# The Role of Bacterial Vaginosis-Associated Sialidase on HIV-1 Infection

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# Declaration

I, Riley James Traviss, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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# Abbreviations

| | |
|---|---|
| Sec | Seconds |
| min | Minutes |
| hr(s) | Hour(s) |
| L | Litre |
| mL | Millilitre |
| µL | Microlitre |
| µg | Microgram |
| ng | Nanogram |
| M | Molar |
| mM | Millimolar |
| µm | Micromolar |
| nm | Nanometre |
| $^{o}$C | Degrees Celsius |
| U | Unit(s) |
| kDa | Kilo-Daltons |
| API | Atmospheric Pressure Ionization |
| APCI | Atmospheric Pressure Chemical Ionization |
| AS | Ammonium Sulphate |
| ATCC | American Type Culture Collection |
| BCA | Bicinchoninic Acid |
| BHI | Brain-Heart Infusion |

| | |
|---|---|
| BSA | Bovine Serum Albumin |
| BV | Bacterial Vaginosis |
| BVAB | Bacterial Vaginosis-Associated Bacteria |
| CVL | Cervical Vaginal Lavages |
| cDNA | Cloned DNA |
| CM | Culture Medium |
| $CO_2$ | Carbon Dioxide |
| CV | Column Volume |
| CVF | Cervical-Vaginal Fluid |
| DMEM | Dulbecco Modified Eagle high glucose medium |
| DNA | Deoxyribonucleic Acid |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| Env | Envelope |
| FBS | Fetal Bovine Serum |
| FGT | Female Genital Tract |
| GV | *Gardnerella vaginalis* |
| HEK293T | Human Endocrine Kidney Cells |
| HIV | Human Immunodeficiency Virus |
| LA | Luria Agar |
| LB | Luria Broth |
| LC/MS | Liquid Chromatography Mass Spectrometry |
| $MgCl_2$ | Magnesium Chloride |
| NaCl | Sodium Chloride |

| | |
|---|---|
| NADNA | N-acetyl-2,3-dehydro-2-deoxyneuraminic acid |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PBMCs | Peripheral Blood Mononuclear Cells |
| PEI | Polyethylenimine |
| PFT | Pore-Forming Toxins |
| PSV | Pseudovirus |
| RFU | Relative Fluorescence Units |
| RLU | Relative Light Units |
| RT | Reverse Transcriptase |
| SEC | Size-Exclusion Chromatography |
| SDS | Sodium Dodecyl Sulphate |
| SGA | Single Genome Amplification |
| STI | Sexually Transmitted Infection |
| TBS | Tris-Buffered Saline |

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# Abstract

Studies suggest that women with Bacterial Vaginosis (BV) are at a higher risk of being infected by HIV-1. One possible reason is that bacterial vaginosis-associated bacteria (BVAB) release sialidases which alter either the virus and host cells directly or the viscosity of the female vaginal tract (FGT) mucosal barrier, increasing the likelihood of infection. This study aimed to determine the impact of bacterial sialidase on HIV-1 infection focussing specifically on the BVAB, *G. vaginalis*. Pseudovirus was produced by transfection of HEK293T cells and infection of TZM-bl cells was measured by luminescence. In the presence of purified commercial sialidase, pseudovirus infection increased significantly (2-fold) suggesting that sialidase enhanced HIV infection. The sialidase appeared to act on both the virus and the host cell, although the former interaction appeared to play a more important role in enhancing pseudovirus infection. Two *G. vaginalis* strains, 3H6 and 2HI, isolated from the FGT were cultured and tested for production of sialidase. Sialidase was purified from the culture medium of these strains by anion-exchange and gel filtration. Sialidase was successfully purified, as confirmed with a sialidase activity assay as well as SDS-PAGE. Analysis by SDS-PAGE showed two distinct bands, with approximate molecular weights of 40 – 45 kDA. Inclusion of the purified sialidase in a pseudovirus entry assay resulted in a 1.5-fold increase in HIV entry and LC/MS mass spectrometry confirmed the presence of sialidase. As not all *G. vaginalis* strains express sialidase, we tested whether the ATCC 14018 strain, which does not produce sialidase, could also impact HIV infection. Pseudovirus entry of TZM-bl cells was measured in the presence of the growth medium used to culture *G. vaginalis*. However, instead of an increase in pseudovirus infection, the culture medium inhibited virus entry. We then determined whether *G. vaginalis* growth altered the pH of the culture medium and/or produced an inhibitor that reduced HIV entry. When the culture medium was heated and the volume reduced, pseudovirus infection was restored albeit to a level lower than the virus-only control. Increased culture medium pH did enhance infectivity but was less effective compared to heating. Overall, we concluded that BHI medium components reduced cell viability above 5ul in the absence of *G. vaginalis* growth and that ATCC 14018 secreted a heat-labile factor that inhibited HIV infection. Therefore, it seems as though the expression of sialidase might be a very important determinant of how *G. vaginalis* affects HIV infection.

# Chapter 1: Background and Literature Review

## 1.1 Introduction

It is estimated that 66% of women will be affected by bacterial vaginosis (BV) at some point in their life. BV is associated with other sexually transmitted infections (STIs), as well as medical conditions such as pelvic inflammatory disease (Schwebke, 2009). Studies have shown women with BV have a 60% increase in risk of being infected by human immunodeficiency virus type-1 (HIV-1) (Atashili et al., 2008). There are an estimated 37.9 million people infected with HIV-1 ("Global HIV & AIDS statistics — 2019 fact sheet | UNAIDS," 2019) and an estimated 7.5 million people living with HIV reside in South Africa (STATS SA, 2018).

The cause of BV remains unknown, but it is linked with the loss of bacteria associated with a "healthy" female genital tract (FGT) microbiota. Healthy FGT microbiota are dominated by *Lactobacillus* sp., with *Lactobacillus crispatus* being the most prominent bacterium (Ravel et al., 2011). *Lactobacilli* help maintain vaginal homeostasis through the production of lactic acid, which results in the lowering of the vaginal pH (Aldunate et al., 2015; Rönnqvist, Forsgren-Brusk, & Grahn-Håkansson, 2006). *Lactobacilli sp.* also produce hydrogen peroxide, bacteriocins and various other antimicrobial compounds. These factors also allow the *Lactobacilli* to adhere to the vaginal epithelial cells and are thus able to compete with other bacteria for nutrients, preventing dysbiosis (Barbés & Boris, 1999; Eschenbach et al., 1989; Ocaña, De Ruiz Holgado, & Nader-Macías, 1999; Stoyancheva, Marzotto, Dellaglio, & Torriani, 2014; Tachedjian, O'Hanlon, & Ravel, 2018).

*L. crispatus* is thought to provide protection against pathogens (Aldunate et al., 2015; Rönnqvist et al., 2006), however studies suggest that women of different ethnicity have differences in their genital tract microbiota. Whilst the majority of Caucasian women's microbiota are dominated by *L. crispatus*, Hispanic and African women have been found to have microbiota that are typically dominated by *L. iners* (Dols et al., 2012). As *L. iners* has been linked with an increased risk of progression to symptomatic BV (Zheng et al., 2019), women from these race groups appear to be at greater risk of acquiring BV and therefore potentially at greater risk of HIV infection.

The association between BV and HIV infection is still not fully understood. There are several proposed mechanisms such as a greater cervicovaginal pH, lack of lactic acid and hydrogen peroxide-producing bacteria such as *Lactobacilli*, an increase in HIV target cells through activation of monocytes via increase in proinflammatory cytokines, and release of bacterial enzymes which are involved in increasing the infectivity of HIV (Sobel, 2000). The reason for the increased risk of HIV infection is most likely due to a combination of these mechanisms.

One class of enzymes that is commonly released by bacterial vaginosis-associated bacteria (BVAB) and associated with BV is sialidases. Sialidases catalyse the hydrolysis of sialic acid moieties from surface glycoproteins and have been found to disrupt the mucosal barrier, which plays a key role in the protection of the FGT from incoming pathogens (Briselden, Moncla, Stevens, & Hillier, 1992a; Howe et al., 1999). In addition to targeting the mucosal barrier of the FGT, sialidase has also been found to increase the infectivity of HIV by targeting the CD4-positive T cells and the virus directly. Infectious molecular clones (IMCs) (Hu et al., 1996) and pseudovirus (Sun, Barbeau, Sato, & Tremblay, 2001) treated with sialidase have been found to have increased infectivity. This review will focus on the role of bacterial-associated sialidase on the infectivity and pathogenesis of HIV.

## 1.2 HIV Diversity, Genome Diversity and Life Cycle

HIV can be classified into nine different subtypes (A, B, C, D, E, F, H, K and L). These subtypes were found to be associated with different regions of the world. Subtype C is the predominant HIV subtype found in sub-Saharan Africa, accounting for 50% of all HIV infections worldwide (Hemelaar, Gouws, Ghys, & Osmanov, 2006; Hemelaar, Gouws, Ghys, Osmanov, et al., 2011).

HIV infection involves the delivery of two copies of single-stranded viral RNA into the host cell, each containing nine genes (Varmus, 1988). Upon release into the cell, the viral genome (9.7 kilobases in length) is first converted into complementary DNA (cDNA) by a viral enzyme called reverse transcriptase (RT). Once converted into cDNA, it integrates into the host cell's chromosomal DNA through the action of the viral enzyme, integrase. The viral cDNA is then transcribed into messenger RNA (mRNA), and then synthesised into three structural proteins and six accessory proteins. The six accessory genes (*rev, nef, tat, vif, vpu and vpr*) encode the

HIV accessory proteins. These accessory proteins function as trans-activators, as well as gene expression regulators (Fanales-Belasio, Raimondo, Suligoi, & Buttò, 2010).

The three structural genes encode *env, pol* and *gag* required for the assembly of infectious viral particles (Varmus, 1988). The *pol* gene encodes integrase, reverse transcriptase and protease. Protease carries out proteolytic cleavage and processing of the polyprotein precursor, Gag-p55, resulting in mature, functional proteins (Mervis et al., 1988). Gag-p55 is encoded by the *gag* gene, and cleavage results in four individual Gag proteins: matrix protein (p17), which associates itself with the host cell membrane, thereby initiating the formation of budding viral particles (Ono et al., 2000); capsid (p24), which assists in the formation of new viral particles, as well as the enclosing of the viral genome into the new viral particles (Göttlinger, Sodroski, & Haseltine, 1989); nucleocapsid (p7), which assists in viral RNA transport (Levin, Guo, Rouzina, & Musier-Forsyth, 2005); and HIV-1 p6, which allows for the incorporation of the accessory protein, Vpr, into new viral particles.

Expression of the *env* gene results in a 160 kDa polyprotein called gp160. The resulting gp160 is then proteolytically cleaved to form two individual glycoproteins: gp120 and gp41. Gp120 is associated with the surface of the viral particle, whilst gp41 is a transmembrane glycoprotein. These two glycoproteins interact to form a non-covalent trimer of heterodimers on the surface of viral particles (Didigu & Doms, 2012; Staropoli, Chanel, Girard, & Altmeyer, 2000). Env is responsible for mediating the initial infection of T lymphocytes. It does so by binding to the host cell CD4 receptor and co-receptor (CCR5 or CXCR4), depending on Env tropism, followed by fusion of the viral and host cell membranes (Berger, Murphy, & Farber, 1999; Kwong et al., 1998). There is evidence that suggests that gp120 can also interact with other co-receptors such as CX3CR1 and CCR3 (Meucci et al., 1998). However, binding to CCR5 and CXCR4 remains the most common co-receptor during infection, as studies have found that inhibition of these co-receptors prevents HIV infection (Berger et al., 1999).

When gp120 binds to CD4 and CCR5 or CXCR4, a conformational change in gp120 allows gp41 to penetrate the target cell membrane allowing fusion between the host and viral cell membranes (Wilen, Tilton, & Doms, 2012). Studies also suggest that Env can bind to dendritic cells (DCs) that are present in the mucosal tissues, which assist in forming the first line of defence against pathogens. Whilst immature DCs have been found to express low levels of CD4 and CCR5/CXCR4, they have been found to express high levels of dendritic cell-specific

ICAM grabbing non integrin (DC-SIGN) receptor on their cell surface which can bind to gp120 (Turville et al., 2001). Whilst binding of gp120 to DC-SIGN does not lead to viral entry into DCs, it does allow for DCs to stably bind Env and transport viral particles from the genital tract mucosa to the lymph nodes, where the viral particles will be able to *trans*-infect T lymphocytes and thus allow for the systemic distribution of virus (C. M. Coleman & Wu, 2009; Geijtenbeek et al., 2000).

## 1.3 Structure of Envelope

The gp120 subunit is comprised of variable loops (V1-V5) and constant domains (C1-C5) (Starcich et al., 1986; Wyatt et al., 1998). There are numerous highly conserved cysteine residues, positioned throughout gp120 and gp41, which form intramolecular bonds that give rise to the trimeric structure of Env (Leonard et al., 1990). The C1 and C5 regions contain residues that are essential in binding to CD4 (Starcich et al., 1986; Wyatt et al., 1998), whilst the C4 region has been implicated in membrane fusion (Starcich et al., 1986; Wyatt et al., 1998). The V2 loop contains residues that play an important role in the infection of memory CD4+ T lymphocytes. The V3 loop has been found to be important in membrane fusion (Freed, Myers, & Risser, 1991) and plays a role in co-receptor interactions (Napier, Wang, Peiper, & Trent, 2006). Studies which looked at mutations in the V3 loop found that this region is also a determinant of Env tropism (Fouchier et al., 1992; Pollakis et al., 2004; Shioda et al., 1994).

The gp41 subunit is comprised of a highly conserved hydrophobic membrane proximal eternal region (MPER), a transmembrane region and a C-terminal tail. The C-terminal tail anchors gp41 to the viral lipid membrane, whilst the MPER has been found to play an important role in the self-assembly of trimers (Fu et al., 2018).

## 1.4 Processing of Envelope

Synthesis of gp160 occurs in the endoplasmic reticulum (ER), where it is processed both co-translationally and at a post-translational level. Following synthesis in the ER, gp160 is modified by Asparagine (N)-linked glycosylation. After trimerization, the N-glycans present on

gp160 are further modified in compartments of the Golgi Apparatus. Gp160 is extensively glycosylated, with approximately 25-30 potential N-glycosylation sites being identified (Allan et al., 1985). N-glycosylation therefore contributes to over 50% of the molecular weight of Gp120 (Lasky et al., 1987). Membrane-bound Oligosaccharyl transferases, found in the ER, carry out N-glycosylation (Leonard et al., 1990) and O-linked glycosylation (Bernstein, Tucker, Hunter, Schutzbach, & Compans, 1994) during translation, yielding high mannose N-glycans present on the surface of the gp160 trimers. Further modification of the high mannose N-glycans occurs in the Golgi, after which the gp160 is cleaved into gp120 and gp41 in the *medial* and/or *cis* Golgi. Following cleavage, the gp120 and gp41 subunits are inserted into the plasma membrane to allow for incorporation, viral particle assembly, maturation and budding (Earl, Moss, & Doms, 1991; Stein & Engleman, 1990). In addition, studies have found that mutation of certain potential N-glycosylation sites (PNGS) resulted in a decrease or loss of cleavage, thereby suggesting that N-glycosylation also plays a key role in the cleavage of gp160 (Dash, McIntosh, Barrett, & Daniels, 1994).

### 1.4.1 N-Glycosylation of Envelope

N-glycosylation plays a crucial role in the functioning of numerous proteins, with over 70% of the eukaryotic proteome undergoing some form of N-glycosylation. N-glycosylation starts in the ER, with the formation of a core oligosaccharide. This oligosaccharide is comprised of three glucose (Glc), two N-acetlyglucosamine (GlcNac) and nine mannose (Man) residues. Whilst the growing polypeptide chain is translocated into the ER, the oligosaccharide core is transferred to asparagine (N) residues within a N-X-T/S sequon (Apweiler, Hermjakob, & Sharon, 1999; Gavel & Heijne, 1990; Welply, Shenbagamurthil, Lennarz, & Naider, 1983). For proper folding and transport of proteins to the Golgi Apparatus (Kornfeld & Kornfeld, 1985), ER α-mannosidase I and glucosidases remove three glucose molecules from the oligosaccharide core, yielding $Man_8GlcNAc_2$ (Hettkamp, Legler, & Bause, 1984).

Once in the *cis*-Golgi, three mannose residues are removed, yielding $Man_5GlcNAc_2$, resulting in high-mannose N-type glycans. These N-glycans are further modified by enzymes present in the Golgi, where two mannose residues are removed in the *medial* Golgi, followed by the addition of three N-acetylglucosamine and one fucose residues, resulting in hybrid type N-

glycans. Additional N-acetylglucosamine residues are added in the *trans*-Golgi, followed by terminal glycosylation where galactose and sialic acid residues are further added via β-1,2-N-acetyleglucosaminyl-transferase I (GnTI), resulting in complex type N-glycans (Vigerust & Shepherd, 2007). The fully processed Envs, carrying high mannose, hybrid and complex type N-glycans, are then transported to the cell membrane.

## 1.5 Role of Sialic Acid in Envelope and Host Cells

Sialic acid residues present in complex type N-glycans carry a net negative charge, thus adding to the overall surface charge of viral particles. Sialic acid residues are also present on the surface of some host cells, as removal of these cell surface sialic acid residues results in increased cell-to-cell contact (Acheson, Sunshine, & Rutishauser, 1991; Rutishauser, Watanabe, Silver, Troy, & Vimr, 1985a). It is suggested that removal of negatively charged sialic acids results in a reduced net negative charge on the surface of cells, thereby enhancing protein-protein interactions. In host cells, the level of sialylation is carefully regulated by sialytransferases and sialidase, an enzyme catalysing the removal of sialic acid residues from various glycoproteins. Sialidase plays a key role in animals, with sialidase being present in nearly every tissue (Tonelli et al., 2010). It has been found that sialidase is important in cell-cell interactions, hematopoetic cell differentiation and T and B cell activation (Keppler et al., 1999; Taylor, 1996).

