Carbon Source and Biofilm Formation: Implications for Bacterial Vaginosis Treatment Strategies

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Carbon Source and Biofilm Formation: Implications for Bacterial Vaginosis Treatment Strategies

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Abstract

Bacterial vaginosis (BV) is the most common vaginal infection in reproductive-aged women[1]. It is a polymicrobial infection caused by the overgrowth of multiple pathogenic anaerobes in the vagina. The dominant genus associated with BV, *Gardnerella*, can form a biofilm. Biofilm formation confers increased antibiotic resistance, resulting in higher risk of recurrent infection. The *Gardnerella* biofilm composition has yet to be characterized. Our project investigated the primary polysaccharide components of the *Gardnerella* biofilm and determined how carbon sources can modulate *Gardnerella* biofilm synthesis. We found that acetate, glutamate, lactate, and succinate significantly decreased biofilm growth for *Gardnerella vaginalis* and *Gardnerella piotti*, while mannitol significantly increased growth for *G. piotti*. Based on this data, we identified and tested enzymes to assess their capacity to disrupt *Gardnerella* biofilms. We found that all five tested enzymes, cellulase, DNase I, α-amylase, lipase, and proteinase-k, significantly disrupted the *G. vaginalis* biofilm. Understanding more about *Gardnerella* biofilm will promote improved BV treatment options and has the potential to decrease the current high rate of recurring infection.

Keywords: bacterial vaginosis, vaginal microbiome, *Gardnerella*, biofilm, carbon source, enzyme disruptor

Introduction

Bacterial vaginosis (BV) is a polymicrobial vaginal infection caused by an imbalance of bacteria present in the vagina. Specifically, there is an increase in pathogenic bacteria, the dominant genus being *Gardnerella*, and a decrease in the beneficial Lactobacillus species, as seen in Figure 1.

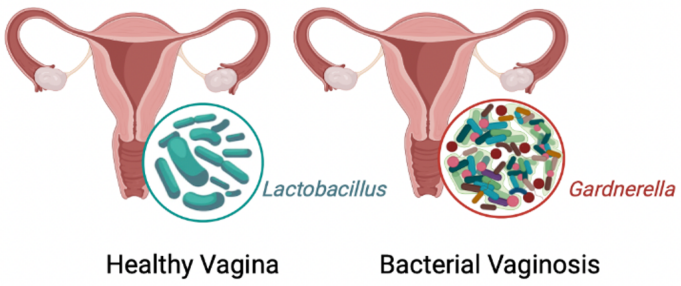

Figure 1. Schematic of healthy vs. BV vaginal microflora

BV is responsible for over 60% of vulvovaginal infections, making it the most common vaginal infection in reproductive aged women[1]. A National Health and Nutrition Examination Survey (NHANES) (2001–2004) showed that 21.2 million women were affected by BV[2]. Prevalence of BV varies among different subpopulations within the United States, with higher rates for black, Hispanic, and pregnant women[3]. Patients with BV can experience physical discomfort such as thin, gray, and foul-smelling vaginal discharge, vaginal itching, and burning during urination[4]. In addition, BV can lead to adverse outcomes when left untreated such as pelvic inflammatory disease, higher rates of certain sexually transmitted infections, higher likelihood for postoperative infections, and preterm birth[1].

While BV can go away on its own, this is not common and people with symptoms should get tested and treated to prevent these adverse outcomes. Current treatment options are antibiotics such as metronidazole, clindamycin, tinidazole, and secnidazole[4]. However, 58% of the successfully treated cases recur within one year of initial treatment[5]. This relapse occurs when BV related bacteria re-colonize the vagina. Additionally, these recurrent BV related bacteria, such as *Gardnerella* and other pathogens may gain higher resistance and become less susceptible to these antibiotics due to beneficial mutations, further emphasizing the problems with current antibiotic

treatment[6]. This high rate of recurrence contributes to half of the high annual cost burden to treat BV in the US, $1.3 billion. The global treatment cost for symptomatic BV cases is also staggering at $4.8 billion[3].

Additional problems arise with current treatment options, because the *Gardnerella* forms a biofilm, a collection of microorganisms enclosed in a sticky layer of carbohydrates, proteins, and nucleic acids called extracellular polymeric substances (EPS), that is associated with decreased antibiotic susceptibility. The EPS components provide a layer of physical protection and promote the combination of multiple species of bacteria, facilitating the creation of heterogeneous populations. This means that the pathogenic bacteria present can utilize the endurance properties of multicellular organisms during antibiotic treatment, increasing survival rates[7].

