 24-well plate were examined by inverted microscope (Nikon TMS) to ensure that cells had been properly dislodged for both undifferentiated and differentiated cells. Contents of each well were transferred to individual sterile microcentrifuge tubes and centrifuged at 12,000 *x g* for 10 min at 4°C to

pellet both the bacteria and the THP-1 cells. The supernatant was discarded and each cell pellet was reconstituted with 500 µL of sterile, distilled water (dH_2O) to lyse the THP-1 cells. Each cell suspension was serially diluted tenfold in dH_2O to a final dilution of 1 x 10^{-5}. A volume of 100 µL of each of the 1 x 10^{-3}, 1 x 10^{-4}, and 1 x 10^{-5} dilutions was spread evenly onto the surfaces of freshly prepared BHI agar plates and the inoculated plates were incubated for 72 - 120 h at 37°C and 5% CO_2. Colony-forming units (CFUs) were counted from plated dilutions with 30-300 colonies and averaged to yield a total cell concentration at each harvest time, for each strain, and THP-1 cell type, independently. Total cell counts were compared against the previously recorded mean to yield the fold increase using the following formula:

(# of current CFUs - # of CFUs at 0 h) / (# of CFUs at 0 h)

With the exception of the time = 0 point, which was used as the baseline, fold changes are displayed with error bars that correspond with the standard error associated with the differences in fold-increase or decrease among samples. The statistical analysis of data was processed via two-tail, heteroscedatic t-test, and analyzed via Prism7 (GraphPad Software), using the untreated sample as a control and statistical significance at $p < 0.05$.

THP-1 viability in Gardnerella vaginalis *co-cultures*

At 0, 12, 24, and 48 h, cells in co-culture wells were triturated and scraped with a pipette to ensure that THP-1 cells were dislodged from the wells. Contents of each well were transferred to a microcentrifuge tube and spun at 400 *x g* for 5 min at 4°C to pellet the THP-1 cells. Supernatant was transferred to a new microcentrifuge tube and the cell

pellet was reconstituted in 500 µl of sterile antibiotic-free RPMI medium. Reconstituted cell pellets were serially diluted 10-fold to reach a final dilution of 1×10^{-2} to adjust cell density for counting via hemocytometer by trypan blue exclusion.

Cell viability was recorded for all three replicates of both undifferentiated and differentiated THP-1 cells for each treatment. The statistical analysis of data was processed via two-tail, heteroscedatic t-test, and analyzed via Prism7 (GraphPad Software), using the untreated sample as a control and statistical significance at $p < 0.05$

In situ *genomic analysis*

The genome sequences of *G. vaginalis* strains ATCC 14018 (NCBI:txid585528), ATCC 14019 (NCBI:txid525284) and ATCC 49145 (NCBI:txid2702) were obtained from the GenBank database at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/genome) and annotated with Rapid Annotation using the Subsystem Technology (RAST; http://rast.nmpdr.org) server. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation server (KAAS; www.genome.jp/kegg/kaas/) was used to analyze metabolic pathway differences between the three strains. Protein coding sequences of the *G. vaginalis* strains, obtained from the RAST server, were submitted to KAAS for comparison against manually curated KEGG genes using the GHOSTZ homology search tool (www.bi.cs.titech.ac.jp/ghostz/) and bi-directional best hit method. Venn diagrams were generated using Venny software (version 2.1; http://bioinfogp.cnb.csic.es/tools/venny/) to compare the numbers of unique and overlapping orthologs between the three strains.

III. RESULTS

Growth characteristics of Gardnerella vaginalis *using BHI media*

To assess phenotypic differences between *G. vaginalis* strains 14018, 14019, and 49145, bacterial growth characteristics were observed when bacteria were grown on BHI agar plates and in BHI broth. BHI is a medium routinely used to culture *G. vaginalis* and contains bovine muscle and milk peptone mixtures, protein extracts from bovine brain and heart, D-glucose, and sodium chloride (Thermo Fisher Scientific, 2018). Morphology of bacterial growth such as colony size, color, density, texture, and elevation can be distinguishing characteristics of bacteria, including the appearance of growth in broth cultures. After 48 h of growth on BHI agar in a 37°C humidified atmosphere with 5% CO_2 without shaking, strains 14018 and 49145 grew as small-medium, white, opaque, domed colonies with smooth edges, while strain 14019 exhibited smaller, white, translucent, domed colonies with smooth edges (Figure 2). There were also visible differences when culturing in BHI broth. Strain 14018 grew as a confluent biofilm on the bottom of glass tubes with very few cells in suspension. Strain 14019 exhibited pinpoint colony-like growth on tube bottoms with some cells adhered to the bottom of the glass tubes. Strain 49145 grew in suspension resulting in a dense, cloudy appearance to the medium, and a confluent biofilm was also visible on the bottom of the tube.

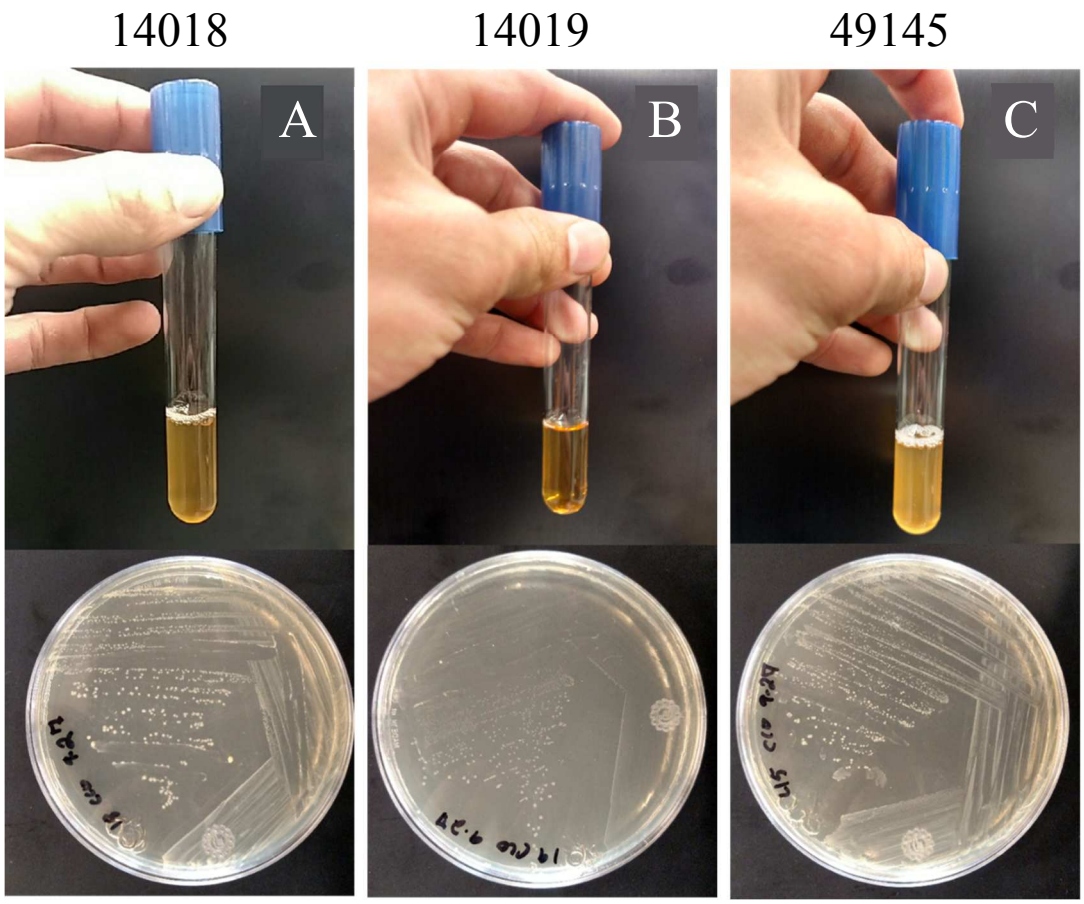

Figure 2. Qualitative growth analysis of *G. vaginalis* on BHI agar and in BHI broth media. Prior to inoculation, freshly streaked plates were incubated for 3-5 days to ensure for purity of the cultures before subculture to 5 ml of BHI broth or on BHI agar plates for 48 h. Incubation of the cultures was continued at 37°C in a humidified atmosphere with 5% CO_2 and 5 ml BHI broth started at an initial volume of 1 x 10^7 cells/ml. Images were taken with an Apple iPhone 4S.