## 1.6 Bacterial Sialidases

Many dysbiotic bacteria have been found to produce high levels of sialidase. It is thought that the release of sialidase aids in the removal of sialic acid residues from the terminal glycans present in secretions and on the mucosal cell surfaces, including that of the mucus membrane of the FGT (Hardy et al., 2017; W. G. Lewis et al., 2012; Severi, Hood, & Thomas, 2007; Stafford, Roy, Honma, & Sharma, 2012). The removal of sialic acid has been suggested to be used by pathogenic bacteria to facilitate adherence to different cellular surfaces, modify normal mucus barriers and immune responses, as well as to provide the bacteria with a source

of nutrients (Amith et al., 2009; A. L. Lewis & Lewis, 2012; W. G. Lewis, Robinson, Gilbert, Perry, & Lewis, 2013; Stafford et al., 2012; Vick et al., 2014).

## 1.7 Microbiota of the Female Reproductive Tract

FGT microbiota have been studied extensively in order to assess the identity of the different microorganisms present, and their subsequent impact in reproductive pathophysiology. In recent years, sensitive molecular techniques such next generation sequencing (NGS) of bacterial DNA have catalogued FGT microbial diversity. These studies have shown that the FGT microbiota account for approximately 9% of the total bacterial load in humans. The biomass of the microbiome of the vagina in healthy, reproductive-age women is approximately one billion bacteria per gram of vaginal fluid, with *Lactobacilli* representing 90-95% of the total bacteria in the reproductive tract (Delaney & Onderdonk, 2001; Srinivasan et al., 2010).

The microbiota of the FGT varies over the course of a woman's lifetime. In childhood, *Escherichia coli* and obligate and facultative anaerobes dominate the microbial community (Hammerschlag et al., 1978). Rising oestrogen levels during puberty leads to the production of glycogen, the carbohydrate source for *Lactobacilli* and these bacteria begin to colonize the vaginal epithelium, thus *Lactobacilli* remain dominant throughout the reproductive age. Once menopause has been reached and oestrogen levels drop, *Lactobacilli* numbers also decrease. Interestingly, women who experience menopause and underwent hormonal replacement therapy were found to have higher levels of *Lactobacilli*, with lower FGT pH compared to untreated women (Brotman et al., 2015; Galhardo et al., 2006; Hillier & Lau, 1997).

The female reproductive tract microbiota plays a key role in the different stages of the reproductive process, including gamete production, fertilisation, the initiation and maintenance of pregnancy, as well as the microbial colonisation of the foetus (Franasiak & Scott, 2015). This most likely explains the relationship between BV and complications during pregnancy.

## 1.8 Bacterial Vaginosis

The FGT of most healthy women are predominantly colonised by *Lactobacilli* species such as *L. crispatus*, *L. iners*, *L. jensenii* and *L. gasseri* (Antonio, Hawes, & Hillier, 1999). BV is an STI characterised by the outgrowth of anaerobic bacteria (BVAB) and is the most common vaginal disorder affecting women of a reproductive age. It is estimated that BV is prevalent in over 55% of women in South Africa (Lennard et al., 2017). BV commonly manifests as abnormal vaginal discharge, as well as the presence of a foul odour. BV has been associated with an increased risk of acquiring additional STIs, as well as pelvic inflammatory disease (Schwebke, 2009). The cause of BV remains unknown; however, it is associated with the loss of the predominant *Lactobacilli* and the outgrowth of facultative and obligate anaerobes within the FGT (Ravel et al., 2011). Anaerobes that are commonly associated with BV include *Gardnerella vaginalis*, *Prevotella* sp., *Mycoplasma hominis*, *Mobiluncus* sp., *Bacteroides* sp., and *Peptostreptococcus* sp. (Onderdonk, Delaney, & Fichorova, 2016). *Lactobacilli*, most commonly *L. iners*, are also sometimes present in women with BV; however, these make up a very small percentage of the total microflora (Bukusi et al., 2006; Cohen et al., 2012).

### 1.8.1 Diagnosis

The most common diagnosis of BV in developing countries is based on four physiological Amsel criteria (high pH, discharge, malodour and clue cells) and Nugent score. To determine the Nugent score, a gram-stain is performed that determines the relative amount gram-negative rods to that of gram-positive *Lactobacilli*. A low score of 0-3 indicates a predominance of *Lactobacilli*, whereas a high score of 8-10 shows an increased level of gram-negative rods, thus indicating the presence of BV. Recent findings have found that these tests were unlikely to be able to identify BV-positive women if they lack the dominant *Lactobacilli* sp., as their BV-positive status might not correlate to three out of the four Amsel criteria (Gautam et al., 2015).

### 1.8.2 Treatment

BV is typically treated with the antibiotic, metronidazole; however, this treatment does not prevent recurrent BV infections (Sassi et al., 2011). This is likely due to the biofilm barrier that is associated with BVAB. Thus, any successful treatments will need to overcome this barrier in order to gain access to the adherent BVAB. Another reason for treatment with metronidazole not preventing recurrent BV infections is due to the lack of reconstitution of the protective *Lactobacilli* population (Falagas, Betsi, & Athanasiou, 2007). Probiotics could be beneficial, as they could replenish the *Lactobacilli* that are lost due to BV. These *Lactobacilli* sp. could help restore a healthy cervicovaginal microflora, maintain a healthy epithelial barrier and help regulate immune responses. *Lactobacilli* have also been found to produce hydrogen peroxide, which may be protective against future bacterial infections (Rolfe, 2000; Zabihollahi et al., 2012), as well as HIV infection (Licciardi & Tang, 2011). Thus, it has been suggested that treatment with metronidazole should be taken in conjunction with probiotics (Vicariotto, Mogna, & Piano, 2014). However, additional studies have suggested that treatment of BV with the use of probiotics may be ineffective as there is insufficient evidence to suggest that probiotics have a long-lasting impact in treating BV (Mirmonsef et al., 2012). It is possible that probiotic dosage might need to be optimised, thus further studies are required to determine the optimal concentration of *Lactobacilli* sp. that is able to effectively colonize the FGT and outcompete the BVAB (Rolfe, 2000).

## 1.9 Role of *Lactobacilli*

### 1.9.1 Phenotypic and Genotypic Variation

*Lactobacilli* in the genital tract offer protection against the outgrowth of dysbiotic bacteria by several mechanisms. The protective effect of *Lactobacilli* seems to be species-specific. Studies of Caucasian women suggested that *L. crispatus* was significantly associated with the absence of BV (Verstraelen et al., 2009), whereas the opposite was true for *L. iners* (Zheng et al., 2019). It was suggested that *L. crispatus* offers a greater level of protection against the outgrowth of *L. iners* compared to that of *L. gasseri* and *L. jensenii*, thus suggesting that *L. crispatus* helps protect against vaginal dysbiosis (Verstraelen et al., 2009). Interestingly, metagenomic sequencing of seminal microbiota indicated that *Lactobacillus* sp. was the predominant

bacterium similar to that in the FGT. It has been suggested that *Lactobacilli* aid in protection against the outgrowth of pathogenic bacteria such as *Prevotella* sp. and *Pseudomonas* sp.. As the presence of these bacteria were found to be associated with male infertility, it appears as though the presence of *Lactobacilli* plays a crucial role in the maintenance of healthy seminal quality (Weng et al., 2014).

In contrast, studies performed on sub-Saharan countries, in particular South Africa, showed conflicting results. A study showed that *L. crispatus* was most prominent in Southern African women, supporting the findings found in Caucasian women (Pendharkar et al., 2013). Another study (Dols et al., 2012) identified *L. iners* and *L. salivarius* as being the most dominant *lactobacilli* in the FGT of South African women. Further studies by Damelin et al. (2011) found that *L. jensenii* was responsible for protecting against dysbiosis and not *L. crispatus*. Studies performed on American women have found that black females are at greater risk of acquiring BV compared to that of white females (Allsworth & Peipert, 2007; Peipert et al., 2008). The reason for these findings is unknown, although it might be due to differences in microbiota composition amongst different race groups. A study of black American females found that most asymptomatic women carry non-*Lactobacillus* species (Ravel et al., 2011) and a study looking at asymptomatic black South African females also identified non-*Lactobacillus* sp.. In addition, it was found that *L. iners* was the dominant *Lactobacillus* sp. amongst these women (van de Wijgert et al., 2014). These results suggest that black South African women are less likely to have protective *Lactobacillus* sp. such as that of *L. crispatus*, thereby putting them at elevated risk of acquiring BV.

### 1.9.2 Mechanisms of Protection

As women with healthy FGT microbiota have an abundance of *Lactobacilli*, it has been postulated that the *Lactobacilli* help protect against BV as well as HIV infection (Aldunate et al., 2015; Rönnqvist et al., 2006). Comparative functional genomic studies have suggested that *Lactobacilli* associated with the female reproductive tract have evolved to survive in the cervicovaginal environment. In doing so, they have developed adaptive factors that might influence the health of the genital tract by multiple mechanisms (Branco et al., 2010). The mechanisms include the production of lactic acid which results in the lowering of the genital

tract pH, bacteriocins and/or the release of hydrogen peroxide (Cicenia et al., 2014; Klebanoff & Coombs, 1991). Possible bacteriocins that aid in protection of the genital tract include lactocepin, produced by *L. casai* and *L. paracase*, and reuterin produced by *L. reuteri* (O'Hanlon, Moench, & Cone, 2011). Whilst hydrogen peroxide is produced by *Lactobacilli*, the levels of hydrogen peroxide produced in the FGT are thought to be too low to have any significant inhibition of HIV (Hawes et al., 1996).

High Nugent scores correlate with a higher pH (Ravel et al., 2011) and low pH has been found to prevent HIV infection. However, studies have shown that the lactic acid produced by *Lactobacilli* is directly responsible for the inhibition of HIV and BVAB and not the lowering of the pH by the presence of lactic acid (Hawes et al., 1996; Sha et al., 2005). Women with any species of *Lactobacilli* present have been found to have a lower risk of HIV infection (Martin et al., 1999) and decreased levels of viral RNA in cervical vaginal lavages (CVLs) (Borgdorff et al., 2014). Additional studies have suggested that the level of protection by *Lactobacilli* is species-dependent, with the presence of *L. crispatus* being associated with lower HIV RNA levels compared to that of *L. iners* (Mitchell & Marrazzo, 2014). *Lactobacilli* have been found to produce either L- or D-lactic acid, with only the L-isoform showing inhibition of HIV infection (Witkin et al., 2013). The levels of L- or D-lactic acid have been found to differ between *Lactobacilli* sp., which is most likely due to the different number of copies of the L- and D-lactate dehydrogenase (LDH) genes across the species (Klaenhammer, Barrangou, Buck, Azcarate-Peril, & Altermann, 2005). Therefore, it can be inferred that the level of protection is dependent on the species of *Lactobacilli*, and it is likely that additional commensals could also offer similar mechanisms to help protect against HIV infection.

## 1.10 *Gardnerella vaginalis*

One of the most well-studied and prominent BVAB is *Gardnerella vaginalis*. *G. vaginalis* is a gram variable anaerobic coccobacillus, which possess a thin gram-positive cell wall (Castro et al., 2015; Cauci, McGregor, Thorsen, Grove, & Guaschino, 2005; W. G. Lewis et al., 2013; Marrs et al., 2012). *G. vaginalis* is the most frequently isolated bacterium associated with BV, with strong evidence suggesting that this bacterium produces sialidases which participate in mucosal degradation in the FGT (Briselden et al., 1992; B. J. Moncla, Braham, & Hillier, 1990;

Hubertus Von Nicolai, Hammann, Salehnia, & Zilliken, 1984); however, little is known about the relationship between *G. vaginalis* and the human host. Some women who do not have BV and possess "healthy" microflora may also carry low levels of *G. vaginalis* (Briselden & Hillier, 1990), suggesting that *G. vaginalis* might not be the causative agent of BV. However, *in vitro* studies of *G. vaginalis* have shown that there is pathogenic potential in cell adhesion and entry, biofilm formation and cytolytic toxin production (Gelber, Aguilar, Lewis, & Ratner, 2008; Harwich et al., 2010; Patterson, Stull-Lane, Girerd, & Jefferson, 2010). Furthermore, computational studies have found a strong correlation between *G. vaginalis* and the clinical phenotypes of BV (Srinivasan et al., 2012). Therefore, it appears that *G. vaginalis* might be directly involved in causing BV, although other bacterial species might also have a role.

### 1.10.1 Pathogenic Determinants of *Gardnerella vaginalis*

#### 1.10.1.1 Vaginolysin

An early study suggested that *G. vaginalis* releases a cytolytic toxin which mediates FGT colonisation and virulence (Rottini et al., 1990). Gelber et al. (2008) further characterised this toxin and suggested that Vaginolysin was a member of the cholesterol dependent Cytolysin family (CDC) and formed pores within cellular membranes (Gelber et al., 2008). The CDC family is comprised of 15 proteinaceous toxins which are produced by several Gram-positive bacteria and some Gram-negative commensals (Christie, Johnstone, Tweten, Parker, & Morton, 2018; Hotze et al., 2013). Genotypic analysis found that not all *G. vaginalis* isolates produced Vaginolysin (Yeoman et al., 2010) and that its presence could be used as a phenotypic marker to classify strains into specific clades or subgroups (Janulaitiene et al., 2017).

At high concentrations, pore-forming toxins (PFTs) have been found to cause cellular lysis (Los, Randis, Aroian, & Ratner, 2013). Lower concentrations (sublytic) of PFTs have been found to allow cellular survival, but it may compromise the cellular barrier and function (Cassidy & O'Riordan, 2013; Gurcel, Abrami, Girardin, Tschopp, & van der Goot, 2006). Randis et al. (2013) found that exposure to high concentrations of Vaginolysin caused cellular lysis, whereas exposure at lower concentrations resulted in cellular blebbing (Randis et al., 2013). Cellular blebbing has been described as a protective response to the disruption of cellular

membrane integrity (Keyel et al., 2011). Interestingly, it is believed that upon cellular lysis by Vaginolysin, putrescine is produced which results in BV having its characteristic odour. As there is an odour test used to diagnose BV and due to the cytolytic nature of Vaginolysin, it is therefore possible that it can be used as a determinant for BV and virulence.

#### 1.10.1.2 Biofilms

Bacteria are rarely planktonic and instead adhere to surfaces as biofilms which comprise multiple bacterial species embedded within a matrix of polysaccharides, proteins and/or nucleic acids (O'Toole, Kaplan, & Kolter, 2000). As *G. vaginalis* has a high propensity to form biofilms (Alves, Castro, Sousa, Cereija, & Cerca, 2014; Machado, Jefferson, & Cerca, 2013; Patterson et al., 2010), research suggests that it is responsible for initiating colonisation of the FGT by acting as a scaffold for other BVAB species to attach (Hardy et al., 2015; Machado & Cerca, 2015). A study by Castro et al. (2019) described distinct biofilm structures composed of *G. vaginalis* and 15 anaerobic BVAB. It was suggested that *G. vaginalis* does not directly cause BV but that it is its ability to encourage the survival of other BVAB species that determines its virulence (Castro et al., 2019). Therefore, efficient biofilm formation could determine the link between *G. vaginalis* and BV. Biofilms have also been suggested to reduce the efficacy of antibiotics such as metronidazole, leading to recurrent BV (Bradshaw & Brotman, 2015). Understanding the role of biofilm production and the relationship between *G. vaginalis* and other BVAB could provide insight into the aetiology of BV for the development of future treatments that prevent BV relapse.

#### 1.10.1.3 Sialidase

Sialidases are enzymes which cleave sialic acid residues from glycoprotein chains (Hardy et al., 2017; W. G. Lewis et al., 2012; Severi, Hood, & Thomas, 2007; Stafford, Roy, Honma, & Sharma, 2012). Sialidase is one of the most prevalent virulence factors associated with BV and has been suggested to play a vital role in nutrient acquisition and thinning of FGT mucus to allow for increased mobility of *G. vaginalis* (Lewis et al., 2013). BVBlue, a common test used

to diagnose BV, tests Sialidase activity as a diagnostic marker (Bradshaw et al., 2005; Myziuk, Romanowski, & Johnson, 2003).

Bacterial sialidases are classified into two groups according to their molecular weight: small and large (Vimr, 1994), and their amino acid sequences can differ by 80% (Hoyer, Hamilton, Steenbergen, & Vimr, 1992). There is high variation in sialidase molecular weight across bacterial species, suggesting phenotypic and functional differences (Table 1).

Table 1: Bacterial sialidase are divided into two groups: small and large

| Bacterial Species | Molecular Weight (kDa) | Reference |
|---|---|---|
| *Pasteurella multocida* | 80 | Mizan, Henk, Stallings, Maier, & Lee, 2000 |
| *Clostridium perfringens* | 40 | Roggentin, Kleineidam, & Schauer, 1995 |
| Vibrio *cholerae** (NanH1) | 82 | S. Crennell, Garman, Laver, Vimr, & Taylor, 1994 |
| *Clostridium septicum* | 80 | Rothe, Rothe, Roggentin, & Schauer, 1991 |
| *Salmonella typhimurium* | 40 | S. J. Crennell, Garman, Graeme Layer, Vimr, & Taylor, 1993 |
| *Tannerella forsythia** (NanH1) | 60 | Thompson, Homer, Rao, Booth, & Hosie, 2009 |
| *Gardnerella vaginalis** (NANH2 and NANH3) | 80 | Robinson, Schwebke, Lewis, & Lewis, 2019 |

*NanH1-3 are three genes shown to produce bacterial sialidases

Three genes have been identified that express sialidase isoforms: NanH1, NanH2 and NanH3; with the latter two identified as being responsible for *G. Vaginalis*-associated sialidase activity. NanH2 and NanH3 encode proteins with transmembrane domains, suggesting they are membrane-bound and this affects their purification from cell lysates and culture medium (Robinson et al., 2019). Most characterized bacterial sialidases are secreted or are cell-bound (Corfield, 1992). Not all strains produce sialidase, but in those that do, GV sialidases can be surface bound or secreted (Lewis et al., 2013). It has been suggested that a soluble, active

region is cleaved and released into the surrounding environment, similar to the sialidase produced by *Streptococcus pneumoniae* (Lock et al., 1988). Studies have linked the presence of NanH3 (sialidase A) gene to biofilm production (Hardy et al., 2017), suggesting that sialidase could play multiple roles in determining the pathogenicity of *G. vaginalis* and development of BV.