Despite its prevalence and lack of effective treatment, there continues to be insufficient information regarding the metabolic mechanisms of BV and the mechanisms underlying the high rate of recurrent infection. In order to be able to develop more efficient treatment options, there needs to be a better understanding of the *Gardnerella* biofilm composition. We focused on two different strains of *Gardnerella*, *Gardnerella vaginalis* and *Gardnerella piotti*. We aimed to use Scanning Electron Microscopy (SEM) to visualize the EPS layer and compare the two strains of bacteria. We then aimed to do carbon source experimentation to determine the impact of different nutrients on biofilm growth. Finally, we performed enzyme disruption experimentation to analyze the enzymes' ability to disrupt the biofilm. This provides more information about the composition of the *Gardnerella* biofilm, which is still uncharacterized and not well understood. This will allow for the improvement of treatment options for BV, hopefully lowering costs for patients and preventing harmful side effects.

Results

SEM

We conducted SEM in order to visualize the surface topography and composition of the biofilm formed by *G. vaginalis* and *G. piotti.* Figure 2 shows the bacteria and the EPS layer that is formed. The EPS takes up the intercellular space in order to form the matrix that provides structure and protection (Figure 2a). Higher magnification images show these components in greater detail (Figure 2b). Figure 2a also shows the three-dimensional nature of the biofilm, giving insight into growth patterns and adhesion properties. No visually apparent differences were observed between the *G. vaginalis* and *G. piotti* biofilms.

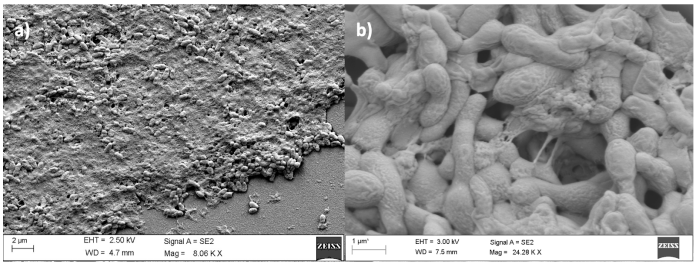

Figure 2. SEM images of (a) *G. vaginalis* and (b) *G. piotti* biofilm

Lectin Staining

N-acetylneuraminic acid (sialic acid) and N-acetylglucosamine are monosaccharides that compose the peptidoglycan layer of bacterial cell walls[8]. These compounds have both been associated with *Gardnerella* biofilms[9]. Wheat germ agglutinin (WGA) is a lectin that has high affinity for both of these carbohydrates[10]. Studies have shown that L-fucose is another monosaccharide that is utilized by *Gardnerella*[11]. Ulex europaeus agglutinin (UEA) is a lectin produced by the gorse plant that selectively binds to fucose[12].

We used FITC conjugated WGA and TRITC conjugated UAE in order to determine if these metabolites are incorporated into the EPS of *Gardnerella* biofilm. Mature *G. vaginalis* and *G. piotti* biofilms were stained with each fluorescent lectin, and a fluorescent plate reader was used for absorbance measurements. Our results did not show increased absorbance compared to control groups.

Carbon Source Experimentation

We then compared *G. vaginalis* and *G. piotti* biofilm growth with ten different carbon sources; galactose, maltose, mannitol, mannose, pyruvate, sucrose, acetate, glutamate, lactate, and succinate. These metabolites were chosen due to their association with BV, *Gardnerella*, and similar organisms[13]. After growing the biofilm with synthetic vaginal media (SVM) supplemented with 1% of each carbon source, the biofilms were stained with crystal violet in order to quantify biofilm, and the results are shown in Figure 3. A two-tailed t-test was performed comparing each carbon source to the control SVM. For *G. vaginalis* and *G. piotti*, acetate (p = 5.65E-10, 1.44E-09), glutamate (p = 7.34E-09, 0.01), lactate (p = 5.81E-09, 4.44E-10), and succinate (p = 3.39E-10, 3.46E-09) significantly decreased biofilm growth, and for *G. piotti* mannitol (p = 5.53E-05) significantly increased growth. Although the increase in growth was not statistically

significant for *G. vaginalis* growth with mannitol, each carbon source had a similar effect on both strains of *Gardnerella*.