Gardnerella vaginalis ***growth curves in BHI and RPMI 1640 media***

To quantify growth differences between strains, growth curves of *G. vaginalis* strains, in both BHI and complete RPMI 1640 without antibiotics (hereafter referred to as RPMI) media were generated by measuring the optical density at 600 nm (OD_{600}) at 0, 4, 8, 12, 16, 20, and 24 h post-inoculation (Figure 3). In BHI, strains 14018 and 49145 began exponential growth by 8 h and reached maximum growth by 18 h, increasing at least 50-fold in optical density. Strain 49145 exhibited an increase in density of over 70-fold, which was significantly higher than the growth of strains 14018 and 49145 as determined by t test ($p < 0.05$). Conversely, strain 14019 did not begin to grow exponentially until after 12 h and exhibited a slower growth rate, increasing less than 10-fold by 24 h (Figure 3A).

Growth in complete RPMI medium without antibiotics was also assessed to determine if this this medium type would influence strain growth, since it is a common medium type used for culturing mammalian cells. RPMI 1640 is a buffered medium used for the growth of cell lines with which *G. vaginalis* has been co-cultured (Vick *et al.* 2014). The medium contains 20 amino acids, D-glucose, growth factors and salts (Thermo Fisher Scientific 2018). The medium is supplemented with 10% FBS. FBS is collected sera from fetal calves and the composition of the serum can vary; however, the serum is generally rich in proteins and also contains glucose and other carbohydrates, as well as growth factors, hormones, cytokines, and nitrogenous compounds such as urea (Brunner *et al.* 2010).

In RPMI, strain 14018 began exponential growth by approximately 8 h and significantly surpassed the growth of the other strains by 12 h ($p < 0.05$), but only

increased by 20-fold in density by 24 h and did not appear to have reached stationary phase (Figure 3B). Strain 49145 began increasing exponentially between 8-12 h but had increased less than 10-fold by 24 h, also without reaching stationary phase. Strain 14019 exhibited very little increase in growth by 24 h. When comparing the two media, all three strains grew much better in BHI, so BHI was chosen as the growth medium used to grow *G. vaginalis* strains prior to all THP-1 experiments to ensure substantial cell numbers.

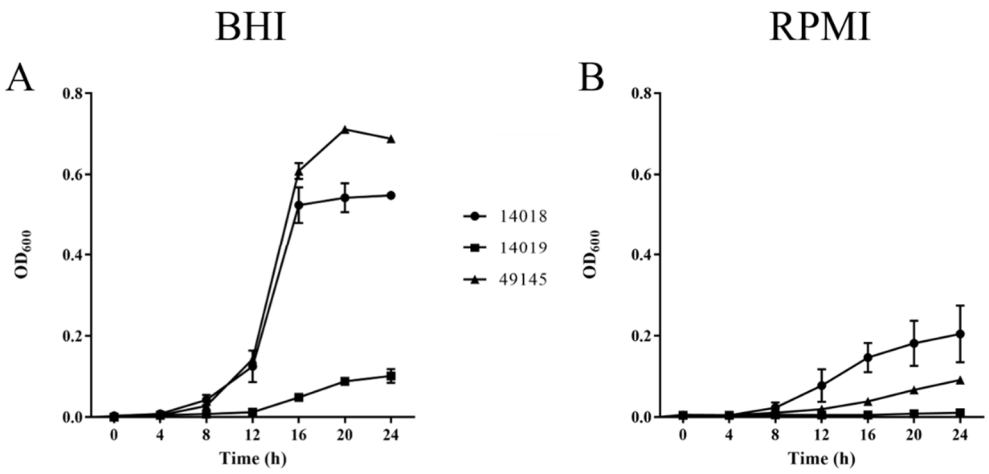

Figure 3. Quantitative growth of *G. vaginalis* strains cultured in BHI and RPMI liquid media. Sterile tubes of BHI (A) and serum-free, antibiotic-free RPMI (B) were inoculated with 2 x 10^6 CFU/mL of *G. vaginalis* as determined spectrophotometrically. After inoculation into the medium, an initial spectrophotometer measurement was taken, after which cultures were incubated at 37°C in a humidified atmosphere in 5% CO_2 without shaking. Optical density at 600 nm (OD_{600}) was measured by removing tubes from the incubator, vortexing for 30 seconds to resuspend cells for accurate readings, and placing tubes directly into the spectrophotometer, such that no volume was removed from the tubes. The OD_{600} was measured every 4 h until 24 h. Results are displayed as a mean of three, independent replicates, accompanied by standard error.

Growth of* Gardnerella vaginalis *in co-culture with human THP-1 cells

To determine whether co-culture with a human monocyte cell line could influence *G. vaginalis* growth in a strain dependent manner, growth rates of each strain of *G. vaginalis* were investigated in co-culture with undifferentiated and differentiated THP-1 human monocytes. These cells were derived from a male child with leukemia and can be maintained as monocytic cells in suspension, or differentiated into macrophage-like cells (Tsuchiya *et al.* 1980). For these studies, it was necessary to measure bacteria by the viable plate method, because the presence of the THP-1 cells would interfere with optical density measurements. For these treatments, individual strains of *G. vaginalis* were adjusted to 1 x 10^7 CFU and diluted such that cells would be infected with an MOI of 5. Bacteria were incubated with undifferentiated and differentiated THP-1 cells for 0, 12, 24, and 48 h. Following incubation, cells and supernatants were collected as described in the Materials and Methods, and dilutions of the concentrated cells were plated onto BHI agar plates and incubated for 48 h before counting bacterial colonies. The bacterial cell number in CFU/mL and the fold increase in bacterial cell number in reference to the time = 0 point were calculated.

When co-cultured with both undifferentiated and differentiated THP-1 cells, strain 49145 exhibited the greatest amount of growth with an almost 6-$\log_{10}$ increase in bacterial concentration in culture with undifferentiated cells and a 5-$\log_{10}$ increase in differentiated cell co-culture. When strain 14018 was co-cultured with THP-1 cells, the bacteria exhibited only a 10-fold increase in cell number by 24 h in co-culture with both undifferentiated and differentiated cells, after which time, bacterial cell numbers decreased by 48 h. Strain 14019 exhibited a 2-$\log_{10}$ increase in cell number in culture

with undifferentiated THP-1 cells, but did not exhibit any significant change in cell number in differentiated cells.