## 1.11 Genotypic and Phenotypic Variation Among *Gardnerella vaginalis* Strains

There is a high level of genetic variation amongst *G. vaginalis*. Genomic studies of *G. vaginalis* isolates identified four phylogenetic groups or clades (Ahmed et al., 2012; Balashov, Mordechai, Adelson, & Gygax, 2014; Janulaitiene et al., 2017; Schellenberg et al., 2016). Genomic analysis by Janulaitiene et al. (2017) found that *G. vaginalis* was present in 87% of women without BV. Of this, Clade 4 was most common (79.4%). Clade 1 and 2 were significantly associated with high Nugent scores (NS 7- 10). All isolates from Clades 1 and 2 were found to carry the sialidase gene, whereas those strains from Clade 4 did not (Janulaitiene et al., 2017). These findings suggest that the presence of *G. vaginalis* itself is not associated with BV, but that it's the presence of strains that produce sialidase. As Clade 4 is not associated with high Nugent scores and sialidase is not present, it is possible that isolates from this clade are not as pathogenic as those from Clades 1 and 2. This emphasises the importance of sialidase to the virulence of *G. vaginalis*. Similarly, Schellenberg et al. (2016) determined that only some Clades expressed sialidase using genomic analysis of FGT microbiota of women from three countries on separate continents (Kenya, Canada and Belgium), although they also showed that the presence of a sialidase gene was not predictive of whether there would be sialidase activity (Schellenberg et al., 2016). Interestingly, all isolates from Clade 2 were found to be positive for the sialidase gene and sialidase activity in both studies by Schellenberg et al. (2016) and Janulaitiene et al. (2017).

A study by Ahmed et al. (2012) also found that *G. vaginalis* isolates clustered into four phylogenetic groups. This study included the *vaginolysin* gene to determine the distribution of Vaginolysin across the four groups. They found that the *vaginolysin* gene was not associated with any particular group, as it was present in isolates from all four groups (Ahmed et al., 2012). Janulaitiene et al. (2017) on the other hand showed that Vaginolysin was present

in all isolates from Clade 4 but not from Clades 1 and 2, although the distribution was not statistically significant (Janulaitiene et al., 2017). These authors suggest that the four *G. vaginalis* groups should be defined according to sialidase activity and Vaginolysin production and that these characteristics are determinant of bacterial pathogenicity.

## 1.12 Mucus in the Female Reproductive Tract

Mucus in the FGT plays a crucial role in human reproductive physiology. It provides lubrication and prevents desiccation within the reproductive tract (Fahrbach, Malykhina, Stieh, & Hope, 2013; Gipson et al., 1995; Idris & Carraway, 1999; Lagow, DeSouza, & Carson, 1999), whilst preventing fluid loss and microbial colonisation. It also assists with sperm survival, as well as migration of sperm to the upper reproductive tract (Argüeso, Spurr-Michaud, Tisdale, & Gipson, 2002; Carlstedt, Sheehan, Ulmsten, & Wingerup, 1982; Lagow et al., 1999; Linford, 1974). The amount and viscosity of mucus varies during the female reproductive cycle. (Mall, Habte, Mthembu, Peacocke, & De Beer, 2017).

One of the major constituents of mucus in the female reproductive tract is glycoproteins carrying terminal sialic acid residues (sialoglycoproteins). Sialoglycoproteins participate in the exclusion of potential pathogens from mucosal surfaces (A. L. Lewis & Lewis, 2012). Mucins are mucosal glycoproteins which contain large amounts of sialic acid residues, up to 16% by weight. Their main function is to offer a thick, lubricated barrier that prevents contact between pathogens and the epithelial cells (Moran, Gupta, & Joshi, 2011; Slomiany, Murty, Piotrowski, & Slomiany, 1996). Studies have found that mucus degradation through the presence of various hydrolytic activities, especially that of sialidase, is a key factor in the etiology of BV and BV-associated health outcomes (Briselden et al., 1992; Howe et al., 1999). Sialidase activity has been associated with increased risk of preterm birth (Bradshaw et al., 2005; Cauci & Culhane, 2011; Cauci et al., 2005).

### 1.12.1 The Depletion of Mucus Sialoglycans by *G. vaginalis*

The function of bacterial sialidase secreted by *G. vaginalis* with respect to mucus depletion was investigated in a study by W. G. Lewis et al., (2013). It was found that free sialic acid residues released from sialoglycoproteins in the culture medium were consumed by sialidase-positive isolates, whereas levels of sialic acid in culture medium with sialidase-negative isolates remained normal. The study also found that sialic acid was released from sialoglycoproteins outside of the cell, thus indicating that hydrolysis of sialoglycoproteins occurred before consumption of the free sialic acid (W. G. Lewis et al., 2013). They suggest that isolates that were negative for sialidase activity did not possess the necessary uptake and/or catabolism mechanisms downstream of sialidase. In addition, the level of free sialic acids in vaginal fluids from women with BV was found to be over 3-fold higher compared to women with a healthy microbiota (W. G. Lewis et al., 2013). It is therefore possible that *G. vaginalis* releases sialidases as a source of nutrition which allows for colonisation of *G. vaginalis* and the displacement of *Lactobacill* sp., favouring BV.

### 1.12.2 Importance of Mucin in Trapping of HIV

Studies by Lai et al. (2009) have shown that Cervical-vaginal fluid (CVF) gathered from women with healthy microbiota efficiently trap HIV. The trapping of HIV in CVF lead to a 1000-fold decrease in HIV mobility compared to mobility in water (Lai et al., 2009). CVF collected from HIV positive and negative women contain both innate and adaptive factors which lead to inhibition of the virus (Ghosh et al., 2010). *In vitro* studies have found that crude CVF collected from HIV negative women did not have any inhibitory effects on HIV. However, purified mucin from the same CVF samples did lead to viral inhibition (Habte et al., 2008). In women with a healthy microbiota, the lactic acid released by the *Lactobacilli* could lead to protonation of negative charges, reducing the overall net negative charge on the surface of the viral particles, whilst not resulting in viral lysis (Mall et al., 2017). This decrease in the net negative charge most likely results in increased protein-protein interactions between the mucin and HIV, resulting in decreased mobility through the reproductive tract. In women with BV, the presence of bacteria-associated sialidases could result in the hydrolysis of the sialic acid groups present in mucin, thereby decreasing the ability of mucin to bind and entrap HIV.

## 1.13 BV Increases the Risk of HIV Infection

A cross-sectional study involving females from seven different African countries showed that the presence of BV increases the risk of HIV infection (Cohen et al., 2012). Additional cross-sectional studies performed in Malawi (Taha et al., 1999, 1998), Uganda (Sewankambo et al., 1997) and Thailand (Hillier et al.; 1995) showed increased incidence of HIV in women with BV, however these studies did not identify a cause and effect relationship between BV and HIV infection. A study performed with Kenyan women (Martin et al., 1999) showed that the absence of *Lactobacilli* and the presence of BV was associated with HIV infection. A meta-analysis of published studies by Atashili et. Al (2008) showed that there was a 60% increase in the risk of HIV infection in women with BV (Atashili et al., 2008).

There is also evidence to suggest that BV increases the risk of acquiring additional STIs, including *Chlamydia trachomatis*, *Trichomonas vaginalis*, gonorrhoea and herpes simplex virus type 2 (HSV-2) (Martin et al., 1999; Wiesenfeld, Hillier, Krohn, Landers, & Sweet, 2003). The presence of these STIs has been suggested to increase the likelihood of HIV infection (Sobel, 2000). Thus, it is likely that BV increases HIV transmission both directly and indirectly by increasing the possibility of additional STIs. Other studies have found that BV results in increased expression of HIV in the lower genital tract in women that are already infected with HIV, therefore explaining the increased levels of viral RNA found in women with BV compared to women without BV (Cu-Uvin et al., 2001; Sha et al., 2005). An additional study showed that women that have lower levels of *Lactobacilli* present in the genital tract have increased levels of genital tract HIV (J. S. Coleman et al., 2007).

Several mechanisms have been suggested which could account for the increase in HIV transmission in women with BV. These include an increase in cervicovaginal pH, decreased levels of hydrogen-peroxide and lactic acid producing *Lactobacilli*, production of enzymes or substances by BVAB that inhibit anti-HIV immunity and increase HIV expression in the cervicovaginal tract in women already infected with HIV (Sobel, 2000). Another potential mechanism is the production of pro-inflammatory cytokines such as interleukin (IL)-8 and IL-1β. IL-8 helps recruit immune cells and IL-1β has been found to induce production of additional pro-inflammatory cytokines, thereby increasing the number of cellular targets for HIV (Dinarello, 1996). Therefore, it is most likely that a combination of all these mechanisms

could result in the increased risk of HIV transmission in women with BV. In particular, the production of bacterial enzymes such as sialidase by BVAB is of interest.

## 1.14 The Effect of Bacterial-Associated Sialidase on HIV Infection

Sialidases that are secreted by BVAB could play an important role in increasing the risk of HIV infection in women with BV. A study found that treatment of peripheral blood mononuclear cells (PBMCs) with sialidase resulted in increased p24 and RT titres, without an increase in DNA or cells (Stamatos et al., 1997). In addition, desialylation also increased production of IL-6, macrophage inflammatory protein (MIP)-1α and MIP-1β. These results suggest that loss of sialic acids from cell surface glycoproteins influences the immune responses of monocytes (Stamatos, Curreli, Zella, & Cross, 2003). Treatment of infectious molecular clones (IMCs) (Hu et al., 1996) and pseudovirus (Sun et al., 2001) with sialidase was found to result in increased single-round infection of human CD4+ cell lines. Both studies also observed an increase in HIV-mediated syncytium formation, showing increased levels of cell-to-cell conjugate formation following treatment with sialidase. Hu et al. (1996) postulated that the reason for this increase in HIV infectivity was due to the loss of the overall negative charge present between the viral particle and host-cell receptors (Hu et al., 1996).

## 1.15 Conclusion

Studies have found that most black women have genital tract microbiota dominated by *L. iners* (Dols et al., 2012). This bacterium has been found to lack some of the protective factors associated with other *Lactobacilli* such as *L. crispatus* (Aldunate et al., 2015; Rönnqvist et al., 2006). Therefore, these women are potentially at greater risk of getting BV and women with BV are at a greater risk of acquiring an HIV infection (Atashili et al., 2008). Understanding the prevalence of *G. vaginalis* strains that produce sialidase and Vaginolysin in this vulnerable population might provide additional insight into their risk of HIV infection. Sialidase is not only linked to FGT dysbiosis but can also directly increase HIV infection.

Therefore, further study is required to determine whether there might be a potential advantage to include specific sialidase inhibitors in treatment of BV as inhibition of these sialidases might help decrease the high risk of women with BV acquiring HIV.

## 1.16 Study Rationale and Objectives

### 1.16.1 Aim

The overall aim of this study was to determine whether bacterial sialidase released from *Gardnerella vaginalis* influences HIV entry.

### 1.16.2 Objectives

1. Determine whether the presence of commercially available sialidase influences HIV entry
2. Investigate the mechanism of how purified bacterial sialidase influences HIV infection
3. Purify sialidase from *G. vaginalis* isolates and determine whether it influences HIV entry
4. Investigate the impact of *G. vaginalis* culture medium on HIV infection

# Chapter 2: Methods and Materials

## 2.1 Envelope Construct

An HIV subtype C *env* clone, CAP210 E8 (donated by Gama Bandawe, IIDMM, UCT) was obtained from the Centre for the AIDS Programme of Research in South Africa (CAPRISA) 002 study cohort. This *env* clone was isolated from a participant five weeks post-infection and single genome amplification (SGA) was performed to characterise the *env* gene. Viral RNA was extracted from participant samples which tested positive for HIV infection. Viral RNA was used to prepare cDNA with limiting dilutions to obtain single templates and the amplicons were sequenced. The SGA-derived *env* sequences were analysed and a consensus *env* sequence was generated. The *env* SGA amplicon was then cloned into the pcDNA3.1 V5-His Topo® (Life technologies™) mammalian expression vector.

## 2.2 Mammalian Cell Culturing

Human embryonic kidney cell line (HEK 293T) and TZM-bl cells (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID) were maintained in Dulbecco Modified Eagle high glucose growth medium (DMEM) (Sigma Aldrich®) supplemented with 10% fetal bovine serum (FBS) (Biocom Biotech®), 1 U/mL penicillin and 1 µg/mL streptomycin (Pen-Strep) (Sigma Aldrich®) (Full-DMEM). Mammalian cells were grown and incubated at 37 $^{o}$C in a water-jacket incubator (90% humidity, 5% CO2).

## 2.3 Pseudovirus Preparation and p24 Assay

### 2.3.1 Plasmid preparation

Plasmid DNA used to produce pseudovirus was extracted from transformed *E. coli* cells. These cells were initially inoculated, either from picked colonies or glycerol stocks, into 5 mL Luria Broth (LB) [1% tryptone, 0.5% yeast extract, 1% NaCl in distilled water with 100 µg/mL carbenicillin disodium salt (Sigma Aldrich®)] and grown at 30 $^{o}$C for 10 hrs with shaking. The 5 mL bacterial culture was used to inoculate 50 mL LB containing 100 µg/mL carbenicillin

disodium salt and incubated at 30 $^{o}$C for 16 hrs with shaking. Plasmid DNA was extracted and purified using the Qiagen® Plasmid Midi Kit (Qiagen®) according to the manufacturer's instructions.

### 2.3.2 Transfection

Mammalian cells were maintained in 75cm² cell culture flasks (NEST®). HEK 293T cells were lifted with 1% trypsin (Lonza™) and seeded at a density of 4x10$^{5}$ cells/well in a 6-well plate (NEST®) with 2 mL of Full-DMEM. The plates were then incubated overnight at 37 °C in a water-jacket incubator (90% humidity and 5% CO2). When the cell monolayers were approximately 60% confluent, the HEK293T cells were co-transfected with Polyethylenimine (PEI) (Sigma Aldrich®) at a PEI to DNA ratio of 1:3. Each transfection mixture contained 400 µL serum-free DMEM, 7.5 µg of DNA [2.5µg *env* and 5 µg viral backbone (pSG3Δ*env*)] and 21.5 µL PEI (1 mg/mL). The mixture was mixed vigorously by vortexing and incubated at room temperature for 10 min to allow for DNA:PEI complex formation. Growth medium was discarded and 1.5 mL Full-DMEM was added to each well containing cells and the DNA:PEI mixtures were added drop wise. The plates were incubated for 6 hrs to allow for DNA incorporation and the growth media was replaced with 2 mL Full-DMEM followed by incubation for 48 hrs to allow for pseudovirion production. After incubation, the growth media was collected and clarified through a 0.22 µm pore size filter. The pseudovirus stocks were supplemented with 20% FBS and aliquoted before being stored at -80 °C. Pseudovirus stocks were inactivated with 1% Empigen® (Sigma-Aldrich®) in Tris-buffered Saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.5) in order to determine concentration of p24.

### 2.3.3 p24 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of viral p24 was determined using a chemiluminescent p24 enzyme-linked immunosorbent assay (ELISA) and the TROPIX® detection system (CDP-Star®, Applied Biosystems). High binding 96-well plates (Whitehead Scientific™) were coated with lyophilised sheep anti-HIV p24 Gag coating antibody (Alto Bio Reagents) diluted to a final concentration of 2 µg/mL in coating buffer (100 mM NaHCO3, pH 8.5). The plates were

incubated overnight at room temperature and unbound antibody was removed and wells were washed with 100 µL TBS three times. The coated p24 plates were blocked with 5% BSA (Biocom Biotech®) in TBS for one hour at room temperature and stored at -20 °C until required. The coated plates were washed with TBS four times to remove any unbound BSA, and inactivated pseudovirus was serially diluted with 1% Empigen®-TBS and 100 µL was added the necessary wells. A standard curve was constructed by serially diluting purified p24 protein (0-32 ng/µL) (Aalto Bio Reagents) in 1% Empigen®-TBS. Following the addition of the p24 standards and pseudovirus in duplicate to the appropriated wells, the plates were incubated at room temperature for three hours. Unbound p24 was discarded and the plate was washed with 100 µL TBS four times. An alkaline phosphatase-conjugated mouse anti-HIV-1 p24 antibody [EH12AP (Aalto Bio Reagents)] was diluted 1:64000 in TBS with 0.1% Tween-20 (TBS-T), 20% sheep serum (Lasec®) and 2% BSA before 100 µL was added to each well and incubated for 1 hr at room temperature. The plates were washed with 100 µL TBS-T eight times to remove any unbound antibody before two washes with 100 µL 1X TROPIX® buffer (200mM Tris, 10mM $MgCl_2$, pH 8.9). ELISA CPD Star/Sapphire II (Applied Biosystems®) was diluted 1:4 in 1x TROPIX® buffer and 50 µL was added to each necessary well. A multi-well plate reader (Turner Biosystem® Modulus Microplate) was used to determine luminescence and relative light unit (RLU) readings were converted to a p24 concentration (ng/µL) through the construction of a standard curve and non-linear regression analysis in Microsoft Excel™.