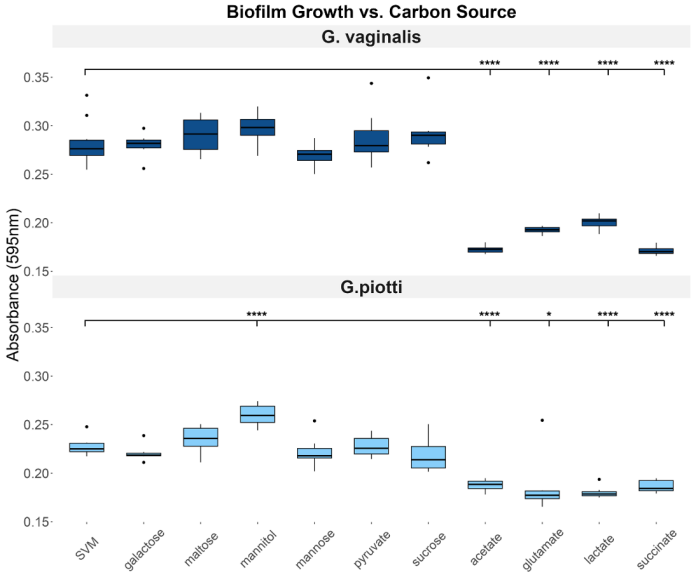

Figure 3. Biofilm growth measured by crystal violet staining for *G. vaginalis* and *G. piotti* in Synthetic Vaginal Media (SVM) + 1% each carbon source

Enzyme Disruption

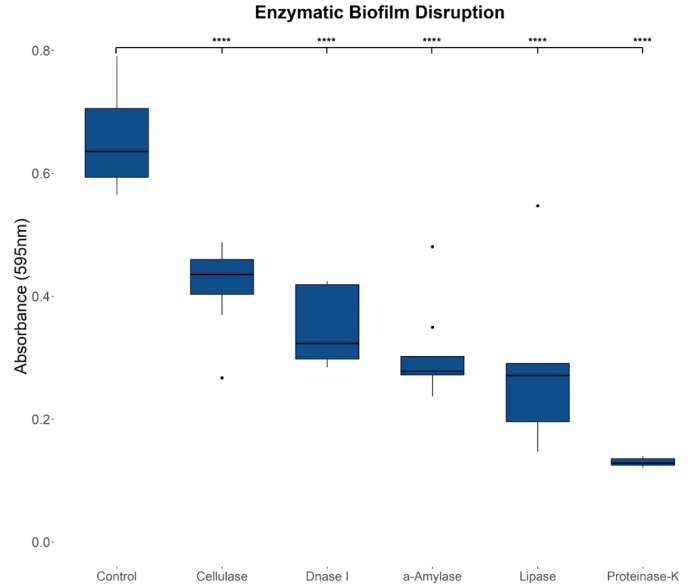

Figure 4. *G. vaginalis* biofilm present measured by crystal violet staining following two-hour incubation with each enzyme

Based on prior results and similar organisms, five enzymes were chosen that we hypothesized could disrupt *G. vaginalis* biofilm. Mature biofilms were treated with cellulase, DNase I, α-amylase, lipase, and proteinase-k each at a concentration of 2 U/µL. Crystal violet staining showed the biofilm present after disruption and this was compared to the control biofilm with no treatment. As shown in Figure 4, cellulase (p = 6.97E-05), DNase I (p = 6.16E-06), α-amylase (p = 1.76E-06), lipase (p = 1.15E-05), and proteinase-k (p = 2.58E-06) all significantly disrupted the *G. vaginalis* biofilm. Proteinase-k was the most effective with a 5-fold decrease in the amount of biofilm present following treatment compared to the control.

Discussion

This preliminary exploration offers some new information about *G. vaginalis* and *G. piotti* biofilms that is helpful for understanding BV and crucial for the development of more effective BV treatment. Despite the fact that there are phenotypic differences between the *Gardnerella* strains, our SEM results that there were no observable differences were not surprising. This is due to the subgroups being >98.5% identical and there having no signature sequences differentiating them when comparing the 16S rRNA sequences[14]. This implies that the formation of the biofilm by each strain would be similar, resulting in no observable differences. However, this technique of visualization has limitations as it was qualitative and not quantitative data collection and results could reveal observer biases.

As mentioned earlier the SEM images allowed us to visualize the three-dimensional properties of the biofilm such as adherence. The bacteria can be seen in clumps, as *Gardnerella* has aggregative properties. This is beneficial for the progression of BV as adherence of the bacteria is necessary in order to avoid clearance by vaginal secretions and in order to form a strong biofilm[15]. One observation that was unexpected from these images were the wrinkles on the higher magnification images. A scanning electron microscopy technical review paper found that this wrinkling was due to shrinkage from the dehydration step in the SEM preparation step as the cell wall loses turgor from the drying[16].