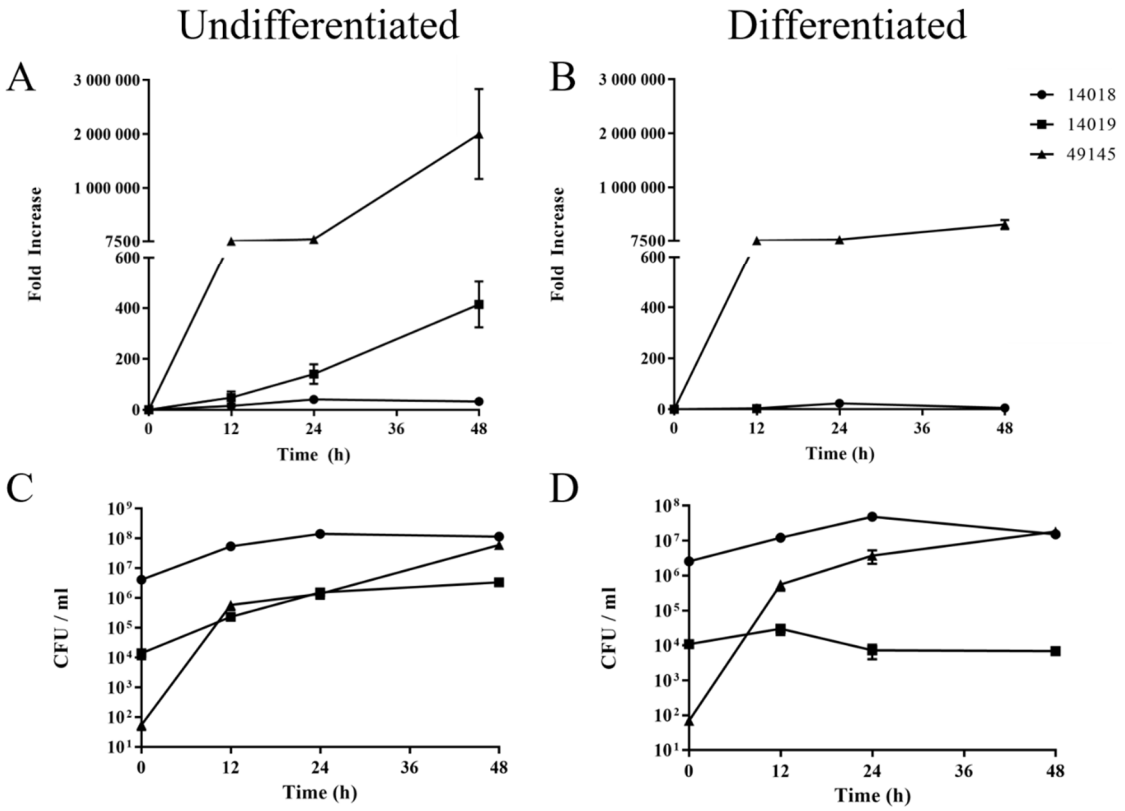

Figure 4. Growth of *G. vaginalis* during co-culture with undifferentiated and differentiated human THP-1 cells. *G. vaginalis* strains 14018, 14019, and 49145 were incubated with both undifferentiated (A and C) and differentiated (B and D) THP-1 cells in RPMI. Cells were collected at 0, 12, 24, and 48 h and assessed for bacterial cell numbers. Cell numbers are shown as concentrations of bacteria (CFU/mL) and fold increase in bacteria compared to the time 0 point. The concentration (C) and fold

increase (A) of *G. vaginalis* strains in undifferentiated THP-1 co-culture, and the concentration (D) and fold increase (B) of *G. vaginalis* strains in differentiated THP-1 co-culture. Error bars represent the standard error of triplicate cultures.

Viability of human THP-1 cells in co-culture with* Gardnerella vaginalis *strains.

While strain-dependent growth of the bacteria has been the focus of this study, strain-specific cytotoxicity for human cells may be a factor in the immune response to *G. vaginalis* and consequently the onset of BV. It was therefore of interest to determine THP-1 cell viability for both monocyte and macrophage cell types when co-cultured with *G. vaginalis* strains. Viability of THP-1 cells was determined at 0, 24, and 48 h post-treatment. At the time = 0 point, there were statistically significant differences between untreated and bacterial-treated THP-1 cells for both undifferentiated and differentiated cultures. However, viability of all cells was greater than 93% and differences in viability may represent initial plating errors. For example, the viability of strain 14018-treated cells is statistically significantly greater than for untreated cells (Figures 5A-B).

At 24 h of bacterial exposure, which represents the time point at which Vick (2014) assessed differences in inflammatory responses to *G. vaginalis* strains, the viability of bacterial-treated THP-1 cells was significantly decreased compared to untreated controls for both undifferentiated and differentiated cells ($p < 0.001$); however, viability of THP-1 cells for all bacterial treatments remained above 85% (Figure 5A-B).

The viability of undifferentiated cells treated with the three different strains were all significantly different from one another ($p < 0.01$, Figure 5A), but strain 14019-treated cells were significantly lower in viability compared with the two other strains. At 24 h, bacterial cell concentration averaged 1.43 x 10^8 CFU/mL for strain 14018, but 1.56 x10^6 CFU/mL for strain 14019 and 1.39 x10^6 CFU/mL for strain 49145 (Figure 4A), such that THP-1 cell viability did not correspond to bacterial concentration.

For differentiated THP-1 cells at 24 h, cells treated with strains 14019 and 49145 were significantly lower in viability than cells treated with 14018 (Figure 4B, $p < 0.001$), and the viability of strain 14019-treated cells was significantly less than for strain 49145 (Figure 4B, $p < 0.01$), although bacterial cell concentrations ranged from approximately 7.32 x 10^3 CFU/mL for strain 14019 to 3.74 x 10^6 CFU/mL for 49145, while strain 14018 which was at the highest concentration of approximately 4.89 x 10^7 CFU/mL. These results imply that bacterial cell concentration may not be the only factor affecting viability of the THP-1 cells.

By 48 h, viability of all treated THP-1 cells decreased to less than 80% with all treatment groups being significantly less viable than untreated cells ($p < 0.001$, Figures 5A-B). For undifferentiated THP-1 cells, the viability of strain 14018-treated cells was significantly less at 75% ($p < 0.001$) than for strain 14019 and 49145 treatments, although there had been no increases in strain 14018 bacterial cell numbers (Figures 4A and 5A). The viability of strain 49145-treated cells had also significantly decreased relative to strain 14019 treatments ($p < 0.01$, Figure 5A); strain 49145 did increase in cell number from 24 to 48 h, while strain 14019 did not (Figures 4A and 4C). For

differentiated THP-1 cells at 48 h of bacterial treatment, strain 14018-treated cells were significantly less viable compared to the other two strains ($p < 0.05$, Figure 4B), even though bacterial concentration for strain 14018 had decreased (Figures 4B and 4D). There were also no significant differences in viability between treatments with strains 14019 and 49145despite the cell concentration of strain 14019 being 2 $\log_{10}$-fold lower (Figures 4B and 4D).

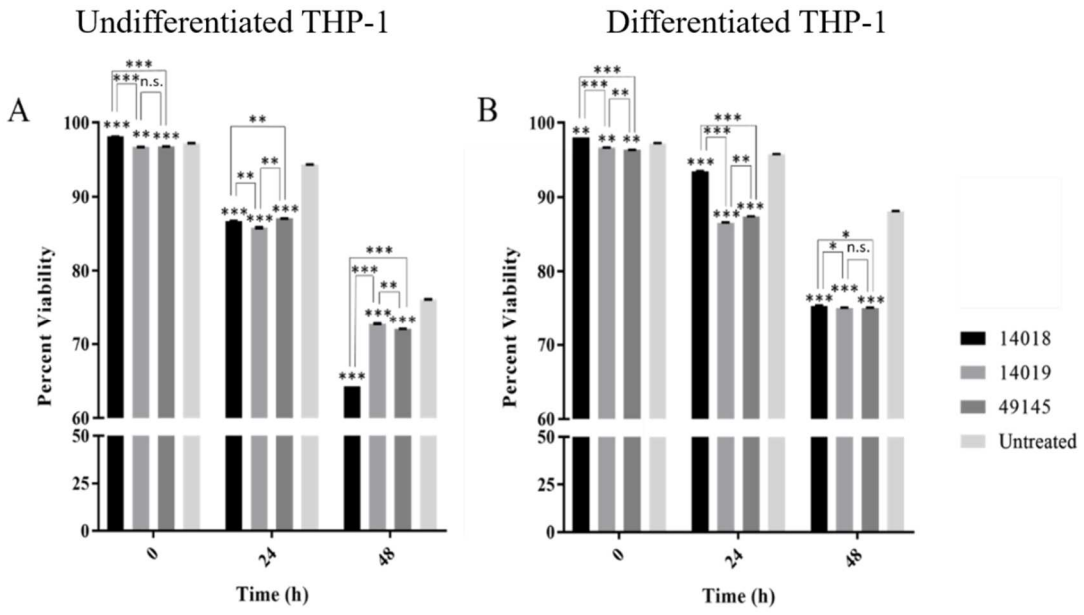

Figure 5. THP-1 cell viability when cultured with *Gardnerella vaginalis*. *G. vaginalis* strains were incubated with both undifferentiated (A) and differentiated (B) THP-1 cells and harvested at 0, 24, and 48 h for cell viability staining using trypan blue exclusion and a hemocytometer for microscopic enumeration. Values are the mean viability observed with error bars representing standard deviation. Statistical difference between co-culture viability compared to untreated samples of the same time-point was determined by a two-tailed t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant).