## 2.4 HIV Entry Efficiency Assay

The entry efficiency of pseudovirus was determined by infecting TZM-bl cells, a type of HeLa cell that has been modified to express CD4 and co-receptors CCR5 and CXCR4 on their cell surface. TZM-bl cells were lifted using 1% trypsin and $1x10^4$ cells were seeded in 200 µL Full DMEM in a 96-well plate (NEST®) before being incubated at 37 $^oC$ overnight in a water-jacket incubator (90% humidity and 5% CO2). Once cell monolayers were approximately 60% confluent, the pseudovirus stocks were thawed and diluted to 200 ng/mL in Full-DMEM. Growth medium (150 µL) was removed followed by the addition of 100 µL of Full DMEM and 50 µL of diluted pseudovirus (50 ng/mL) in triplicate. The plates were incubated at 37 $^oC$ for 48 hrs, 5% CO2 in order to allow for a single round of infection to occur. Following infection,

150 µL of growth medium was removed from the appropriate wells and 50 µL of BriteGlo® lysis buffer (Promega®) was added. The 96-well plate was left at RT for 2 minutes to allow for cell lysis and 100 µL was transferred to an opaque 96 well plate (Porvair®). A luminometer (Glomax® 96 Modulus Microplate) was used to measure luciferase activity in RLU. The mean and standard deviation of the triplicate values was determined and normalised to the positive control (virus-only) to give the fold-change. The virus-only control contained the cells, pseudovirus and growth medium and underwent no treatment of any sort to indicate normal infection levels. A negative (medium only) control consisted of cells and growth medium to control for the effect of Full DMEM on luminescence. These data are plotted using GraphPad Prism™ 5.

## 2.5 Sialidase Treatment

### 2.5.1 HIV Entry Efficiency Assay in the Presence of Sialidase

For experiments that determined the effects of sialidase on HIV entry, TZM-bl cells were infected with 200 ng pseudovirus as outlined in Section 2.4 with the following modifications: 2 µL of commercial sialidase (1 U/µL) (NEB®) isolated from *Clostridium perfringens* (40kDa) was added to each HIV infection assay in a final well volume of 200 µL.

### 2.5.2 HIV Entry Efficiency Assay with Sialidase-Treated Pseudovirus and Cells

To determine whether sialidase targets the viral particle or the cell, an entry efficiency assay was performed as outlined in Section 2.4 with modifications. To treat the pseudovirus with sialidase, 0.5 µL of commercial sialidase (1 U/µL) was added to 50 µL of pseudovirus (200 ng/mL) and incubated at 37 $^{o}$C, 5% $CO_2$ for 12 hrs. To inhibit sialidase activity, 2 µL of N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (NADNA) (30 µg/µL) (Sigma Aldrich®) was added to the treated pseudovirus and incubated at 37 $^{o}$C, 5% $CO_2$ for 2 hrs. To determine whether sialidase targets the cells, 150 µL of growth medium was removed from the wells and 0.5 µL of commercial sialidase (1 U/µL) was added to the TZM-bl cells in 50 µL of Full-DMEM. The cells were incubated at 37 $^{o}$C, 5% $CO_2$ for 12 hrs and inhibited with 2 µL of NADNA for 2 hrs. Pseudovirus was incubated under the same conditions in order to control for the additional

time taken before infection. The treated pseudovirus was used to infect TZM-bl cells and the treated cells were infected with the incubated pseudovirus and Full-DMEM was added to give each well to a final volume of 200 µL. Pseudovirus was also incubated for 14 hrs in the virus-only control. These infections were performed in triplicate and the RLU values were normalised to the virus-only control.

## 2.6 Preparation of Abiotic *G. vaginalis* Culture Medium

Two strains of *Gardnerella vaginalis* (3H6 and 2I3) were obtained as a gift from Dr Remy Froissart (French National Centre for Scientific Research, Paris) that were isolated from French women with BV (Unpublished). The American Type Culture Collection (ATCC) (14018) strain of *G. vaginalis* was obtained as a gift from Dr Lindi Mason (IDMM, UCT). Frozen stocks in 50% Glycerol were recovered on BHI Agar plates [3.7% BHI powder (Sigma Aldrich®), 1% yeast extract, 0.1% soluble starch, 1.5% Agar] and incubated at 37 $^{o}$C for 48 hrs. The purity of the *G. vaginalis* strains were determined by streaking from -80 °C stocks onto BHI plates and assessed visually for consistent colony size, colour, and morphology as outlined by other studies (Robinson et al., 2019). Gram staining was also performed by a colleague to ensure purity of strains.

The *G. vaginalis* cultures were used to inoculate 15 mL of BHI growth medium (3.7% BHI powder (Sigma Aldrich®), 1% yeast extract, 0.1% soluble starch) and incubated at 37 $^{o}$C for 48 hrs without shaking. Anaerobic conditions were ensured by filling the tube with medium, minimising exposure of the bacteria to air. The bacterial cells were removed from the cell medium by centrifugation at 6000 rpm for 10 min followed by further clarification through a 0.22 µm pore size filter. The abiotic culture medium was stored at 4 $^{o}$C until required.

## 2.7 Purification of Sialidase

### 2.7.1 Sialidase Sequence Analysis

Peptide sequences of sialidase produced by ten *G. vaginalis* isolates were acquired from the NCBI Protein Database with accession numbers: RFD76680.1, RFD75533.1, RFD74547.1, RDW99899.1, RDW98445.1, RDW97464.1, RDW97411.1, RIY22936.1, RIY20495.1

andRFT30369.1. The predicted MW and estimated isoelectric point of these sequences was calculated using an online ExPASy tool (https://web.expasy.org/compute_pi/).

### 2.7.2 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation was performed as a first step in the sialidase purification process. Abiotic *G. vaginalis* culture medium was cultured as outlined in Section 2.6 with the following modification: the 3H6 and 2I3 strains were used to inoculate 50 mL fresh BHI media and incubated at 37 $^{o}$C for 48 hrs without shaking to scale-up the total amount of culture medium. The bacterial cells were removed from the cell medium by centrifugation at 6000 rpm for 10 min followed by further clarification through a 0.22 µm pore size filter. To perform the precipitation, ammonium sulphate was added to the 50 mL abiotic culture medium in 10% cuts, starting with 20%. The solution was stirred rapidly at 4 $^{o}$C. Once the salt had dissolved, the solution was left on ice for 20 minutes in order to allow the proteins to come out of solution. The samples were then transferred to SS34 centrifuge tubes and centrifuged at 10 000 g for 10 min at 4 $^{o}$C. The supernatants were kept aside for additional salt to be added. The pellet was resuspended in 10 mL buffer (20 mM Tris, 50 mM NaCl, pH = 8.0) and stored at 4 $^{o}$C. The volume of the supernatant was measured and used to determine the amount of salt to add to increase the %saturation from 20% to 30%. This process was repeated until the 80% cut had been pelleted and resuspended in buffer. All fractions were stored at 4 $^{o}$C until sialidase activity was measured.

### 2.7.3 Sialidase Activity Assay

Sialidase activity was measured using a Neuraminidase Assay Kit (Fluorometric - Blue) (ab138888) (Abcam®). The assay was performed according to the manufacturer's instructions. Sialidase standards (0.312 mU/mL to 20 mU/mL), test samples and the blank control were added (50 µL) to a solid black 96-well microplate (NEST®) followed by addition of 50 µL of sialidase assay reaction mixture to each well. The plate was incubated at 37 $^{o}$C, 5% $CO_2$ for one hour. Fluorescence was determined using a fluorometer at an Excitation/Emission wavelength of 320/450 nm with a 20 nm slit width. Enzyme activity was determined as

relative fluorescent units (RFU) and test sample RFU was converted to units using the standard curve. A unit of enzyme corresponds to the total amount of enzyme required to catalyze the conversion of one micromole of substrate per minute (*Enzyme Nomenclature: Recommendations*, 1964). The neuraminidase assay is based on the principle that change in RFU is proportional to the conversion of one micromole non-fluorescent neuraminidase substrate per minute to fluorescent product enabling the calculation of units of enzyme present.

### 2.7.4 Desalting

Desalting was necessary due to the presence of high levels of salt in the samples following the ammonium sulphate precipitation step. The pooled protein samples were desalted using a HiTrap® Desalting Column (Sigma Aldrich®) using the Akta Explorer FPLC System and UNICORN 4.11 software (GE Healthcare Life Sciences). The column was equilibrated using 25 mL of equilibration buffer (20 mM Tris, 50 mM NaCl, pH = 8.0) at a flow rate of 5 mL/min to completely remove ethanol from the column and 10 mL of the precipitated protein sample was loaded into the column. Total protein and conductivity were observed using a UV and conductivity monitor. The $A_{254nm}$ readings indicated protein level, and elution was stopped once the level of protein dropped and the conductivity level began to increase. The desalted protein was collected in 5 mL fraction sizes. This process was repeated until the entire protein sample (50 mL) had been desalted.

### 2.7.5 Anion-Exchange Chromatography

Anion-exchange chromatography was performed due to sialidase carrying a net-negative charge. It was used as an intermediate step in the sialidase purification process. A HiTrap™ Capto Q HiRes column (Sigma Aldrich®) with an Akta Explorer™ system and UNICORN 4.11 software was used. The column volume was 5 mL, and the column was first equilibrated using 10 column volumes (CV) of equilibration buffer and loaded with 25 mL of the desalted protein sample using a 50 mL superloop. Once the sample was applied, the column was washed with 10 CV of equilibration buffer and the protein was eluted over 20 CV of a linear salt gradient

(from 0% to 100%) of elution buffer (20 mM Tris, 1 M NaCl, pH 8.0). The column was washed with 5 CV of elution buffer to elute any remaining ionically bound material. All fractions were collected in 5 mL aliquots and stored at 4 $^{o}$C. Protein fractions from anion-exchange chromatography that were found to contain sialidase activity were pooled and concentrated using an Amicon® Ultra-15 Centrifugal Filter Unit. The pooled protein sample (15 mL) was loaded into the centrifugal filter unit and centrifuged at 10000 g for 30 min. This process was repeated until the 50 mL of the pooled protein sample was loaded and centrifuged, resulting in a 5ml sample volume (10-fold increase in concentration).

### 2.7.6 Size-Exclusion Chromatography (SEC)

As protein samples had been separated by charge, SEC was the final step of the sialidase purification process using a HiLoad™ 16/600 Superdex™ 200 pg column (GE Healthcare Life Sciences) with a Gilson UniPoint™ system and UniPoint™ software. The column was equilibrated by applying 120 ml of SEC buffer (20mM Tris, 50 mM NaCl, pH = 8.0) at a flow rate of 1 mL/min. The concentrated sample (5 mL) was loaded into the column. All fractions were collected in 1.5 mL aliquots and were stored at 4 $^{o}$C. Following size exclusion, protein samples with sialidase activity were pooled (8mL) and concentrated using the same concentration method listed above, resulting in 2mL sample volume (4-fold increase in concentration).

### 2.7.7 SDS-PAGE and Coomassie Staining

Protein samples were separated using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). 10 µL of protein samples was mixed with loading buffer (1% SDS, 8% glycerol, 1% β-mercaptoethanol, and 0.01% bromophenol blue) before being denatured by heating at 100 $^{o}$C for 10 min. The denatured samples were loaded into a 5% Bis-Tris polyacrylamide stacking gel and a 10% Bis-Tris polyacrylamide resolving gel in the presence of SDS-PAGE running buffer (25mM Tris, 190 mM glycine, 0.1 % SDS, pH 8.3) and separated by electrophoresis at a constant current of 20 mA using a BioRad® PowerPac™. The gels were

stained with Coomassie stain [1% Coomassie Brilliant Blue R (Sigma Aldrich®), 50% Methanol, 10% acetic acid, 40% $dH_2O$] and destained using destaining solution (25% Ethanol, 10% acetic acid, 65% $dH_2O$) to allow for visualisation of protein bands.

### 2.7.8 Bicinchoninic Acid (BCA) Assay

A BCA assay kit (ThermoFischer®) was used to determine the concentration of total protein present in the purified sialidase samples. The assay was performed according to the kit manufacturer's instructions. Briefly, bovine serum abumin (BSA stock) was serially diluted from 20 to 2,000 µg/mL. One part of Copper Reagent was mixed with 50 parts of BCA Reagent and 100 µL was added to each sample. Standards and sample (50 µL) were added per well and gently mixed before incubating for 30 min at 37°C, 5% $CO_2$. The assay mixture was cooled to ambient temperature and OD was measured at 562nm.

### 2.7.9 Mass Spectrometry

In order to identify the proteins, the protein samples (356 ng/mL) were sent to the service provider, Centre for Proteomic and Genomic Research (CPGR) for liquid chromatography and mass spectrometry analysis (LC-MS/MS) for peptide mass fingerprinting. The sample had a low concentration and was concentrated before analysis. The sample was digested with trypsin and ionised, either by electrospray ionisation or atmospheric pressure chemical ionization (APCI), before passing over a mass analyser. The mass analysis involved the separation of fragments created in the atmospheric pressure ionisation (API) interface and then detection by the mass analyser. The fragment ions were plotted according to the mass-to-charge ratio (m/z) vs relative abundance.

## 2.8 HIV Entry Assay in the Presence of *G. vaginalis* Culture Medium

*G. vaginalis* was cultured as outlined in Section 2.6. An HIV entry efficiency assay was performed as outlined in Section 2.4 with modifications. Following seeding of the TZM-bl cells in triplicate, 150 µL of growth medium was removed, 50 µL of pseudovirus (200 ng/mL) and varying volumes of culture medium (5 µL, 10 µL, 25 µL) were added. An appropriate volume of Full-DMEM was added to each well to ensure that the total well volume was 200 µL. To optimise the pH of the assay, the pH of the culture medium was measured (pH = 4.6) and adjusted using 1M sodium hydroxide until the pH matched that of Full DMEM (pH = 8.74). For heating of the medium, abiotic culture medium and BHI were heated at 100 $^{o}$C for 10 min using a heating block.

## 2.9 Cell Viability Assay

To determine cell viability, 1x$10^4$ TZM-bl cells were seeded in 100 µL Full-DMEM in triplicate in a 96-well plate and incubated overnight at 37 $^{o}$C, 5% $CO_2$. The next day, 150 µL of growth medium was removed and varying volumes of abiotic *G. vaginalis* culture medium (5 µL, 10 µL, 25 µL) was added. An appropriate volume of Full DMEM was added to ensure that each well had a 200 µL volume. The cells were incubated at 37 $^{o}$C, 5% $CO_2$ for 48 hrs. Following incubation, 100 µL was removed and 20 µL of the MTT labelling agent was added to each well. MTT is a colorimetric assay that measures the formation of insoluble, purple formazan through the reduction of tetrazolium dye MTT by mitochondrial NADPH-dependent cellular oxidoreductase. Dead cells lack functional mitochondria and are thus unable to reduce the tetrazolium and do not become purple (Green, Reade, Ware, 1984). The plates were incubated for 4 hrs to allow for MTT reduction, followed by the addition of 100 µL solubilisation solution to each well and incubated at 37 $^{o}$C, 5% $CO_2$ overnight. The plates were checked for complete solubilisation of the purple formazan crystals and if complete solubalisation had not occurred, then the wells were mixed by pipetting until it did occur. Absorbance was measured at a 595nm wavelength using a Gen5 microplate reader.

## 2.10 Statistical Analyses

Analysis of variance (ANOVA) is an extension of an unpaired t-test and both compare differences between means of groups treated independently. A one-way ANOVA with multiple comparison Tukey post-test were performed using GraphPad Prism™ 9 on the mean of two independent biological experiments done in triplicate that determined whether the addition of commercial sialidase, purified sialidase or abiotic G. vaginalis culture medium increased pseudovirus infection compared to when only culture medium was added or the pseudovirus infection assay was left untreated. An unpaired t-test was performed using GraphPad™ Prism 9 on the means of three independent biological experiments done in triplicate to determine whether 1) viral entry increased when the cells were treated with sialidase, 2) viral entry increased when the pseudovirus were treated with sialidase, and 3) whether cell viability changed when G. vaginalis culture medium was added. Statistical significance was indicated as *, ** and *** for p-values less than 0.05, 0.01 and 0.001, respectively.

# Chapter 3: The effect of bacterial-associated sialidase on HIV entry

## 3.1 Introduction

Bacterial vaginosis is the most common vaginal disorder affecting women of a reproductive age. It is highly prevalent in sub-Saharan Africa, where it is estimated that 66% of women will be affected at some point in their life. BV is a disorder whereby the healthy vaginal microflora, which is dominated by *Lactobacillus sp.*, is disrupted and facultative anaerobic bacteria begin to outgrow the healthy *Lactobacilli* (Ravel et al., 2011). One of the most prevalent anaerobes found in women with BV is *Gardnerella vaginalis* (Briselden et al., 1992; Moncla, Braham, & Hillier, 1990; Von Nicolai, Hammann, Salehnia, & Zilliken, 1984).

Previous studies have found that women who have BV are at a greater risk of being infected with HIV-1 (Atashili, Poole, Ndumbe, Adimora, & Smith, 2008). As sub-Saharan Africa has one of the highest prevalence of HIV in the world (Hemelaar, Gouws, Ghys, & Osmanov, 2006; Hemelaar, Gouws, Ghys, Osmanov, et al., 2011), it is of great importance to study and understand the relationship between BV and HIV.