Contrary to previous studies, our results indicated that sialic acid and fucose are not incorporated into *Gardnerella* biofilm. However, there are many other reasons why we did not get a fluorescent signal after lectin treatment. Most notably, the procedure that was followed did not include a fixation step, and the biofilms may have been washed away due to multiple wash steps prior to staining. In the future, this procedure should be optimized and these lectins should be tested again to understand the actual participation of these metabolites in *Gardnerella* biofilm.

Some of the carbon sources impacted biofilm growth as we hypothesized, while some had unexpected effects. Lactate and Glutamate are compounds that are associated with *Lactobacillus* species of bacteria[17]. As previously mentioned, a healthy vagina is usually dominated by *Lactobacillus*, which creates an acidic environment that prevents the adherence and growth of pathogenic bacteria. In dysbiosis, the environment becomes more basic which contributes to the growth of *Gardnerella* in BV. Because of this, we hypothesized that these compounds would decrease biofilm growth. They have also been found in decreased concentrations in BV patient secretions, indicating that this could be one of the reasons for the vaginal dysbiosis[13]. Our analysis showed that both lactate and glutamate did significantly decrease *G. vaginalis* and *G. piotti* growth, which was consistent with expectations and previous studies.

However, some of our results conflict with other studies that we have encountered. Acetate and succinate are both compounds that we expected would increase *Gardnerella* biofilm growth. Both acetate and succinate have been found in increased concentrations in BV patient secretions and are thought to be negatively associated with *Lactobacillus* bacterial species[13]. Yet our results indicated that *G. vaginalis* experienced the great inhibition to form a biofilm in the presence of acetate and succinate (Figure 4). *G. piotti* biofilm growth was also significantly decreased by acetate and succinate. Additionally, we expected mannitol to decrease biofilm growth because studies have shown an inverse relationship between mannitol and BV[13]. However, *G. piotti* biofilm growth was significantly increased with mannitol supplemented media.

There are many reasons why these results could differ from other studies. BV is a polymicrobial infection which means that a community of bacteria is formed that includes many different species. Even though *Gardnerella* is the most dominant bacteria in BV, our results show how *Gardnerella* alone utilizes and produces different metabolites. They do not give the full picture of how these metabolites are used during a BV infection, because many different bacteria may affect these metabolites concentrations more than *Gardnerella*. Also, studies that find that a metabolite is present in increased concentrations in BV patient secretions, cannot conclude that *Gardnerella* and the other pathogens are consuming these compounds as a means for growth. Increased acetate and succinate concentrations in BV secretions could just be a byproduct of one or more of the reactions happening among the bacteria present in the vagina at that time. It could also be a byproduct of another BV symptom such as higher epithelial cell turnover.

Finally, all five enzymes that were chosen significantly disrupted the *G. vaginalis* biofilm. As previously stated, biofilms are a collection of microorganisms enclosed in a collection of carbohydrates, proteins, and nucleic acids. In most bacterial biofilms, the polysaccharide components provide the protection and strength that allows these biofilms to continue growing and colonizing different surfaces. For this reason, we hypothesized that cellulase and α-amylase would be the most effective biofilm disruptors because these enzymes catalyze the decomposition of carbohydrates. Interestingly, cellulase was the least effective biofilm disruptor out of the five enzymes that were tested, followed by DNase I and then α-amylase. Proteinase-k, which targets amino acids, was the most effective enzyme tested, resulting in a 5-fold decrease in the amount of *G. vaginalis* biofilm. Lipase, which targets fats, was the second most effective, but had a much larger standard deviation than proteinase-k. This may indicate that Gardnerella biofilm is unlike many other bacterial biofilms and does not rely heavily on its carbohydrate components for protection. These results, however, are similar to results seen in other biofilm studies[18]. Another possible reason for these results could be the different activity levels affecting normalization across different types of enzymes.

In the future, computational metabolic modeling can be used to validate some of these experimental results. Exploring the metabolites, reactions, and genes that are necessary for *Gardnerella* biofilm growth computationally would provide a better starting point for experiments that can then be tested experimentally. Utilizing computational data to inform experimental data and vice versa will optimize efforts to understand more about *Gardnerella* and BV. Also, treating *G. piotti* biofilms with the same five enzymes would be interesting in order to see if there are any differences between the two strains. SEM could also be performed on the biofilm that is left over after enzyme treatment to visualize how that affects its structure. Additionally, SEM procedures could be optimized, for example, using ionic liquid infiltrated preparation for smoother appearance of bacteria[16]. Finally, this information can be used to continue developing biofilm disruptors to pair with antibiotics in order to hopefully improve BV treatment methods and decrease reinfection rates.