To discriminate against "natural" untreated cell death, the difference of cell viability was subtracted from the 24 h and 48 h time point from time point 0 h to determine the amount of cell death due to strain exposure (Table 1).

Table 1. *G. vaginalis* toxicity on THP-1 cells after accounting for untreated death.

Undifferentiated Adjusted % Cell Death			
Time	14018	14019	49145
24 h	8.52%	7.98%	6.81%
48 h	12.75%	2.88%	3.65%

Differentiated Adjusted % Cell Death			
Time	14018	14019	49145
24 h	3.05%	8.67%	7.5%
48 h	13.45%	12.38%	12.08%

In silico *analysis of Gardnerella vaginalis strains.*

In order to better understand genetic differences that might be responsible for differences in growth and ability to influence mammalian cell viability between these three *G. vaginalis* strains, a genomic comparison of the strains was performed. The genomes of strains 14018 and 14019 have been sequenced through the Human Microbiome Project and some comparative genomics have been completed on these strains (Yeoman *et al.* 2010). The draft genome of strain 49145 has recently been published, although the virulence or metabolic features of this genome have not been explored (Kidane *et al.* 2017).

All of the genes identified in *G. vaginalis* strains are putative, with the exception of the vaginolysin gene, which has been cloned and expressed (Gelber *et al.* 2008). Further study is necessary to identify the activity of the gene products, especially those that differ between these and other strains of *G. vaginalis,* in order to assess their roles in *G. vaginalis* growth in different environmental conditions.

The genome of strain 49145 is larger (1,706,848 bp) compared with the genomes of 14018 and 14019 (1,667,406 and 1,667,350 bp, respectively), and has more putative coding regions for genes (1363) compared to genes for strains 14018 (1309) and 49145 (1324) (Table 1). Strain 49145 also possesses more genes with putative assigned functions (1013) compared to strains 14018 (983) and 14019 (997). All three strains have similar GC content, ranging from 41.2-41.4% and similar average protein sizes, ranging from 361-366 amino acids (Table 2). Approximately 25% of the genes for all three strains are as yet hypothetical. For this study, only those genes with putative functions were considered. The genome sequences for each strain were annotated and

inserted into the Venny software and organized the orthologs into a Venn diagram with the numbers of unique and overlapping orthologs (Figure 6). The three strains share 658 assigned coding regions. The two strains, originally deposited by Gardner and Dukes in 1955 as *Haemophilus vaginalis* 14018 and 14019, share 15 assigned gene coding regions, with strain 14018 having three unique genes and strain 14019 possessing one unique gene. Strain 49145, which has the largest genome of the three strains, shares four genes with strain 14018. No genes are shared between 14019 and 49145. Strain 49145 has an additional 21 unique gene coding regions compared to the other two strains.

Table 2. General genomic features of *G. vaginalis* strains 14018, 14019, and 49145.

General Genomic Analysis	14018	14019	49145
Size	1,667,406	1,667,350	1,706,848
GC Content	41.4	41.4	41.2
Average protein size (aa)	366	361	363
Genes	1308	1324	1363
Assigned Function	983	997	1013
Hypothetical	325	327	350

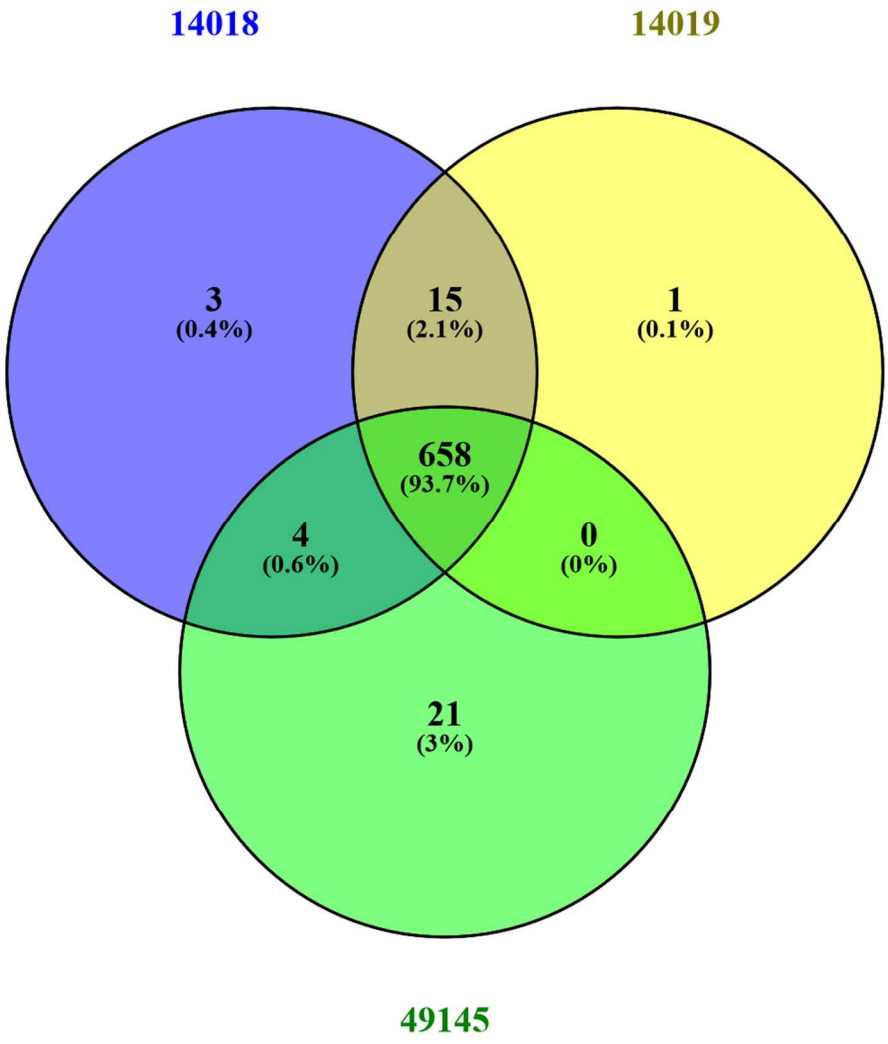

Figure 6. Orthology distribution between *G. vaginalis* strains 14018, 14019, and 49145. Genomic orthology was executed using Venn diagram software (Venny) by comparing orthology of classified proteins of the three *G. vaginalis* strains. The genome sequences obtained from NCBI for each strain were annotated via RAST and inserted into the Venny software and organized the orthologs into a Venn diagram with the numbers of unique and overlapping orthologs

Since growth influencing genes were of particular interest, KEGG analysis was used to predict metabolic pathways in all three strains. The genomes of all three strains include pathways for glycolysis as well as the metabolism of other sugars including glycogen, which is abundant in the vaginal tract. Differences in metabolic pathway genes between the strains were clearly present (Table 3). Genes for the enzymes for a complete tricarboxylic acid cycle were not identified, but all three strains possess putative genes for enzymes for the pentose phosphate pathway. Strain 49145 has a putative gene for a deoxyribose-phosphate aldolase, which could give this strain the ability to use extracellular deoxynucleosides as a unique carbon source via the pentose phosphate pathway (Lomax and Greenberg 1968). Strain 49145 also possesses a putative gene for an evolved β-D-galactosidase beta subunit not found in the other two strains, although all three strains have the β-galactosidase gene for lactose metabolism. Strain 49145 also contained a gene for a putative bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetyl-mannosamine kinase enzyme. In bacteria, this enzyme is typically associated with synthesis of sialic acid-containing carbohydrates found in capsules (Campbell *et al.* 2000). Capsule formation has not been reported in *G. vaginalis* strains, although the production of capsule by this strain in different environments should be investigated (Greenwood and Pickett 1980).