The reason for the increased likelihood of HIV infection in women with BV is not yet known. However, prior research suggested that the presence of BVAB induces an inflammatory response that increases the abundance of HIV-permissive cells at the site of infection, thereby increasing the likelihood of viral transmission (Sobel, 2000). Alternatively, it has been suggested that BV dysbiosis reduces levels of 'healthy' *Lactobacilli*, such as *L. crispatus* that release compounds such as lactic acid and hydrogen peroxide which help protect against HIV infection (Sobel, 2000). Finally, it has also been hypothesised that BVAB, in particular *G. vaginalis*, produce bacterial sialidases which target the virus directly and increase HIV infectivity.

Sialidases are a family of enzymes which cleave sialic acid residues from O- and N-glycosylated glycoproteins. Previous studies have found that desialylation of pseudovirus (Sun et al., 2001), IMCs (Hu et al., 1996) and PBMCs increased HIV infection (Stamatos et al., 1997). These findings suggest that sialidase might alter glycan structures on the surface of the virus and/or host cells, promoting receptor interactions and thus HIV infection. Interestingly, sialidase activity in the secretions of the female genital tract was associated with BV and is used as a

diagnostic marker of dysbiosis (Myziuk et al., 2003). An early study suggested that the source of sialidase was the BVAB, *Prevotella sp.* and *G. vaginalis* (Briselden et al., 1992). Therefore, we hypothesized that BV-associated *G. vaginalis* secrete sialidase into the FGT environment and enhance HIV infection. This study isolated the sialidase present in the culture medium of *G. vaginalis* and determined whether both purified and commercially available sialidase (NEB®) increased the infection of the reporter cell line, TZM-bl cells by pseudovirus. Finally, we investigated whether sialidase targeted the viral particle and/or the host cells.

## 3.2 Results

### 3.2.1 Commercially Available Sialidase Increases HIV Entry

Previous studies have found that the addition of commercially available sialidase resulted in increased HIV entry using RT assays (Hu et al., 1996), as well as entry efficiency assays (Sun et al., 2001). In this study, when commercially available sialidase was included in a pseudovirus entry efficiency assay, there was a 2-fold increase in pseudovirus entry compared to the virus-only control ($p<0.001$) (Figure 3.1), confirming earlier reports. The sialidase could potentially remove the negatively charged sialic acid moieties present on Env, thus increasing the potential interaction between the negatively charged host cell membrane and the virus particles (Acheson et al., 1991; Rutishauser et al., 1985). Alternatively, it has been suggested that as sialidase treatment of PBMCs increased HIV replication (Stamatos et al., 1997), it is possible that the removal of sialic acids from the surface of host cells could also increase viral entry.

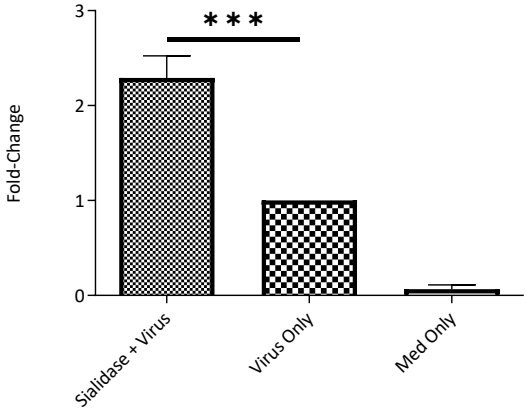


Figure 3.1: **Effect of sialidase on pseudovirus infection.** TZM-bl cells ($5x10^4$ cells/ml) were infected with pseudovirus equivalent to 200 ng p24 in the presence (Virus + Sialidase) and absence (Virus Only) of bacterial sialidase (NEB®). Fold-change of relative light units (RLU) relative to the virus only (positive) control is shown. The medium only (negative) control containing only cells and growth medium is also shown. Bars represent the mean of three independent experiments with error bars indicating standard deviation. Statistical analysis was carried out using GraphPad Prism unpaired t-test. *, **, *** represent P<0.05, P<0.01 and P<0.001, respectively.

### 3.2.2 Commercial Sialidase Primarily Targets the Viral Particle

In order to determine whether the sialidase acted on sialic acids found on the viral particle and/or the host cell membrane, pseudovirus and TZM-bl cells were treated independently with sialidase prior to adding a sialidase inhibitor, N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid. Following inhibition with NADNA, TZM-bl cells were infected with pseudovirus to determine whether desialylation of cells or virus was responsible for the increase in pseudovirus entry.

Controls were included that confirmed that NADNA successfully inhibited the effects of the sialidase and did not have any effect on viral entry (Figure 3.2). There was a significant increase in viral entry after treatment of pseudovirus (1.5-fold) ($p<0.001$) and TZM-bl cells (1.2-fold) ($p=0.0134$) compared to the virus-only control. These results indicate that the sialidase appears to influence both the pseudovirus and host cells. However, desialylation of the viral particle appeared to have a greater influence on viral entry. These results were similar to the findings of Hu et al. (1996), who showed that treatment of virus with a number of different bacterial sialidase increased the replication capacity of HIV strains (Hu et al., 1996).

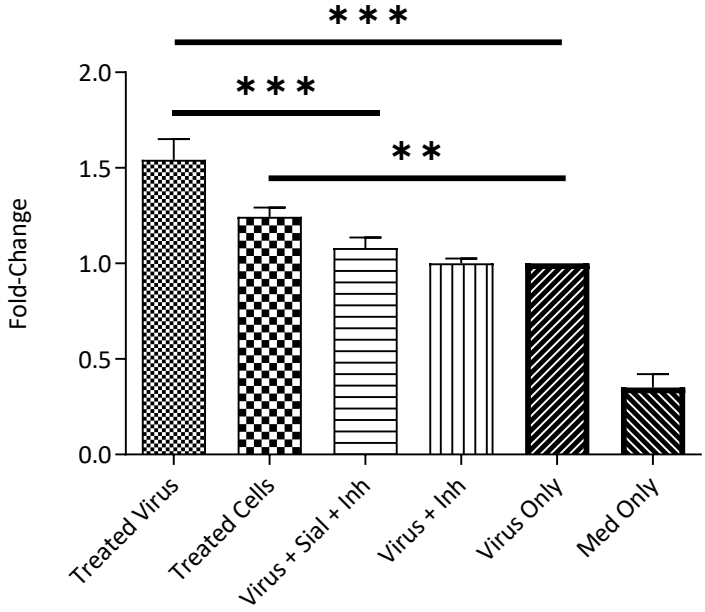


Figure 3.2: **The impact of desialylation of cells and pseudovirus on HIV-1 entry.** TZM-bl cells were infected with the equivalent of 200 ng p24 in the presence/absence of commercial bacterial sialidase. Pseudovirus (Treated Virus) and TZM-bl cells (Treated Cells) were treated with 2 U of commercial sialidase (NEB®) separately for 24 hrs before being inhibited with 300 ng/µl NADNA for 2 hrs followed by incubation for 48 hrs at 37 °C, 5% $CO_2$. Fold-change of relative light units (RLU) normalised to the virus only control is shown. Bars represent the mean of three independent experiments with error bars representing standard deviation. Statistical analysis was carried out using GraphPad Prism unpaired T-test. *, **, *** represent P<0.05, P<0.01 and P<0.001, respectively.

### 3.2.3 Purification of *G. vaginalis* Sialidase

#### 3.2.3.1 Selection of *G. vaginalis* Strains

Six *G. vaginalis* strains isolated during a cohort study in France (donated by Dr Remy Froissart, French National Centre for Scientific Research, Paris) were successfully plated on BHI plates. *G. vaginalis* was confirmed morphologically due to the presence of white, shiny circular colonies. BHI medium was inoculated with each strain and cultured under anaerobic conditions. Of the six strains, only two (2I3, 3H6) grew successfully in BHI medium.

### 3.2.3.2 Ammonium Sulphate Precipitation of *G. vaginalis* Culture Medium

Culture medium of 2I3 and 3H6 strains was centrifuged and an ammonium sulphate precipitation was carried out for crude fractionation of the abiotic culture medium. Ammonium sulphate was added in 10% cuts to the culture medium, starting from 20% to 80% salt concentration and the fractions were tested for sialidase activity. Both strains tested positive for sialidase activity (Figure 3.3). Fractions from 3H6 (50% - 70%) and 2I3 (60% - 70%) that tested positive for sialidase activity were pooled in order to limit loss of enzyme activity and maximize the concentration of sialidase for subsequent purification steps.

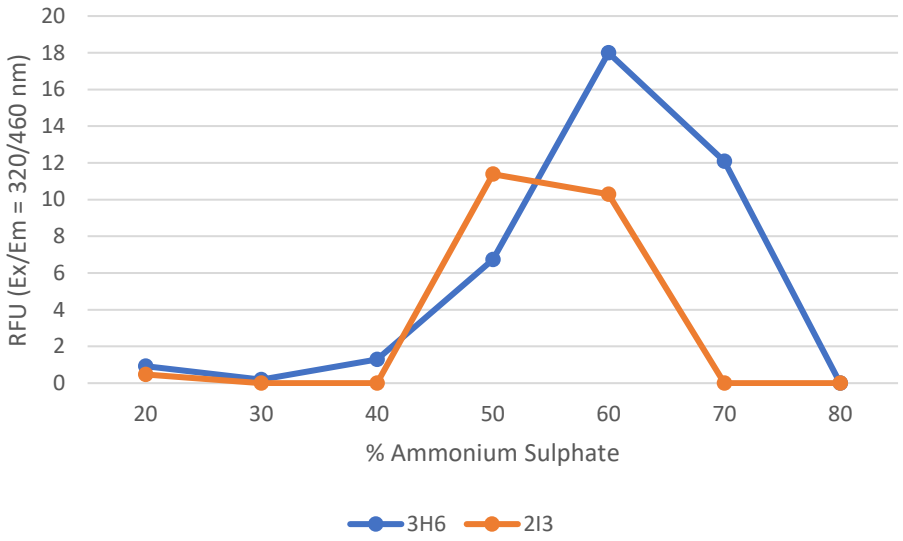


Figure 3.3: **Sialidase activity following ammonium sulphate precipitation of *G. vaginalis* cultures**. Two strains of *G. vaginalis* (3H6 and 2I3) were cultured in BHI medium for 48 hrs at 30°C under anaerobic conditions. The bacterial cells were removed by centrifugation and an ammonium sulphate precipitation was performed with 10% cuts (20% - 80%). Following the precipitation, sialidase activity was measured using a sialidase activity assay kit (Abcam®) for each individual strain by reading the relative fluorescence units (RFU) at an excitation/emission wavelength of 320/450 nm with a 20 nm slit width. The blue and orange lines represent 3H6 and 2I3, respectively. Data shown is a representation of a single experiment.

### 3.2.3.3 Anion-Exchange Chromatography

The pooled sample was desalted using a HiTrap® desalting column to prepare the analyte for ion exchange chromatography. During desalting, the $A_{254nm}$ readings were monitored in real-time to determine whether the protein had successfully flowed through the column. Once the salt concentration began to increase and intersect with the $A_{254nm}$ line, the flow into the desalted sample was stopped.

Anion-exchange was chosen due to the low estimated isoelectric point of *G. vaginalis* sialidase (pI = ~ 5.94) by analysing published *G. vaginalis* sialidase sequences. At pH 8.0, sialidase would be expected to bind to the positively charged column and not elute during the flow through (Figure 3.4A). Sialidase eluted in the second peak as fractions 27-30 tested positive for sialidase activity (Figure 3.4B). To ensure that enzyme was not lost, fractions 25-32 were pooled and analysed by SDS-PAGE. The SDS-PAGE showed no visible bands, most likely due to high sample volume of eluted fractions and thus low protein concentration.

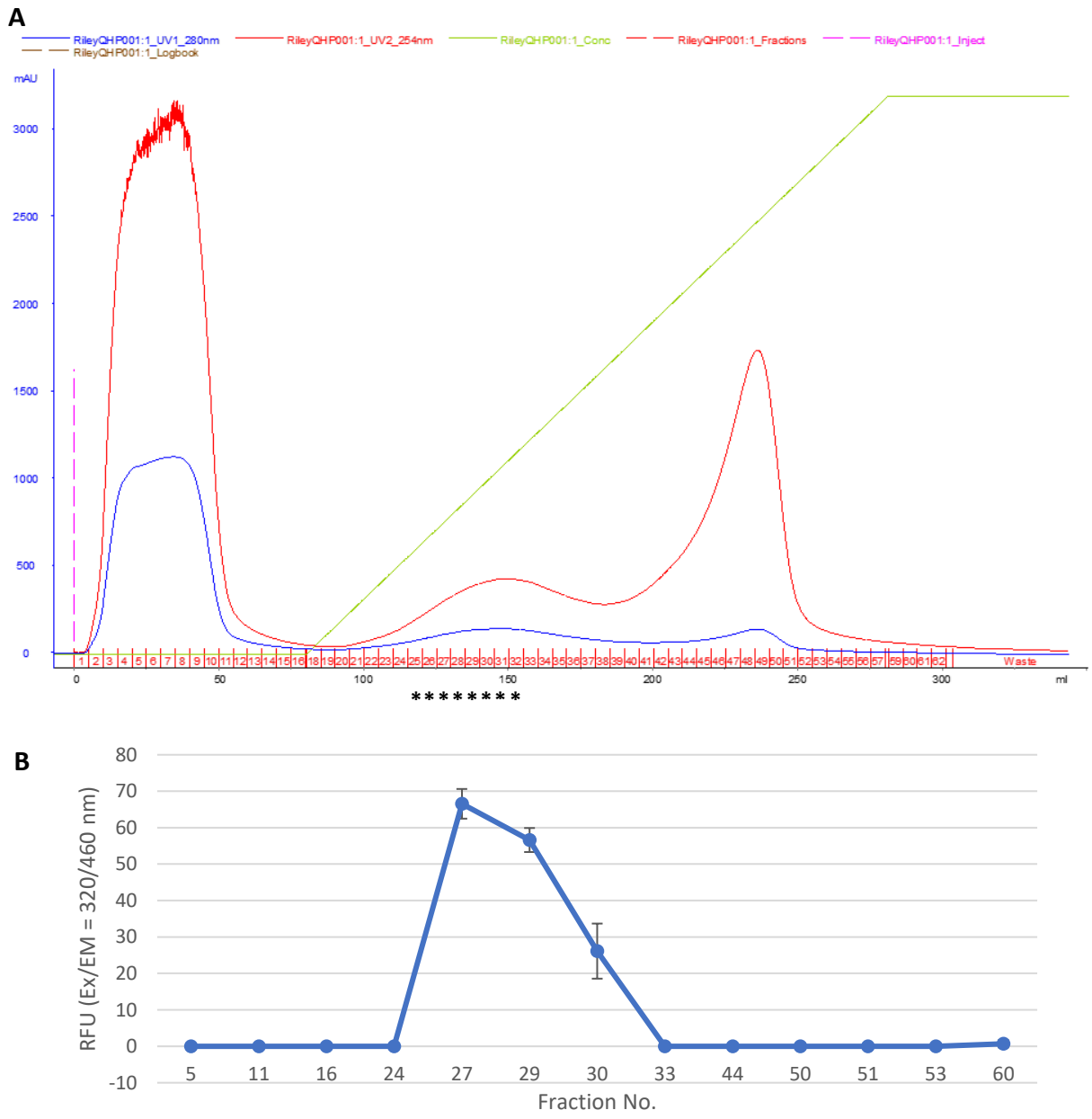


Figure 3.4: **Anion-exchange chromatography of pooled *G. vaginalis* cultures following desalting**. The desalted sample was fractionated using anion-exchange chromatography on a HiTrap™ CaptoQ HiRes column, with a 1 mL/min flowrate and a fraction size of 5mL. (A) Shows the chromatogram representing the anion-exchange, with the red, blue and green lines representing $A_{254nm}$, $A_{280nm}$ and salt concentration (%conductivity), respectively. Fractions 25-32 (indicated with an asterisk) were used in further studies. (B) Sialidase activity was measured in duplicate using a sialidase activity assay kit (Abcam®) by reading the relative fluorescence units (RFU) at an excitation/emission wavelength of 320/450 nm with a 20 nm slit width. Error bars represent the standard deviation between the duplicate values.

#### 3.2.3.4 Size-Exclusion Chromatography

The pooled sample was further fractionated by size-exclusion chromatography into 78 fractions. Figure 3.5A shows the size-exclusion chromatogram, with the green line indicating the $A_{254nm}$ readings and the brown line indicating the $A_{280nm}$ readings. The eluted fractions were tested for sialidase activity and separated by SDS-PAGE. The sialidase activity assay showed that there was sialidase activity in fractions 53 - 56 (Figure 3.5B). It was determined that the average molecular weight of the proteins in these fractions was approximately 45kDa using a calibration curve of the S200 column with protein standards. So as not to lose enzyme by discarding fractions, fractions 50 – 60 were pooled and analysed by SDS-PAGE. However, due to low protein concentration, there were no apparent bands. As seen in Figure 3.6A, the relative protein abundancy in the fractions containing sialidase is low compared to other fractions which do not contain any sialidase. Thus, the proteins were concentrated using an Ultra-15 Centrifugal Filter Unit.