Materials and Methods

Growing G. vaginalis and G. piotti biofilm

The bacterial strains, *Gardnerella vaginalis* and *Gardnerella piotti*, were grown in the anaerobic chamber at 37°C. After growth, the cultures were centrifuged at 7500 rpm for five minutes, and the supernatant was discarded. The cells were then resuspended in one mL PBS and centrifuged at 7500 rpm for five minutes, and this wash step was repeated two more times. The cells were then resuspended in 10 mL reduced NYC III media and incubated in the anaerobic chamber for one hour. In tandem, a 96 well plate was prepared with 200 µL of media per well and incubated. Next, 50 µL of inoculum were added to each well, and the plate was incubated at 37°C in the anaerobic chamber for 48 hours.

Scanning Electron Microscopy Sample Preparation

For Scanning Electron Microscopy, biofilms must be grown on round glass coverslips, and so they can be mounted onto the specimen stubs. In a six well plate, coverslips were placed in the center of each well using forceps. *G. vaginalis* and *G. piotti* biofilms were grown on the coverslips for 48 hours. Similar wash and preparation procedures as 96 well plate biofilm growth were followed, but after putting 50 µL of inoculum on each coverslip, 100 µL of NYC III media was put on the coverslip carefully, without breaking the surface tension which would cause the inoculum to spill off the coverslip. After 48 hours of growth, the samples were rinsed three times for one minute each in a phosphate buffer (PBS) (10mM, pH 7). The specimens were then fixed in 2% glutaraldehyde for 30 minutes. After fixation, the specimens were rinsed three times for one minute each in PBS. Graded ethanol dehydration was then carried out, consisting of sequential immersions in 30%, 50%, 70%, 80%, 90%, and 100% ethanol, with each step lasting five minutes. Finally, the samples were subjected to a final dehydration step with Hexamethyldisilazane (HMDS) for five minutes. The glass coverslips were then coated with gold, mounted on specimen stubs, and biofilms were visualized.

Lectin Staining

FITC conjugated WGA and TRITC conjugated UEA lectins were diluted to a concentration of 100 µg/mL in sodium bicarbonate buffer. Following 48 hours of growth, the media was dumped off the mature *G. vaginalis* and *G. piotti* biofilms, and the wells were washed three times with deionized water. 200 µL of non-fat dry milk (NFDM) was used as a blocking buffer for 30 minutes at room temperature. The samples were rinsed with a sodium bicarbonate buffer, followed immediately by 200 µL of lectin solution. The biofilms were then incubated with the lectin solution under tinfoil for 15 minutes at room temperature. After removal of the lectin solution, samples were gently washed three times with the sodium bicarbonate buffer. Finally, 200 µL of the buffer was added to the wells and absorbance was read using a fluorescent plate reader. FITC samples were excited with 492 nm wavelengths and TRITC samples were excited with 554 nm wavelengths. Absorbance values for emission wavelengths of 517 nm and 570 nm, respectively, were observed.

Growing G. vaginalis and G. piotti biofilm with Carbon Sources

The same procedure was followed for inoculum preparation as normal biofilm growth, including three PBS washes followed by a one-hour incubation period. A 96 well plate was prepared with 200 µL of SVM with 1% of each carbon source. Next, 50 µL of inoculum were added to each well, and the plate was incubated at 37°C in the anaerobic chamber for 48 hours.

The crystal violet staining procedure began with a wash with 0.9% sodium chloride solution and the biofilms were then allowed to dry for 15 minutes at room temperature. Then 200 µL of 100% methanol was added to each well and incubated at room temperature for 20 minutes. Following fixation, 200 µL of 1% crystal violet was added and incubated for another 20 minutes at room temperature. Five washes with deionized water eliminated any non-specific dye, and the plate was left to dry once more for 15 minutes. Finally, 200 µL of 33% acetic acid was added and incubated on a shaker at 300 rpm for ten minutes. The absorbance was quantified with a Tecan plate reader at 595 nm.

Enzyme Disruption of G. vaginalis biofilm

Each enzyme solution was diluted to a concentration of 2 U/mL in PBS. Mature biofilms were washed two times with PBS and then incubated with the enzyme solutions for two hours. This was followed by another two washes with PBS. Next, the same crystal violet staining protocol was followed with methanol fixation, crystal violet incubation, and acetic acid to elute the stain and prepare for absorbance measurements. The absorbance was once again quantified with a Tecan plate reader at 595 nm.

End Matter

Author Contributions and Notes

C.D.G., K.M.G., P.C.J, L.R.D., and J.A.P. designed research, C.D.G., K.M.G., and P.C.J. performed research, C.D.G., K.M.G., and P.C.J. analyzed data; and C.D.G., K.M.G., and P.C.J. wrote the paper.
The authors declare no conflict of interest.

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