The genome of strain 14019 also contained a unique gene for carbohydrate catabolism, a putative gene for 2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase (*kduD*), which is associated with the degradation of pectin and alginate. However, this gene has also been reported as upregulated in *Escherichia coli* in individuals with a high lactose diet, resulting in the breakdown of hexuronates found in gastrointestinal mucus (Rothe *et*

al. 2013). This enzyme could potentially be used by *G. vaginalis* 14019 for the catabolism of vaginal mucus.

All of the strains have genes for fatty acid biosynthesis, although strain 49145 was missing the gene for the holo-[acyl-carrier protein] synthase, which is responsible for post-translational modification of acyl carrier proteins needed for synthesis of lipids and other molecules (Flugel *et al.* 2000). However, this analysis is based on amino acid similarity. This strain may possess a structural orthology that would have to be identified in other ways. Increased fatty acids have been detected in BV secretions in which *G. vaginalis* is present, and fatty acid production may be indicative of strains associated more often with BV (Srinivasan *et al.* 2015).

It has already been established that not all *G. vaginalis* strains possess the putative sialidase gene (Yeoman *et al.* 2010). The sialidase gene is not found in strains 14018 or 14019, but was detected in strain 49145. Sialidases cleave terminal sialic acid residues from large glycolipids or glycoproteins, such as mucins. Mucins are glycoproteins that are either membrane-bound or secreted. Secreted mucins are the primary components of the mucus layer that covers the vaginal epithelium and other mucosal surfaces. Bacteria use sialidases both to acquire nutrients from these complex carbohydrate molecules and as a mechanism to expose binding sites on cell surfaces (Lewis and Lewis 2012). Not much information is available for strain 49145 other than it was a clinical isolate; however, the presence of the putative sialidase is associated with increased virulence (Hardy *et al.* 2017).

Table 3. Differences in genes for metabolic pathways in *G. vaginalis* strains 14018, 14019, and 49145.

Metabolic Gene Analysis	14018	14019	49145	**Details**
ABC Transporters	**Multiple**	**Multiple**	**Multiple**	See Table 4.
Amino sugar Metabolism	Absent	Absent	**Present**	bifunctional UDP-N-acetylglucosamine 2-epimerase / N-acetylmannosamine kinase
Galactose Metabolism	Absent	Absent	**Present**	Evolved β-D-galactosidase, beta subunit
Glycan Degradation	Absent	Absent	**Present**	Sialidase-1
Pantothenate and CoA BioSynthesis	**Present**	**Present**	Absent	holo-[acyl-carrier protein] synthase
Pentose and Glucuronate Interconversions	Absent	**Present**	Absent	2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase
Pentose Phosphate Pathway	Absent	Absent	**Present**	deoxyribose-phosphate aldolase
Ribosomal Proteins	**Multiple**	**Multiple**	**Multiple**	See Figure 5
Sphingolipid metabolism	Absent	Absent	**Present**	Sialidase-1

For bacteria, metabolic activity is dependent upon the use of ATP-binding cassette (ABC) transporters. ABC proteins are transporters that use ATP hydrolysis to export or import large or charged molecules across the hydrophobic cell membrane lipid bilayer. Substrates for ABC transporters include carbohydrates, amino acids, peptides,

cofactors, and metals, all of which are important to bacterial metabolism (Wilkens 2015). Multiple differences were found between types of ABC transporters of the three *G. vaginalis* strains (Table 4). Strains 14018 and 14019 were identical in that they each possessed the same allele (*lplC*) for a putative aldouronate transport system permease and each also possessed genes for glucose/mannose transport and inositol-phosphate transport permeases. Strain 49145 was missing the glucose/mannose (*gtsB)* and inositol-phosphate (*inoF*) transport system permease genes. It did have allele *lplB* for the aldouronate permease but this allele has only 24.4% identity when comparing the amino acid sequence to that for *lplC* and has one less transmembrane domain (Swiss Institute of BioInformatics; Simple Modular Architecture Research Tool). Strain 49145 also had genes for putative ABC transporters not present in the other two strains. Genes for putative cysteine transport system substrate-binding proteins and cobalt/nickle transport system ATP-binding protein were found in strain 49145; both of these ABC transporters have been associated with bacterial pathogens (Tanaka *et al.* 2018).

Table 4. Specific differences in ABC transporters between *G. vaginalis* strains.

ABC Transporter Differences	14018	14019	49145
Putative aldouronate transport system permease protein	lplC	lplC	lplB
Glucose/mannose transport system permease protein	**Present**	**Present**	Absent
Inositol-phosphate transport system permease protein	**Present**	**Present**	Absent
Cysteine transport system substrate-binding protein	Absent	Absent	**Present**
Cobalt/nickel transport system ATP-binding protein	Absent	Absent	**Present**

Because protein synthesis is important for bacterial growth, ribosomal structure and tRNA number were compared between the three *G. vaginalis* strains (Table 5). Strains 14018 and 14019 each possessed two copies of the ribosomal RNA operon, each of which contains a single copy of the 5S, 16S, and 23S genes. Strain 49145 has two copies of the 5S rRNA gene, but only single copies of the 16S and 23S genes. However, because this is a draft genome, it is possible that the other two copies of the 16S and 23S genes may not have yet been assigned or are disrupted by segmentation of the genome during sequencing. Strains 14018 and 49145 each had 45 tRNAs, while 14019 only uses 34. This might imply a different codon usage which could affect growth rate (Sharp *et al.* 2010). Differences were also found in the numbers of ribosomal protein genes. Strains 14019 and 49145 each had 33 large subunit (LSU) genes. Strain 14018 had 32 LSU genes and was missing the gene for L14. All three strains were missing a gene for LSU 36. All strains were also missing the S21 small subunit (SSU) gene, but the genomes of strains 14019 and 49145 both had 20 SSU genes. Strain 14018 was missing subunits S10, S14, and S17 and had only 17 SSU genes.

Table 5. Putative ribosomal differences found in *G. vaginalis* strains.

Ribosomal Differences	14018	14019	49145
Number of tRNAs	45	**34**	45
Total large subunits genes (LSU)	**32**	33	33
Total small subunits genes (SSU)	**17**	20	20

IV. DISCUSSION

These studies were initiated with the goal to determine whether there were differences in growth between *G. vaginalis* strains 14018, 14019, and 49145 *in vitro* using the conditions for laboratory growth and co-culturing with cell lines used for inflammation studies (Vick *et al.* 2014). Phenotypic differences between *G. vaginalis* strains are already well established and include differences in mucin degradation, metabolic pathways, competitive exclusion, biofilm formation, and the production of siderophores, hemolytic toxin, and pili for cell adhesion (Smayevsky *et al.* 2001, Pleckaityte *et al.* 2012). Strain variability is also suggested by the presence of *G. vaginalis* in up to 76% of BV samples as well as in 1% of the same sampled population with no BV symptoms (Hill 1985). Variability in antibiotic resistance has also been demonstrated among strains of *G. vaginalis*, which may also be important in strain-dependent pathogenesis (Tosun *et al.* 2007, Nagaraja 2008, Tomusiak *et al.* 2011). Genotypic studies of *G. vaginalis* strains have also indicated variability in potential virulence genes between strains (Harwich *et al.* 2010, Yeoman *et al.* 2010). For example, a putative sialidase gene, the activity of which is associated with biofilm formation and proposed to be responsible for the destruction of the mucosal layer and subsequent colonization of *G. vaginalis,* has been found in only 75% of *G. vaginalis* isolates (Hardy *et al.* 2017). However, despite numerous studies both *in vivo* and *in vitro* to more clearly define differences in the involvement of *G. vaginalis* strains with BV, no published studies have compared the growth of *G. vaginalis* isolates, which may be important in examining the interactions of these bacteria with host cells.