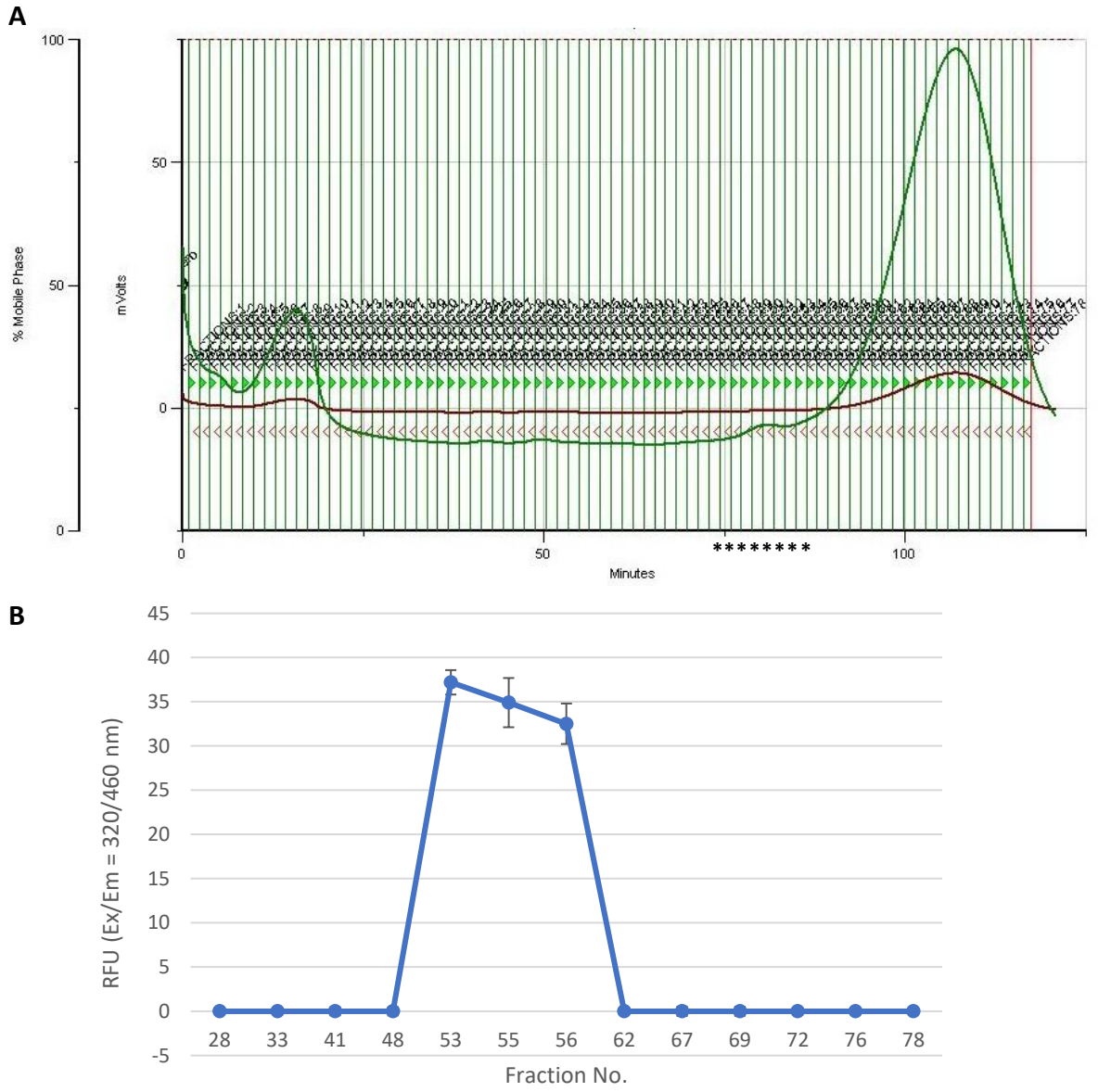


Figure 3.5: **Size-exclusion chromatography data of pooled anion-exchange fractions containing sialidase**. The anion-exchange fractions were pooled and separated according to size using size-exclusion chromatography using a Superdex™ S200 (GE Healthcare Life Sciences®) column, with a 1mL/min flowrate and a fraction size of 1.5mL. (A) Chromatogram representing the size-exclusion data, with the green and brown lines representing the $A_{254nm}$ and $A_{280nm}$ readings, respectively. The fractions (highlighted by asterisks) were used in further studies. (B) Sialidase activity of the fractions were measured in duplicate using a sialidase activity assay kit (Abcam®) by reading the relative fluorescence units (RFU) at an excitation/emission wavelength of 320/450 nm with a 20 nm slit width. Error bars represent the standard deviation between the duplicate values.

### 3.2.4 Analysis of Purified Sialidase

The gel exclusion pooled sample was concentrated 4-fold and had a total protein concentration of 356 ng/ml, with sialidase activity that corresponded to 264.50 RFU (Figure 3.6A). The final volume of purified enzyme was 2 mL with approximately 2 U of purified sialidase. The specific activity was determined to be 2.6 U/mg based on the 0.71 mg of total protein in our sample.

The purified sialidase sample was analysed using SDS-PAGE with 2 U of commercial sialidase (NEB®) included as a control. In the purified sialidase lane, there are two protein bands visible with an approximate size of 40 - 45kDa, similar to that determined by gel exclusion chromatography (Figure 3.5A). The commercial sialidase lane showed a single band with an approximate size of 42.5 kDa. A previous study purified recombinant *G. vaginalis* sialidase from *E.coli* with a molecular weight of approximately 110 kDa that corresponded to its predicted size of 99 kDa , more than twice the size of our protein bands (Janulaitiene et al., 2017). The results obtained from the SDS-PAGE suggested that either: two proteins with sialidase activity were successfully purified, there was protein degradation, contamination by another bacterial species or the bands represented monomers of a multisubunit enzyme. However, both proteins were similar in size to that of the commercial sialidase isolated from *Clostridium perfringens* (40kDa), suggesting that the purified proteins could potentially represent sialidases. However, we cannot exclude the possibility that one or both of the purified proteins are contaminants and that the *G. vaginalis* sialidase concentration was too low to detect by SDS-PAGE. To confirm the identity of the proteins, the sample was sent for mass spectrometry. The protein sample had a low concentration and thus it was further concentrated and analysed using LC/MS. The preliminary analysis suggested that a sialidase was present, but the signal was too low to confirm whether there were two distinct *G. vaginalis* sialidases or a contaminant from other bacteria (data not shown).

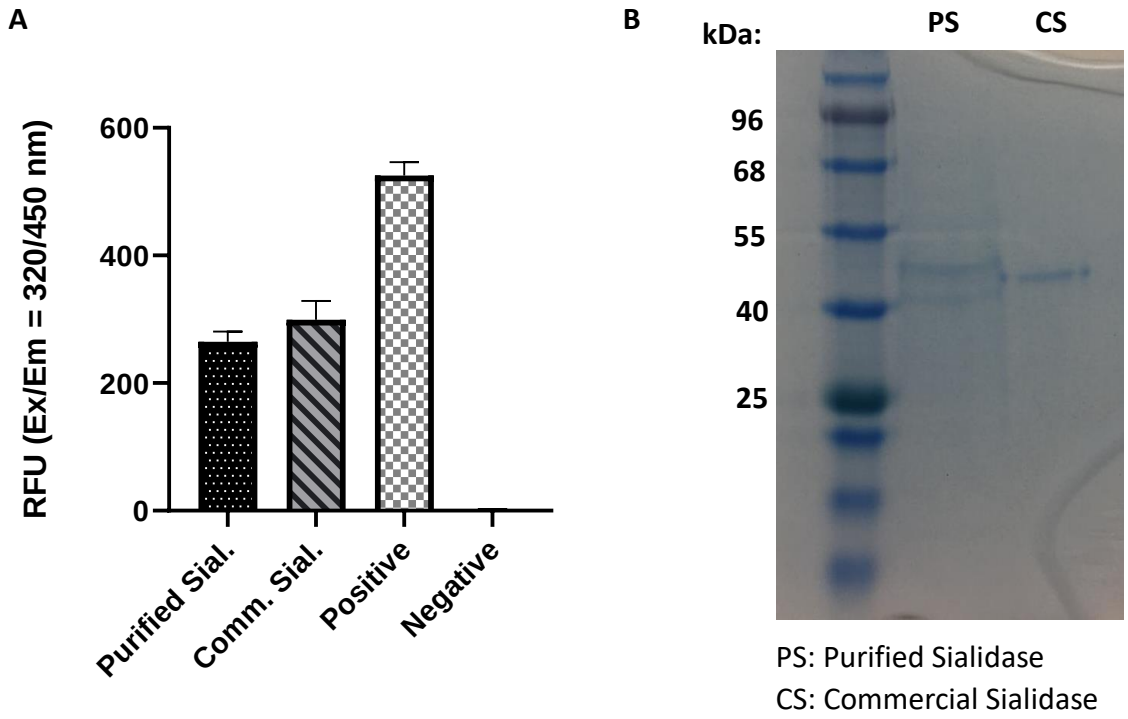


Figure 3.6: **Analysis of purified sialidase of pooled size-exclusion fractions**. (A) Fractions with sialidase activity were pooled and concentrated using an Ultra-15 Centrifugal Filter Unit (Amicon®), which resulted in the volume of the sample decreasing from 15 mL to approximately 1.5 mL. A) Sialidase activity was determined in the purified sialidase sample (Purified Sial.), the commercial sialidase (NEB®) (Comm. Sialidase), 20 mU/mL of sialidase standard (Positive) and a blank containing no sialidase (Negative) using a sialidase activity assay kit (Abcam®) by reading the relative fluorescence units (RFU) at an excitation/emission wavelength of 320/450nm with a 20nm slit width. Error bars represent the standard deviation between duplicate values. B) The purified sialidase was analysed using SDS-PAGE and stained with Coomassie with 2 U of a commercial sialidase (NEB®) included as a positive control. The lanes labelled PS and CS represent purified sialidase and commercial sialidase, respectively. A protein ladder (NEB®) was included to determine the approximate size of the protein bands. The size (kDa) corresponding to each band is written on the left of the gel.

### 3.2.5 Purified Sialidase Increases HIV Entry

As the commercial sialidase increased pseudovirion infection of TZM-bl cells, the putative *G. vaginalis* sialidase(s) was added to the entry efficiency assay. The addition of the putative purified sialidase(s) resulted in a statistically significant 1.5-fold increase in viral infectivity ($P<0.001$), similar to that of the commercial sialidase (Figure 3.7). However, the activity was not confirmed with the sialidase inhibitor (NADNA) as the low volume of the purified sample limited the inclusion of controls. We were thus unable to verify that the sialidase was responsible for the increase in HIV infection.

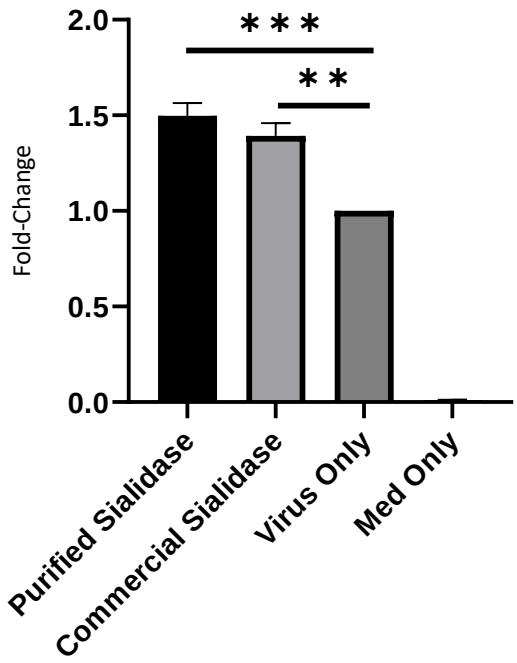


Figure 3.7: **Effects of purified sialidase on HIV entry.** TZM-bl cells ($5x10^4$ cells/mL) were infected with pseudovirus equivalent to 200 ng p24 in the presence of 50 µL of purified sialidase (purified sialidase), 2 U of commercial sialidase (NEB®) (Commercial Sialidase). Fold-change of relative light units (RLU) relative to the virus only (positive) control is shown. Bars represent the mean of three independent experiments with error bars indicating standard deviation. Statistical analysis was carried out using GraphPad Prism unpaired t-test. *, **, *** represent P<0.05, P<0.01 and P<0.001, respectively.

## 3.3 Discussion

Bacterial sialidases facilitate microorganisms to colonise biological niches and destroy tissue (Briselden et al., 1992; Moncla et al., 1990; Von Nicolai et al., 1984). BVAB sialidases cleave sialic acids from a number of different substrates within the cervicovaginal fluid, including that of IgA and mammalian mucins (W. G. Lewis et al., 2012) and might play a very important role in the pathogenesis of *G. vaginalis* (Janulaitiene et al., 2017). The results of this study found that the addition of commercially available sialidase to an HIV entry efficiency assay resulted in a statistically significant increase in viral entry. We then showed that treatment of both the pseudovirus and host cells independently with sialidase resulted in increased viral entry, although exposure of the virus to sialidase resulted in a greater increase in HIV entry compared to when the cells were desialylated independently. These findings are similar to those reported by Hu et al. (1996), who showed that sialidase primarily targets the viral particles (Hu et al., 1996). The increase in HIV entry could be due to the removal of negatively charged sialic acid residues from the surface of the viral particles and/or the cell membrane,

thereby reducing the energetic repulsion induced by the need for two negatively charged molecules to interact (Acheson et al., 1991; Rutishauser et al., 1985).

*G. vaginalis* strains (3H6 and 2I3) were successfully cultured and the abiotic culture medium was fractionated using ammonium sulphate precipitation, anion-exchange chromatography and gel filtration. SDS-PAGE of the purified sample showed two protein bands, approximately 40-45 kDa in size, with associated sialidase activity, suggesting that these two *G. vaginalis* strains produced soluble sialidase. During the purification procedure, enzyme and total protein concentrations were crudely analysed using absorbance readings at 245 nm and not all fractions were tested for sialidase activity. It is thus difficult to construct a purification table and identify steps that led to loss of enzyme and therefore yield and enrichment of sialidase. Based on our rough calculations, the specific activity of the purified protein was calculated to be 2.6 U/mg which is lower than that that of *Clostridium perfringes* (280 U/mg) (Kruse et al., 1996). This suggests that the *G. vaginalis* sialidase was not pure. However, as the substrates used in the sialidase assays were most likely not the same, it is expected that the specific activity would vary. Comparison to the specific activity of purified *G. vaginalis* NanH2 and or NanH3 would have confirmed the successful purification of a sialidase, but *G. vaginalis* sialidase has yet to be purified (Robinson et al 2019). The final total sialidase activity of the purified sample was roughly estimated to be 2 U.

An SDS-PAGE was performed after each of the chromatography steps, however in each case the sample was too dilute and thus there were no visible bands present. This suggests that the purification process could be optimized to increase the yield of protein. This could be achieved by changing the types and/or pH of the buffers or the types of columns used during the desalting, ion-exchange and size-exclusion chromatography stages of the purification process. In addition, silver staining could have been used rather than staining with Coomassie, as this has been found to be 100 times more sensitive and allows one to visualize proteins bands that have very low concentrations (Farrell Jr., 2017). We did not calculate the initial concentration of sialidase activity in the culture medium and growth conditions might need to be optimized to ensure vigorous bacterial growth to increase concentration of secreted enzyme. A better alternative would be to purify the sialidase from *G. vaginalis* cell lysates, as protein concentration would be higher and sample volume would be smaller (W. G. Lewis et al., 2013). However, sialidase secreted into the culture medium is more physiologically

relevant seeing that it would have to desialylate HIV in the FGT environment. Finally, cloning of NanH2 and NanH3 into a bacterial expression vector and purification using His-tagged affinity chromatography might be the best alternative to increase the yield and optimize the purification of the sialidase.

Sample preparation is essential for successful protein fingerprinting by mass spectrometry. The buffer used during the anion-exchange and size-exclusion chromatography contained TRIS which is not an ideal reagent for mass-spectrometry (Shieh, Lee, & Shiea, 2005). Previous studies have shown that the use of volatile buffers is more appropriate during ion-exchange chromatography (van den Eijnden-van Raaij, Koornneef, van Oostwaard, de Laat, & van Zoelen, 1987). Thus, the use of a volatile buffer such as Triethylammonium bicarbonate (TEAB) during the chromatography stages might have improved the mass spectrometry results.

SDS-PAGE indicated two protein bands in the purified sialidase sample which could represent: two sialidase isozymes, an active and inactive form of sialidase, a contaminant, a denatured sialidase multimer or protein degradation. During the process of purifying *Clostridium septicum* sialidase, the authors noticed that the 80kDa protein degraded into bands corresponding to approximately 36 kDa proteins when stored at 4$^{o}$C (Rothe et al., 1991). It is thus possible that the smaller, clear band visible by SDS-PAGE represents a degradation product. The molecular weights of sialidases are highly variable and are grouped according to their size (Vimr, 1994). Therefore, despite the known size of *G. vaginalis* sialidase being 110 kDa, it is possible that smaller isoforms are secreted into the culture medium. The band sizes are very similar to that of the commercial sialidase, suggesting that they could represent enzymes from the group of “small sialidases”. As there were two strains *of G. vaginalis* pooled together before the purification process began, it is possible that each of these strains produced their own unique variation of sialidase which could explain the presence of two bands in the purified sialidase lane. Future studies are required to determine whether each strain produced their own unique sialidase. This could be accomplished by analysing the sialidase gene sequences of G. vaginalis strains to identify potential differences, qPCR to determine expression followed by cloning of the genes, expression and purification.

The purified sialidase significantly increased HIV entry 1.5-fold. This finding suggests that the strains of *G. vaginalis* used for purification produce sialidase that enhance viral entry to a

similar extent as commercially available sialidase. This is the first time to our knowledge that a sialidase purified from *G. vaginalis* has been shown to increase HIV infection, suggesting a mechanism for how BV is associated with HIV infection. Production of sialidase isoforms by different bacterial strains have been linked to differences in the virulence of *G. vaginalis* (Janulaitiene et al., 2017). The *G. vaginalis* 3H6 and 2HI strains were isolated from France and it is possible that the sialidase produced might be different when compared to those produced by strains that have a sub-Saharan Africa origin. Previous studies have found that *G. vaginalis* strains can produce their own unique forms of sialidase, with some expressed more efficiently and/or with greater catalytic potential (Robinson et al., 2019). It is tempting to speculate that *G. vaginalis* strains from sub-Saharan Africa may produce a form of sialidase that is better able to cleave sialic acids from HIV and thus enhance viral infection better than other strains. Therefore, global distribution of *G. vaginalis* strains might explain why women with BV in some regions have higher risk of HIV infection than other areas. This might explain the strong association between BV and HIV acquisition in sub-Saharan Africa. Future studies should determine the structure and catalytic activity of different forms of sialidase produced by BVAB, whether its properties vary between regions and whether they impact HIV infection differently.