In this study, visual analysis of *G. vaginalis* strains 14018, 14019, and 49145 grown in BHI broth or on BHI agar indicated observable differences in growth with strain 14019 exhibiting smaller colonies and less dense growth compared to the other two strains. Use of complete RPMI 1640 medium did not improve the growth of strain 14019 and resulted in decreased growth of strains 14018 and 49145 compared to growth in BHI broth.

To determine whether strain 14019 exhibited less vigorous growth in co-culture with host cells, all three strains were cultured with both undifferentiated and differentiated THP-1 human monocytic cells. Differentiated, macrophage-like, THP-1 cells were used by Vick *et al.* (2014) to characterize the inflammatory response to strain 14018. Vick subsequently characterized inflammatory responses at 24 h to all three strains in undifferentiated and differentiated THP-1 cells, finding that strain 14019 did not induce inflammation and resulted in less THP-1 cell death in undifferentiated cells compared to the other strains. However, that study did not enumerate bacteria (Vick 2014). In the present study, bacterial concentration was assessed at 24 and 48 h after treatment of THP-1 cells with *G. vaginalis* strains. In undifferentiated THP-1 cells at 24 h, bacterial concentration had increased 100-fold and 7,000-fold for strains 14019 and 49145, respectively, and by 48 h, these bacteria had increased 400-fold to 2,000,000-fold, respectively. However, strain 14018 increased only about 20-fold over the 48 h time period. Although all three strains were inoculated at the time 0 point into host cell cultures using the same optical density to produce an MOI of 5, which would be represented by 1.25×10^6 bacterial cells, colony enumeration produced very different numbers of bacterial cells. Strain 14018 was inoculated at a concentration of

approximately 5 x 10^6 CFU/mL (4X greater than expected), whereas strains 49145 and 14019 were recovered at concentrations of 2 and 4-$\log_{10}$ lower than expected. Although the source of these differences is not clear and could be due to inoculation error, the increased concentration of the 14018 strain could have resulted in greater competition for growth factors in the medium and less of an increase in growth.

A similar *G. vaginalis* growth pattern was observed for strain 14018 in the differentiated THP-1 cells in that it exhibited only a 20-fold increase in growth. Strain 49145 increased 300,000 fold in the differentiated cells, which was approximately 10-fold less than in undifferentiated cells. Although strain 14019 increased 400-fold in undifferentiated THP-1 co-culture, it did not show growth in differentiated THP-1 cells. Although it was reported as having been isolated from a patient with BV, the less robust growth of strain 14019 compared to other strains could explain the association of different strains with symptomatic and asymptomatic BV cases in that some strains may not compete as well in different environmental conditions.

In addition to differences in inocula, another factor influencing differences in bacterial cell numbers might be the phagocytic activity of the host cells, which could result in decreases in bacterial cell number, especially if THP-1 cells remained viable in one treatment group compared with another. Additionally, differentiated THP-1 cells have increased phagocytosis compared to undifferentiated cells, which could explain the lower concentration of bacteria for all strains in the differentiated cells (Qin 2012). Another consideration of lower recovery of bacteria, even for the time 0 point, is the potential for induction of the viable but nonculturable state (VBNC) in some of the strains. The VBNC state is often induced in response to stress, including transfer into

culture medium (Li *et al.* 2014). The strains might vary in their response to changes in environmental conditions such that cells may be present but could not be cultured onto solid medium. Further investigation of this is necessary as it could also impact recovery of the bacterium from patient samples.

In order to determine whether the bacterial strain affected THP-1 cell viability, the viability of undifferentiated and differentiated cells was also investigated. In the undifferentiated cells, all strains resulted in approximately a 15% decrease in viability by 24 h, which was a significant decrease compared to untreated cells. Vick (2014) had reported similar decreases in viability for strains 14019 and 49145, but less than 50% viable cells in response to strain 14018, although the bacterial cell numbers are not known for that study. At the 24 h time point in this study, there were approximately 100-fold more bacteria in the strain 14018-treated cells compared to the other two strain treatments, yet THP-1 viability was significantly greater in strain 14018-treated differentiated cells compared to strain 14019 and 49145 treatments. In undifferentiated cells, the viability of strain 14018-treated cells was only significantly less than strain 49145 treatment. By 48 h, the THP-1 cell viability had significantly decreased with strain 14018 treatment of undifferentiated cells compared to strains 14019 and 49145, although at this time point, bacterial concentrations of strains 14018 and 49145 were not significantly different. The increased cell death could have been a cumulative effect of the high strain 14018 concentration over the 48 h, or could result from the increased inflammation and pyroptosis reported for strain 14018 (Vick 2014, Vick *et al.* 2014).

For the differentiated THP-1 cells at 48 h, the viability of strain 14018-treated cells was significantly greater than for THP-1 cells treated with the other two strains

despite high concentrations of strain 14018 bacterial cells, especially considering that strain 14019 was at least 2-$\log_{10}$ fold lower in concentration at this time point. These data would suggest that viability of host cells is not related to bacterial cell number or to the *G. vaginalis* strains used, at least for this study. However, additional factors should also be considered when assessing bacteria-host cell interactions. For example, THP-1 monocytes are not adherent, whereas differentiated macrophages are adherent. Whether *G. vaginalis* remains in suspension, as was observed for strain 49145 in BHI, or forms a biofilm, as observed for strain 14018, might influence interaction with a particular cell type and subsequent inflammatory responses and pyroptosis in that cell. Furthermore, macrophages average 21 μm in diameter and produce pseudopodia, whereas monocytes are slightly smaller and more circular with diameters of 7.72-9.99 μm (Hartwig *et al.* 1977, Hunt 1990, Krombach *et al.* 1997). These variations provide different amounts of surface area to interact with a bacterial cell. *G. vaginalis* interaction with undifferentiated and differentiated cells could also by affected by molecules found on the surfaces of the cells. Monocytes are typically divided into three categories based on surface receptors: classical, with high levels of surface CD14; non-classical, with high levels of surface CD16 but low levels of CD14; and intermediate, with high levels of CD14 but low levels of CD16. Classical monocytes have enhanced phagocytosis and inflammatory responses, intermediate monocytes assist with inflammation, and non-classical monocytes maintain antiviral properties and patrol the body (Italiani and Boraschi 2014). THP-1 cells express various amounts of CD14, depending on culture density, which could affect how well they phagocytize *G. vaginalis*. Vick (2014) also showed that *G. vaginalis* strains 14018 and 49145 induce THP-1 monocyte

differentiation. Differentiated cells cease division which could also affect cell numbers and viability. Similarly, THP-1 cells grown to a higher concentration will differentiate into more macrophage-like cells and at a lower density will differentiate into more granulocytic cells (Aldo *et al.* 2013). The variability in undifferentiated and differentiated THP-1 cells and differences in bacterial cell numbers between co-cultured *G. vaginalis* strains all suggest the importance of assessing bacterial concentration during co-culture studies with host cells.

To better understand observed differences in the growth of *G. vaginalis* strains in this study, comparative genomics was used to provide insight into whether the three strains differed in the presence or absence of genes involved in bacterial growth. The genomes of the three *G. vaginalis* strains used in this study have been sequenced, although strain 49145 is still a draft genome (Kidane *et al.* 2017). One notable feature of the genome of strain 49145 is that it is larger than the other two genomes, has more genes with assigned putative functions, and contains 21 genes not found in strains 14018 and 14019. The larger genome of strain 49145 could be indicative of lateral gene transfer (LGT) events. Genome analysis of this strain is ongoing and although LGT components have not yet been identified, they have been found in the genomes of other *G. vaginalis* strains and include genes for metabolism as well as genes implicated in virulence, such as vaginolysin (Yeoman *et al.* 2010).