In conclusion, this study has purified and identified proteins that might represent an isoform or isoforms of sialidase from two G.vaginalis strains. The addition of the purified protein sample to an HIV pseudovirus assay indicated an increase in HIV infection. This suggests that sialidase purified from primary *G. vaginalis* strains isolated from women with BV can increase HIV infection.

# Chapter 4: The effect of *G. vaginalis* culture medium on HIV entry

## 4.1 Introduction

The role of *G. vaginalis* in BV aetiology remains controversial, as some women are positive for *G. vaginalis* but do not have BV (Criswell, Ladwig, Gardner, & Dukes, 1969). Although *Atopobium vaginae* and *Mycoplasma hominis* have also been suggested as potential causative agents of BV, to date, no single bacterial species has been linked to BV (Ferris et al., 2004; Holst, Svensson, Skarin, Weström, & Mårdh, 1984). However, as the vast majority of BV positive women also carry *G. vaginalis* (Spiegel, Davick, & Totten, 1983; Swidsinski et al., 2005), it is likely that this species plays a role in BV aetiology.

There is high genetic and phenotypic variation amongst different strains of *G. vaginalis,* with some producing sialidase. *G. vaginalis s*ialidase has not only been linked to BV but also HIV infection (Hu et al., 1996; Sun et al., 2001). This study found that sialidase secreted by *G. vaginalis* enhanced pseudovirus infection 2-fold. Studies have shown that *G. vaginalis* strains can fall into four phylogenetic groups: Clades 1-4 with sialidase produced by Clades 1 and 2 only (Ahmed et al., 2012; Balashov et al., 2014; Janulaitiene et al., 2017; Schellenberg et al., 2016), suggesting that only some commensal BVAB could increase the risk of HIV acquisition. Conversely, this suggests that Clade 4 *G. vaginalis* strains might not increase HIV infection and a recent study has suggested that BV is not associated with HIV infection (Heffron et al., 2017). We therefore hypothesized that if women are BV positive but carry *G. vaginalis* that do not produce sialidase, then these women would have lower risk of HIV infection than if they carried strains from Clades 1 and 2.

Harwich et al. (2010) did an extensive genotypic and phenotypic comparison of a *G. vaginalis* strain associated with BV to a commensal strain that was not linked with BV (Harwich et al., 2010). They suggested that adherence factors determined the difference in virulence of the two strains. However, they did not test for the presence of sialidase activity. Another difference between the two strains was the cytotoxicity of Vaginolysin despite differing by only one amino acid. The Vaginolysin of the BV-associated strain was more cytotoxic, causing initial rounding of epithelial cells and then complete lysis after three hours. Vaginolysin is considered a virulence factor as it has been shown to assist in the colonization of *G. vaginalis*

in the FGT (Ragaliauskas et al., 2019) and its lysis of FGT epithelial cells release nutrients to support the growth of *G. vaginalis* (Gelber et al., 2008). Furthermore, it is also suggested that it permeabilises FGT cells which then favours HIV infection. Therefore, strains that do not produce sialidase but are positive for Vaginolysin might still enhance HIV infection. There are conflicting suggestions as to whether Clade 4 strains express Vaginolysin, with Ahmed et al. (2012) suggesting it was found across all clades whereas Janulaitiene et al. (2017) showed that Vaginolysin was present in all isolates from Clade 4 but not from Clades 1 and 2 (Ahmed et al., 2012; Janulaitiene et al., 2017). The ATCC (14018) strain of *G. vaginalis* does not produce sialidase but has been found to express Vaginolysin (Yeoman et al., 2010). Thus, we determined whether the culture medium of ATCC (14018) influenced HIV entry.

## 4.2 Results

### 4.2.1 The Effect of *G. vaginalis* Culture Medium on HIV Entry

#### 4.2.1.1 *G. vaginalis* Culture Medium Decreases HIV Entry

The abiotic BHI culture medium of the *G. vaginalis* strain, 14018, (GV) was added to the pseudovirus entry efficiency assay to determine whether it would enhance HIV infection. Three volumes (25 µL, 10 µL and 5 µL) of GV were added and compared to BHI medium that was not exposed to *G. vaginalis* (BHI). Unexpectedly, addition of *G. vaginalis* culture medium resulted in an overall decrease in viral entry, with a 1.6-, 4.2-, and 7.1-fold decrease, with increasing volume added (Figure 4.1A). Addition of 25 µL and 10 µL BHI medium resulted in a 2.2- and a 1.35-fold decrease in viral entry respectively. However, addition of 5 µL of BHI medium did not appear to have any effect (Figure 4.1B). Although the BHI medium inhibited viral entry, its effect was less severe when compared to the abiotic GV culture medium. This suggested that *G. vaginalis* was either releasing an inhibitory compound or altering the environment to such an extent that HIV entry was inhibited.

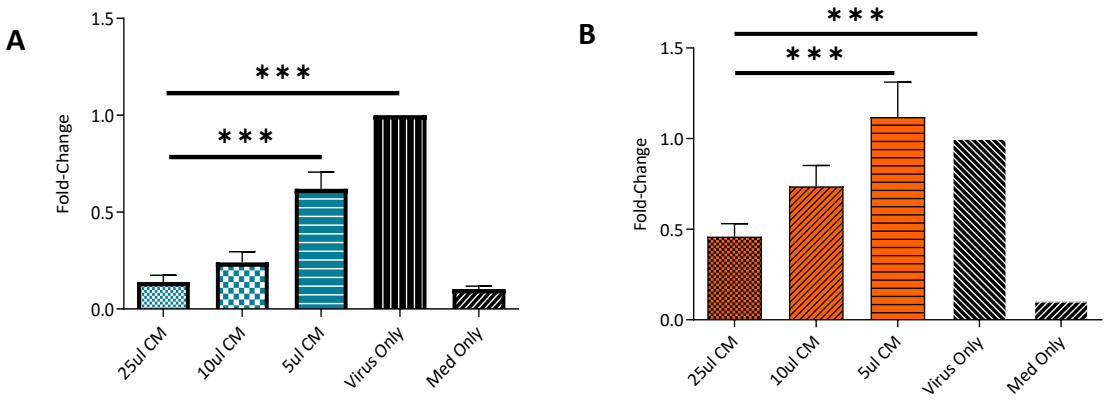


Figure 4.3: **The effects of culture medium on HIV-1 infection.** BHI was cultured for 48 hr with and without *G. vaginalis*. The culture medium (CM) was centrifuged and filter-sterilized. TZM-bl cells ($5x10^4$ cells/mL) were infected with 200 ng pseudovirus with decreasing volumes of (A) GV culture medium and (B) BHI medium and incubated for 48hrs at 37°C, 5% $CO_2$. Fold-change of relative light units (RLU) normalised to virus only control is shown. Bars represent the mean of two independent experiments with error bars showing standard deviation. Statistical analysis was carried out using GraphPad Prism one-way Anova with Tukey's post-test. *, **, *** represent P<0.05, P<0.01 and P<0.001, respectively.

#### 4.2.1.2 ATCC *G. vaginalis* Strain Does Not Secrete Active Sialidase

As the abiotic culture medium of *G. vaginalis* inhibited instead of enhancing HIV infection, we tested whether sialidase was present in the culture medium of the ATCC *G. vaginalis* strain, 14018. The test was performed in duplicate and sialidase activity was not detected in the culture medium (Figure 4.2), as previously shown (Janulaitiene et al., 2017; Bernard J. Moncla & Pryke, 2009).

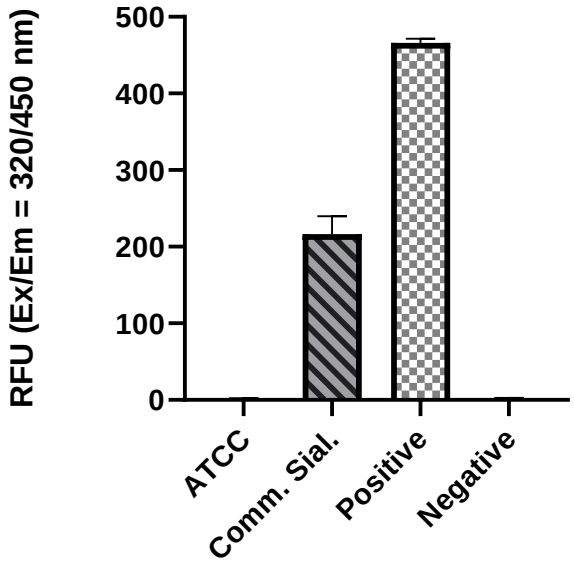


Figure 4.2: **Determination of sialidase activity in ATCC *G. vaginalis* culture medium.** Sialidase activity was determined in ATCC *G. vaginalis* culture medium (ATCC), the commercial sialidase (NEB®) (Comm. Sial), 20 mU/mL of sialidase standard (Positive) and a blank containing no sialidase (Negative) using a sialidase activity assay kit (Abcam®) by reading the relative fluorescence units (RFU) at an excitation/emission wavelength of 320/450nm with a 20 nm slit width. Error bars represent the standard deviation between duplicate values.

#### 4.2.1.3 Investigating the Inhibition of Viral Entry by *G. vaginalis* Culture Medium

As BHI medium lowered pseudovirus entry, a cell viability test was carried out to determine whether the medium itself was cytotoxic. Figure 4.3 indicates that 25 µL of both GV and BHI culture medium resulted in a significant loss in cell viability (1.6- and 1.7-fold, respectively) compared to the medium only control ($p<0.001$). However, 5 µL of GV and BHI did not have any significant impact on cell viability, suggesting that at this volume, the medium was no longer cytotoxic. This supported the previous finding that 5 µL of BHI did not inhibit pseudovirus infection (Figure 4.1A). However, 5 µL of GV culture medium significantly inhibited virus entry, suggesting that *G. vaginalis* growth was required for the decrease in pseudovirus entry.

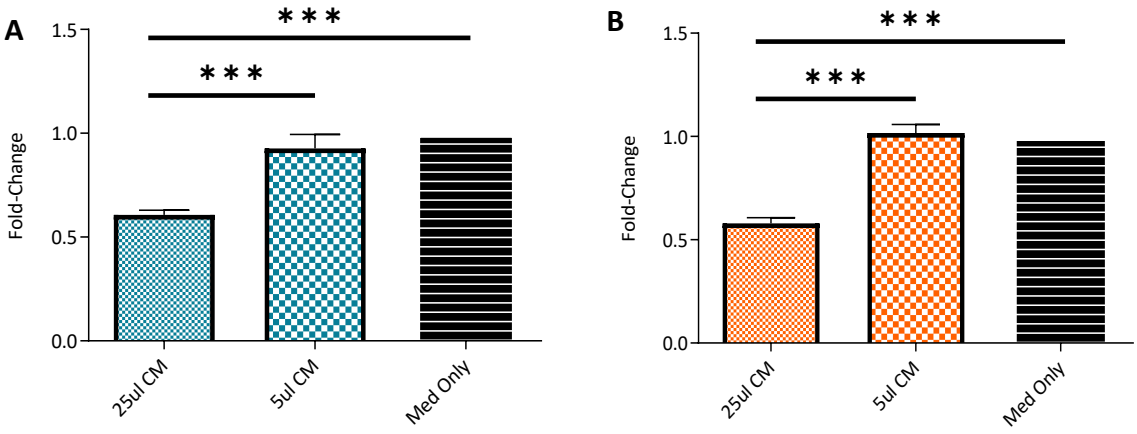


Figure 4.3: **Cytotoxicity of *G. vaginalis* culture medium.** TZM-bl cells ($5x10^4$ cells/mL) exposed to decreasing volumes of (A) GV and (B) BHI medium and were incubated for 48hrs at 37°C, 5% $CO_2$. An MTT assay was performed to determine the effects of the culture medium on TZM-bl cell viability. Fold-change in absorbance normalised to medium only control is shown. Bars represent the mean of three independent experiments with error bars showing standard deviation. Statistical analysis was carried out using GraphPad Prism unpaired T-test. *, **, *** represent P<0.05, P<0.01 and P<0.001, respectively.

#### 4.2.1.4 Effects of pH on Entry Efficiency and Cell Viability

*G. vaginalis* results in the acidification of growth medium to pH 4.7 - 6.0, which might be toxic to mammalian cells (Boskey et al., 1999). As the pH of GV added to the pseudovirus entry assay was pH 4.6, the pH of the culture medium was adjusted to that of complete DMEM medium used to culture TZM-bl cells. The pH of both GV and BHI was adjusted to pH 8.74 and 5 µL was added to TZM-bl cells in the presence of 200 ng of PSV. A cell viability assay showed that increasing the pH did not reduce the cytotoxicity of 25 µL of both GV (Figure 4.4A) and BHI (Figure 4.4B). This suggested that a component of the BHI medium was cytotoxic at higher volumes. Addition of 5 µL of pH-adjusted culture medium did not alter cell viability when TZM-bl cells were exposed to GV and BHI culture medium when compared to the non-adjusted control, suggesting both BHI and GV were not cytotoxic at this volume.

Adjusting the pH of the GV culture medium appeared to restore viral entry, although it remained much lower than the virus only control (Figure 4.4C). This suggested that changes in pH were only partly responsible for the drop in pseudovirus entry. Overall, the results suggested that 5 µL of GV inhibited pseudovirion entry, partly due to low pH and that this relationship was not due to loss of cell viability.

Adjusting the pH of the BHI culture medium did not result in an increase in pseudovirus entry compared to the non-adjusted control (Figure 4.4D). This suggested that the pH of the BHI was not influencing HIV infection. The significant difference between the virus-only control and pH-adjusted treated and untreated samples is likely due to another unknown factor.

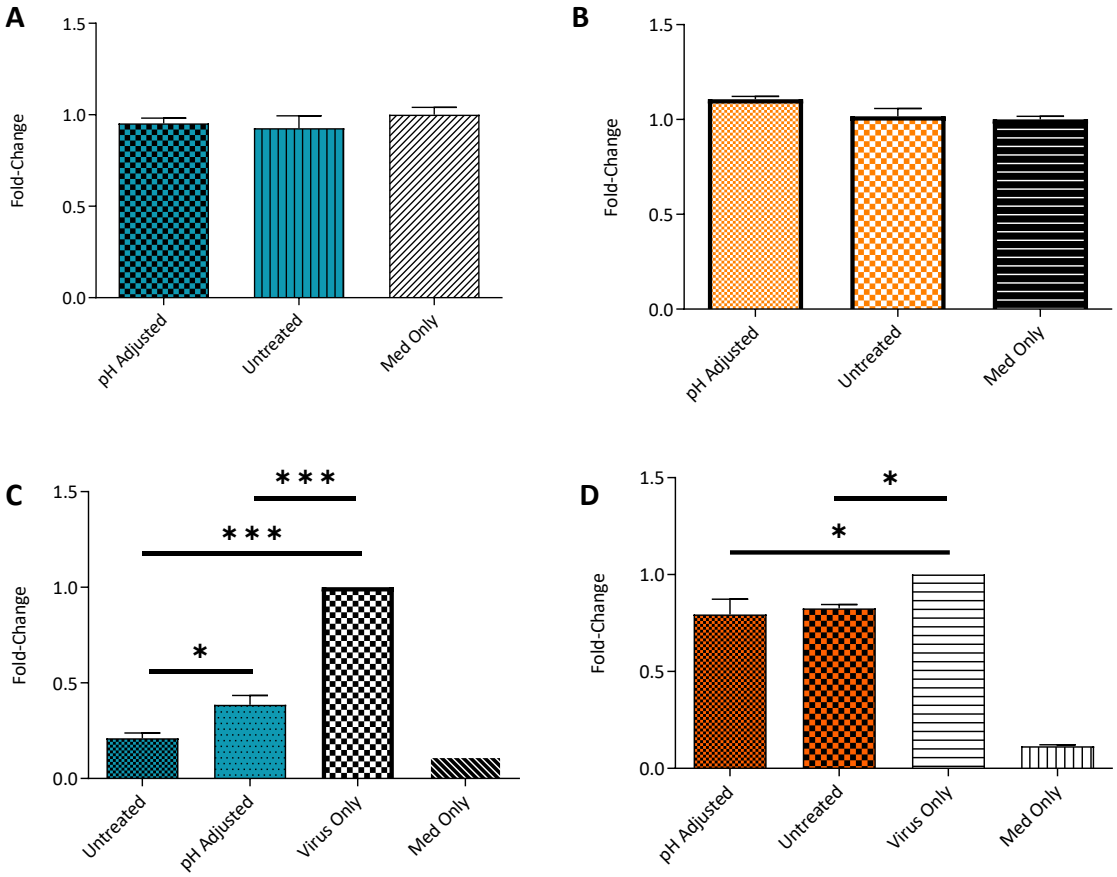


Figure 4.4: **Effect of pH adjustment of culture medium on cell viability and HIV-1 infection.** TZM-bl cells ($5x10^4$ cells/mL) were treated with 5 µL of (A) GV culture medium and (B) BHI medium and cell viability was determined. TZM-bl cells ($5x10^4$ cells/mL) were infected with 200 ng pseudovirus with 5 µL of (C) GV culture medium and (D) BHI medium and incubated for 48hrs at 37°C, 5% $CO_2$. The pH of the GV and BHI medium was adjusted to pH 8.74 using sodium hydroxide. Fold-change of relative light units (RLU) normalised to virus only control is shown. Bars represent the mean of two independent experiments with error bars showing standard deviation. Statistical analysis was carried out using GraphPad Prism, (A) and (B) unpaired T-test (C) and (D) One-way ANOVA with the Tukey's post-test. *, **, *** represent P<0.05, P<0.01 and P<0.001, respectively.