The presence or absence of enzymes for cellular metabolism pathways can alter the growth of bacteria in different environmental conditions as evidenced by both mutation and knockdown studies. For this study, differences in the presence of putative genes involved in metabolic pathways were explored for the three *G. vaginalis* strains

used for these experiments. *G. vaginalis* strain 49145 possessed a higher number of genes associated with metabolism compared to the other strains. Strain 49145 contained a putative gene for the enzyme deoxyribose-phosphate aldolase. Deoxyribose-phosphate aldolase is a catalyst for the reaction, 2-deoxy-D-ribose 5-phosphate <=> D-glyceraldehyde 3-phosphate + acetaldehyde, which allows deoxynucleosides to be used as an additional substrate for the pentose phosphate pathway thus providing this strain with a growth advantage over strains 14018 and 14019 (Racker 1952, Romano and Conway 1996). Although all three strains have genes for a putative β-galactosidase and the evolved β-D-galactosidase alpha subunit (*ebgA*), only strain 49145 also has a gene for the evolved β-D-galactosidase beta subunit (*ebgC*), which is required for full activity of the second β-galactosidase. This enzyme would provide strain 49145 with increased ability to utilize lactose as an energy source (Calugaru et al. 1995). Although lactose is not generally present in large amounts in the vaginal environment, the ability to utilize lactose may provide a survival advantage as the organism passes through the gastrointestinal tract to the vaginal tract, as has been proposed for *Lactobacillus* spp. (Vásquez *et al.* 2005). The putative enzyme could also provide a growth advantage *in vitro* if lactose were supplemented into the medium. Although no lactose-enriched media were used in this study, it would be of interest to determine whether such a medium would increase the growth of strain 49145 relative to the other strains.

The presence of the putative sialidase gene was also found in strain 49145, but not strains 14018 or 14019. Sialidase removes sialic acid from terminal glycans of glycoproteins, including sphingolipids, providing free sialic acid for metabolic breakdown, as well as allowing a bacterium to coat itself with sialic acid, potentially

providing resistance to a host's innate immune response by disguising the bacterium such that it looks similar to host tissue (Severi *et al.* 2007). In strain 49145, there was also a unique gene encoding a putative bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase. This enzyme is essential for initiating and regulating the biosynthesis of a precursor for sialic acid, N-acetylneuraminic acid, which for prokaryotes would be involved in the production of the capsule precursor UDP-N-acetyl-D-mannosaminuronic acid (Keppler *et al.* 1999, Swartley *et al.* 1998). Although *G. vaginalis* has not been observed as having a capsule, this possibility should be investigated (Greenwood and Pickett 1980). This gene may not necessarily provide a growth advantage *in vitro*, but in the host, surface sialic acid on *G. vaginalis*, whether or not in a capsule, could help the bacterium delay the host immune response.

Strain 49145 was lacking a putative gene for holo-[acyl-carrier protein] synthase, which is an enzyme that catalyzes the following reaction: CoA-[4'-phosphopantetheine] + apo-acyl carrier protein <=> adenosine 3',5'-bisphosphate + holo-acyl carrier protein. The adenosine 3',5'-bisphosphate can be later metabolized to ATP (Ramaswamy and Jakoby 1987). The pathway will also form holo-ACP, the active form of the holo carrier involved in lipid synthesis (Mofid *et al.* 2002). Strains 14018 and 14019 both had this gene, which could provide them with a growth advantage over strain 49145. Strain 14018 did exhibit better growth than strain 49145 *in vitro*; however, 14019 did not. Because the genome of 49145 has not been fully annotated, it is also possible that this gene is still classified as a hypothetical protein.

Strain 14018 did not contain any classified genes for metabolic pathways that would seem to provide a distinct growth advantage, and although strain 14019 was the least robust in terms of growth *in vitro*, it did contain a gene that was not present in the other two strains. The unique gene was for the putative 2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase, which participates in the following reaction: 2-dehydro-3-deoxy-D-gluconate + NAD^+ <=> (4S)-4,6-dihydroxy-2,5-dioxohexanoate + NADH + H^+. The 2-dehydro-3-deoxy-D-gluconate can enter the pentose phosphate pathway for catabolism. In *E. coli,* this gene is also responsible for degradation of hexuronate carbohydrates in gastrointestinal mucus, and this enzyme could provide a growth advantage for this strain in the vaginal tract which has mucus rich in glucuronate and galacturonate (Tubeleviciute *et al.* 2014).

ABC transporters are multi domain integral proteins that are a vital part of importing nutrients (Jones and George 2004). All three *G. vaginalis* strains contained an allele for an ABC transporter for aldouronate. Although aldouronates are typically utilized by alginate or pectin-degrading bacteria, as mentioned above, *E. coli* had been able to use the enzyme, 2-deydro-3-deoxy-D-gluconate 5-dehydrogenase enzyme, the putative gene for which was found in the genome of 14019, for hexuronate degradation in mucus under conditions of high osmotic stress (Rothe *et al.* 2013). Enzymes have been reported in cellulose-degrading bacteria that convert aldouronates to hexuronates, such that *G. vaginalis* may be able to use this transporter for mucus degradation (Shulami *et al.* 1999). Although strains 14018 and 14019 both possessed the same allele for this transporter, strain 49145 had a different allele which would result in less than 25% amino acid similarity, and only strain 14019 possessed the gene for putative hexuronate

degradation. The capability of *G. vaginalis* to utilize these putative gene products for degradation of mucus in the vaginal tract, especially in response to environmental stress as for *E. coli,* would be a unique mechanism of pathogenesis for further exploration.

ABC transporter genes found in strains 14018 and 14019, but not in strain 49145, include putative glucose/mannose (*gtsB*) and inositol-phosphate (*inoF*) transport system permeases. *Mycoplasma* spp., which are also reproductive tract pathogens, use GtsB to transport glycerol into the cell for catabolism (Djordjevic *et al.* 2003, Hames *et al.* 2008). The *gtsB* gene has been reported in other genera, primarily environmental isolates of *Pseudomonas, Halomonas,* and *Rhizobium* spp., and has been confirmed as a glycerol transporter for *Pseudomonas fluorescens* (Bailey *et al.* 2014). However, glycerol has been reported as being toxic to *G. vaginalis*, and it is possible that the bacterium uses this transporter for efflux instead of transport into the cell (Noll *et al.* 2012).

The genomes of strains 14018 and 14019 also contained a putative gene for an inositol-phosphate transport permease. The function of this transporter is not well established, but the genes for the ABC transporter have also been found in environmental isolates, including *Bacillus* spp., *Agrobacterium* spp., and *Thermatoga maritima.* In *Bacillus subtilis*, myo-inositol is a precursor in the catabolism to acetyl coA (Yoshida *et al.* 2008, Rodionova *et al.* 2013,). The role of this putative protein for *G. vaginalis* growth is unclear, although another vaginal pathogen, *Candida albicans,* is reported to rely on either the import or synthesis of inositol for pathogenesis (Reynolds 2009).

G. vaginalis strain 49145 contained genes for two ABC transporter proteins that 14018 and 14019 did not. Strain 49145 possesses a gene for a putative L-cysteine-

binding protein (FliY), as well as a putative gene for CbiO, an ABC transporter for cobalt and nickel. The cysteine ABC transporter is also found in the pathogens *Listeria monocytogenes* and *E. coli* (Keevil *et al.* 1986, Tanaka *et al.* 2018). The presence of this substrate binding protein may provide a growth advantage for strains when cysteine is needed for protein synthesis, although all three strains have genes for cysteine synthesis. Strain 49145 grew better in BHI medium compared to the other two *G. vaginalis* strains, whereas its growth was not as high as strain 41018 in RPMI medium. RPMI medium is supplemented with L-cystine, and 19 other amino acids, and would be readily available to the bacteria. The serum might also be a source of amino acids. BHI is a complex medium with infusions and digests of animal tissues. The cysteine and other amino acid composition is variable and the presence of a cysteine transporter could result in the active transport of that amino acid from BHI for strain 49145 providing a growth advantage in that medium. Conversely, in *E. coli,* the FliY transporter has been implicated in the export of reactive oxygen species, such as hydrogen peroxide, bound to L-cystine under stress conditions (Ohtsu *et al.* 2015). Such a response could also increase survival of the strain 49145 bacterium *in vivo.*

CbiO is a cytoplasmic ATP-binding protein that is part of a cobalt/nickel transport system (Rodionov *et al*. 2006). Cobalt and nickel are of importance for certain enzymes and active sites found throughout cells (Ermler *et al.* 1999). Though these pathways have not been explored in *G. vaginalis,* the cobalt/nickel ABC transporter genes and proteins have been found in many bacterial genera, including the pathogens, *Staphylococcus aureus, Streptococcus agalactiae, Enterococcus faecium, Listeria monocytogenes, Klebsiella pneunomiae, Salmonella* Typhimurium, and *E. coli* (Rodionov *et al.* 2006,

Tanaka *et al.* 2018). Transporters for these metals would also provide a growth advantage for strain 49145 in that these metals are generally in trace amounts in culture media. Increasing nickel concentrations in media was shown to increase biofilm formation by *E. coli,* whereas *S. aureus* more readily forms biofilms on cobalt surfaces. Although both of these organisms possess genes for cobalt/nickel ABC transporters, a role for those transporters in these responses has not been reported (Perrin *et al.* 2009, Coraca-Huber *et al.* 2012).

The machinery for protein synthesis, including ribosomal proteins, rRNA, and tRNA molecules are all important for bacterial growth. Genomic comparisons found that although *G. vaginalis* strains 14018 and 14019 each have two copies of the rRNA genes, the draft genome of strain 49145 included two copies of the gene for 5S rRNA, but only single copies for the 16S and 23S. In bacteria, rRNA genes are usually found in an operon with the tRNA genes to transcribe equimolar amounts of the RNA molecules (Klappenbach *et al.* 2001). Because the 49145 genome is a draft genome, the other copies of the rRNA genes may yet to be annotated; however, for bacteria, rRNA copy number has been correlated with the rate of bacterial response to environmental resources; thus fewer genes for rRNA molecules could have a negative effect on growth rate (Klappenbach *et al.* 2000).

Numbers of tRNA genes also differed between strains with strains 14018 and 49145 each having 45 tRNA genes and strain 14019 only 34 tRNA genes. Although a more extensive comparison of these tRNA genes was not completed, the copy number of tRNA has been linked to the codon usage of the bacterium as well as the rate of

translation. In those studies, a greater number of tRNA genes was associated with increased translation and thus increased growth rate (Du *et al.* 2017).

Ribosomes are essential for protein synthesis. The large and small ribosomal subunits of bacteria typically contain about 50 subunits, 34 of which are considered universally conserved among all three domains of cellular life (Yutin *et al.* 2012). For bacteria, genes for 23 SSU proteins have been identified and for LSU proteins, 37 genes have been identified. Some of the ribosomal proteins are considered nonessential for different groups of bacteria, thus not all proteins appear in ribosomal subunits (Lecompte *et al.* 2002). The numbers of ribosomal protein genes differed among the *G. vaginalis* strains. Strains 14019 possessed genes for 20 SSU proteins. Strains 14018 and 49145 were missing genes for subunits S10, S14, and S17. SSU proteins S21, S22, and S31e were absent in all three strains. These three subunits are considered nonessential and are not found in all bacteria (Lecompte *et al.* 2002). In *E. coli* and other bacteria, the S10 and S14 subunits are considered essential (Akanuma *et al.* 2012). The S10 subunit binds tRNA to the ribosome, and acts as a transcriptional elongation factor, and the S14 subunit belongs to the S11P family of ribosomal proteins, and mutations in this protein can resist emetine, a protein synthesis inhibitor (Rhoads and Roufa 1991, Hermann-Le Denmat 1994). With loss of these proteins, a decrease in growth would be expected, but this was not observed for strains 14018 and 49145 in these studies. S17 is suspected to participate in recognition of termination codons in mRNA, and attaches to the 5' end of 16S ribosomal RNA in *E. coli* (Agafonov *et al.* 1997); however, it is considered nonessential.

Strains 14019 and 49145 had genes for 33 LSU proteins, with strain 14018 having 32 LSU genes. All three strains were missing the L36 LSU gene and train 14018 was also missing the L14 LSU gene. The L14 subunit is considered essential, while the L36 subunit is not (Akanuma *et al.* 2012). In bacteria, both the L14 and L36 subunits bind directly to 23S ribosomal RNA, which may act to organize and stabilize ribosome structure to ensure proper translation. L36 is a cystine-rich subunit, and is the smallest of the large subunit proteins (Chandra Sanyal and Liljas 2000.). Compared with *G. vaginalis* strain 14019, strain 14018 was missing four ribosomal subunits, which was unexpected due to its more robust growth compared to strain 14019. It is possible that the loss of these subunits may impact growth *in vivo.* Additionally, although the genome of strain 14018 is complete, the coverage for assigned function is only 46%, such that it is possible that these genes have not yet been identified in the sequence as the genes for these missing subunits are less than 400 nucleotides.

Although genomic comparisons have revealed differences between the bacteria, this study has not determined whether these genes are expressed by the bacteria and under what environmental conditions gene expression occurs. Differences in gene expression between not only these genes, but also other genes involved in growth, could help to explain variation between these three *G. vaginalis* strains.

This study sought to determine whether differences in growth between the three strains of *G. vaginalis* used for studies on inflammation could be a factor in levels of cellular inflammatory responses observed for the three strains. The strains were compared using the environmental conditions for growth in the laboratory and for co-culture with THP-1 human monocytic cells used for the inflammation studies.

Differences in growth were observed between the strains, with strain 14019 having less growth under the conditions used in this study. Despite the low levels of growth, this bacterium still induced significant cell death in THP-1 monocytes. Studies with heat or UV-inactivated bacterial cells at numbers comparable to those achieved for the live bacteria could help to assess whether the observed cell death was due to the presence of bacterial molecules that could be toxic to cells and not necessarily growth of the different strains. However, because the bacteria do grow differently in different conditions, this study highlights the necessity of enumerating bacteria in co-culture studies.

Comparative genomics were also used to assess whether specific genes associated with growth differed between the three *G. vaginalis* strains. These comparisons did reveal differences, with *G. vaginalis* strain 49145, which has a larger genome, having seven unique genes for metabolism that have not yet been identified in strains 14018 or 14019. Strains 49145 and 14018 also possessed 11 more genes for tRNA molecules than did strain 14019 which could positively impact translation and growth of those two strains. Strain 14019 did possess a gene that was not present in either of the strains for a putative enzyme for hexuronate use. This enzyme could prove valuable to the organism *in vivo* for the catabolism of carbohydrates in mucus. Although there are differences in the presence or absence of genes between these organisms, the protein products of these genes are all putative. Additionally, this study has not considered sequence identities in the genes shared between the strains. However, the genomic analyses in this study have highlighted some differences between the three strains that could be considered for future studies, such as supplementation of growth media with lactose, cysteine, or metal ions to improve growth or detection of *G. vaginalis*. It has also provided insight into other gene

products to investigate, for example, those involved in mucin degradation, especially in strains without the sialidase gene. With over 50 genomes for strains of *G. vaginalis* now available in NCBI GenBank, multiple comparisons can also be made with strains other than the three used for this study. These genomes, many of which were obtained through the Human Microbiome Project, can be a valuable resource to better understand not only strain differences, but also roles for different genotypes in BV. Within the past decade, research with the genomes of *G. vaginalis* isolates has shown that isolates can be divided into four separate clades and suggests the establishment of four different species within the genus *Gardnerella* (Schellenberg *et al.* 2017). A greater understanding of these different organisms, both at the genomic and phenotypic levels, could lead to re-defining of the role of "*Gardnerella vaginalis*" in the development of BV.

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