#### 4.2.1.5 Effect of Heating the Culture Medium on Viral Entry Efficiency and Cell Viability

In order to determine whether inhibition was heat-sensitive, samples were heated before filter-sterilisation and addition to the pseudovirus entry assay. We hypothesized that at a high temperature, proteins would be denatured, and inhibition abrogated. Firstly, to ensure that heating of the culture medium did not influence TZM-bl viability, an MTT test was carried out. Heating of the culture medium did not result in any significant change in cell viability for both GV and BHI compared to untreated controls (Figure 4.5A and 4.5B).

However, when the heat-treated culture medium (GV and BHI) was added at a volume of 5 µL, there was a statistically significant increase in viral entry compared to the untreated control ($p= 0.009$) (Figure 4.5C). Heating of the BHI medium did not alter pseudovirus entry when compared to the untreated BHI control (Figure 4.5D). Overall, this suggested that *G. vaginalis* was producing a heat labile inhibitor that was likely to be protein based.

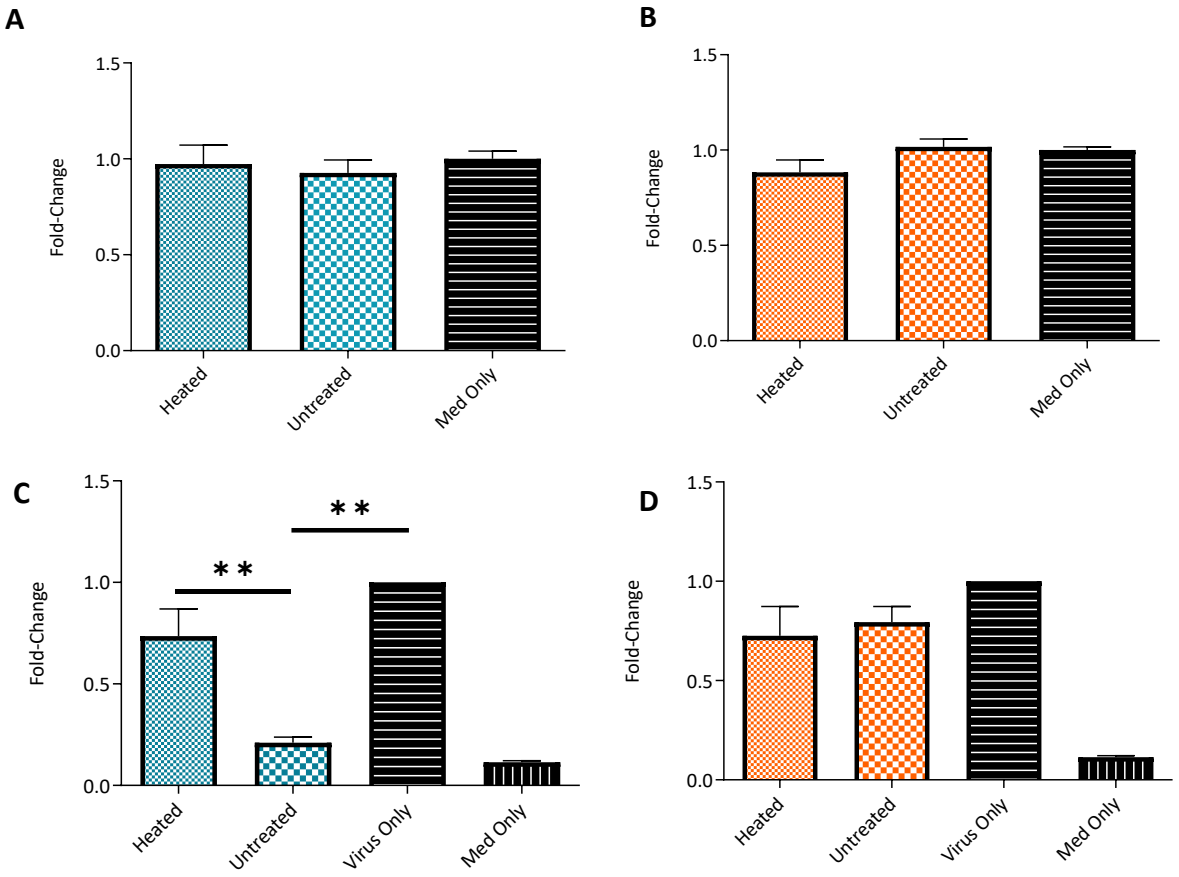


Figure 4.5: **Effect of heating the culture medium on cell viability and HIV-1 infection.** TZM-bl cells ($5x10^4$ cells/mL) were treated with 5 µL of (A) GV culture medium and (B) BHI medium and cell viability was determined. TZM-bl cells ($5x10^4$ cells/mL) were infected with 200 ng pseudovirus with 5 µL of (C) GV culture medium and (D) BHI medium and incubated for 48hrs at 37°C, 5% $CO_2$. GV and BHI medium were heated at 100 °C for 10 min to eliminate inhibition. Fold-change of relative light units (RLU) normalised to virus only control is shown. Bars represent the mean of two independent experiments with error bars showing standard deviation. Statistical analysis was carried out using GraphPad Prism, (A) and (B) unpaired T-test (C) and (D) One-way ANOVA with the Tukey's post-test. *, **, *** represent P<0.05, P<0.01 and P<0.001, respectively.

#### 4.2.1.6 Effect of Adjusting Both the pH and Heating of Culture Medium on Cell Viability and HIV Infection

As adjusting the pH and heating the culture medium separately rescued viral entry compared to untreated culture medium, we hypothesised that combining the two treatments might have an additive effect which might restore viral entry to match that of the virus-only control. The addition of 5 µL pH-adjusted and boiled (pH/boiled) culture medium (GV and BHI) did not appear to have any effect on cell viability for both the GV and BHI (Figure 4.6A and 4.6B). These findings confirmed that adjusting the pH and boiling the culture medium did not result in any significant cell death (Figure 4.5A). The entry efficiency assay indicated that combining pH adjustment and heating the GV culture medium did result in a significant increase in viral

entry compared to the untreated culture medium (Figure 4.5C) ($p<0.001$). However, despite this significant increase, it was still lower than the virus-only control. The combination of adjusting the pH and heating the culture medium did not therefore have any additive significant effect on reducing the ability of GV to inhibit pseudovirus infection.

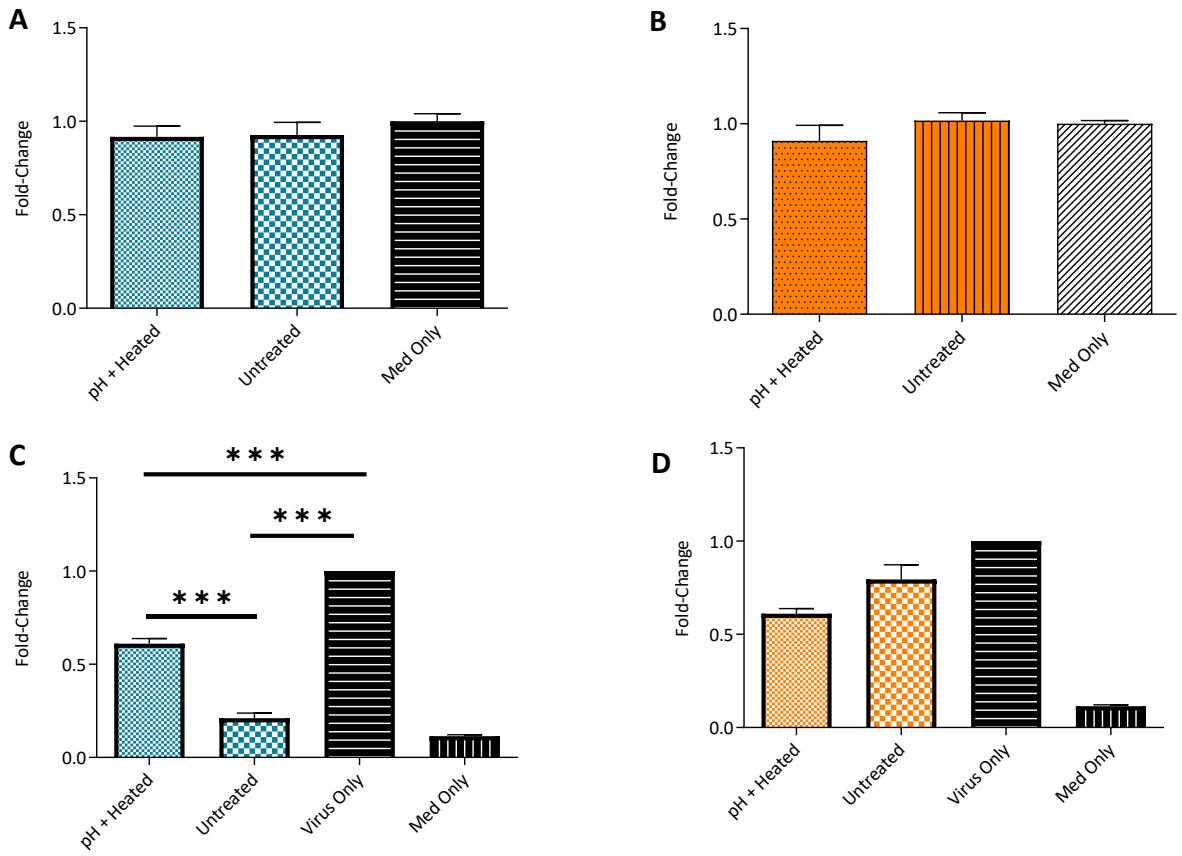


Figure 4.6: **Effect of heating and adjusting the pH of culture medium on cell viability and HIV-1 infection.** TZM-bl cells ($5x10^4$ cells/mL) were treated with 5 µL of (A) GV culture medium and (B) BHI medium and cell viability was determined. TZM-bl cells ($5x10^4$ cells/mL) were infected with 200 ng pseudovirus with 5 µL of (C) GV culture medium and (D) BHI medium and incubated for 48hrs at 37°C, 5% $CO_2$. GV and BHI medium were heated at 100 °C for 10 min to eliminate inhibition and the pH was adjusted to pH 8.74 using sodium hydroxide. Fold-change of relative light units (RLU) normalised to virus only control is shown. Bars represent the mean of two independent experiments with error bars showing standard deviation. Statistical analysis was carried out using GraphPad Prism, (A) and (B) unpaired T-test (C) and (D) One-way ANOVA with the Tukey's post-test. *, **, *** represent $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

## 4.3 Discussion

Research has found that only some *G. vaginalis* strains produce sialidase and Vaginolysin (Janulaitiene et al., 2018), suggesting that different strains of *G. vaginalis* have different levels of virulence. This study wanted to see whether addition of culture medium from a *G. vaginalis* isolate that does not produce sialidase but does produce Vaginolysin would influence HIV entry.

Addition of the abiotic culture medium of *G. vaginalis* to an entry efficiency assay resulted in a statistically significant decrease in viral entry, with GV resulting in a significant decrease in entry compared to medium which contained fresh BHI (Fig 4.1). It was thought that this decrease in entry could be a result of a change in the pH, or the potential release of a proteinaceous inhibitor. Previous studies have suggested that healthy *Lactobacilli* release lactic acid which decreases the pH in a healthy FGT and lowering the likelihood of an HIV infection (Cicenia et al., 2014; Klebanoff & Coombs, 1991). Thus, the pH of the abiotic culture medium was changed to match that of Full-DMEM, the medium used during the entry efficiency assay (pH = 8.74). Changing the pH did result in a statistically significant increase in viral entry compared to the untreated abiotic culture medium, but it was still lower than that of the virus only control. Thus, whilst adjusting the pH did restore some viral entry, it did not appear to remove the inhibitory nature of GV. These findings are similar to those found in a study by Tyssen et al. (2018), who showed that neutralizing the pH of CVF resulted in increased HIV entry for some *G. vaginalis* isolates (Tyssen et al., 2018).

Another reason for the inhibition could be due to the release of a proteinaceous inhibitory molecule. As most proteins are heat sensitive, the abiotic culture medium was heated to denature most of the proteins present. Addition of a protease would have been able to further confirm this suggestion and is an experiment that should be performed in future studies, although these samples would first need to be heated to denature the protease in order to proceed with an HIV entry efficiency assay to proceed. Heating the culture medium resulted in a statistically significant increase in viral entry compared to the untreated culture medium when normalized with the virus only control. Despite this increase in viral entry, heating of the culture medium did not fully restore the level of viral entry that was found in the virus-only control. When BHI was heated, there was no significant change in viral entry,

meaning that heating the culture medium denatured a protein that was released by *G. vaginalis* and not one that was present in the culture medium. Therefore, these data suggests that the *G. vaginalis* ATCC (14018) strain is releasing a heat-labile molecule, most likely a protein, into the culture medium that inhibits pseudovirus infection.

Vaginolysin is a member of the cholesterol dependent Cytolysin family that forms pores within cell membranes leading to lysis and cell death. It is an important virulence factor of *G. vaginalis* that has the potential to be used as a determinant of BV pathogenicity (Gelber et al., 2008). Due to the genetic variation of *G. vaginalis*, not all strains produce Vaginolysin (Yeoman et al., 2010). Studies have found that at high concentrations, Vaginolysin causes cell death, whilst it has been found to cause cell-blebbing at lower concentrations (Randis et al., 2013). In this study, it was determined that higher volumes of GV (25 µL) caused significant cell death and reduced viral entry. Lower concentrations of GV (5 µL) did not have any effect on cell viability, although it still reduced viral entry. Thus, it is possible that the higher volumes of GV had higher concentrations of Vaginolysin causing cell death, whilst lower concentrations resulted in cell-blebbing which might have distorted the membrane and prevented successful viral entry. It has been shown that low concentrations of Vaginolysin causes morphological changes of epithelial cells which might alter the cell membrane (Harwich et al., 2010) so that endocytosis of pseudovirus is more effective (Miyauchi, Marin, & Melikyan, 2011), though further studies are required to determine whether Vaginolysin is the reason for the apparent inhibition of HIV infection observed in this study.

# Chapter 5: Concluding Statements

The aim of this study was to investigate the mechanism of how *G. vaginalis,* a bacterial species associated with BV, might impact HIV infectivity. We initially focused on sialidase because bacterial sialidases are considered virulence factors (Corfield, 1992) and GV sialidase has been linked to BV and severity of dysbiosis (Gelber et al., 2008; Harwich et al., 2010; Patterson et al., 2010). This study confirmed that sialidase enhanced HIV infectivity, as the inclusion of a commercial sialidase from *Clostridium perfringes* resulted in a 2.2-fold increase in pseudoviral entry by primarily targeting the viral particle rather than the host cell. *G. vaginalis* has been found to produce sialidase (Briselden et al., 1992; Howe et al., 1999), thus it is possible that the bacteria release this sialidase which increase HIV entry and contribute to the increased risk of women with BV being infected with HIV.

At least three genes have been identified that express sialidases: NanH1 (also known as sialidase A), NanH2 and NanH3 with a molecular weight of 100, 100 and 77 kDa, respectively (Robinson et al., 2019; Von Nicolai, Hammann, Salehnia, & Zilliken, 1984). Sialidase isozymes also differed in catalysis, with NanHI inefficiently releasing sialic acid from substrates and 9-O-acetylated sialic acid was cleaved preferentially by NanH2 (Robinson et al., 2019). Therefore, the same strain of *G. vaginalis* could produce different sialidases with different molecular and phenotypic properties. Genomic comparison between *G. vaginalis* strains isolated from BV positive women and a strain from a BV negative woman indicated that the BV-associated *G. vaginalis* had higher "pathogenic potential" with mucolytic capabilities (Yeoman et al., 2010).

More specifically, *G. vaginalis* strains that produced sialidase were more likely to be associated with BV (Janulaitiene et al., 2017). This suggests that perhaps those strains that express sialidase are more pathogenic than those who do not. This study compared three *G. vaginalis* strains for their ability to impact HIV infection: two *G. vaginalis* strains obtained from France that produced sialidase and the ATTC strain 14018 that did not. Sialidase was purified from pooled 3H6 and 2I3 abiotic culture and when added to pseudovirus infection of TZM-bl cells, there was significant increase in HIV infectivity. Therefore, one or both of these strains produce a factor that enhances viral entry. The molecular weight of the purified proteins

corresponded to approximately 40 kDa, which is less than that predicted for *G. vaginalis* sialidase. This could be due to degradation, as storage of purified sialidase at 4$^{o}$C led to degradation products of approximately 36 kDa (Rothe et al., 1991). Alternatively, sialidases from other bacterial species have molecular weights of approximately 40 kDa (Roggentin et al., 1995). Mass spectrometry would have confirmed whether the two proteins corresponded to two unique sialidases from 3H6 and 2I3, degradation products of a single sialidase from *G. vaginalis* or enzyme from contaminating bacteria. However, the sample was too dilute to obtain a clear enough signal for confirmation.

Conversely, when we tested whether the ATCC (14018) strain of *G. vaginalis* enhanced HIV infectivity in the absence of sialidase activity, pseudovirus entry of TZM-bl cells was inhibited. Adjusting the pH and heating the culture medium restored viral entry, and it appears as though this strain of *G. vaginalis* releases a heat-labile factor which inhibits HIV infection. Further studies on identifying this inhibitory factor is required, but one potential suspect is the virulence factor, Vaginolysin expressed by the ATCC (14018) strain (Gelber et al., 2008).

Research suggests that at high concentrations, Vaginolysin causes cell death whilst it causes cell-blebbing at lower concentrations (Los et al., 2013). This corresponds to our finding that at high concentrations, the abiotic ATCC culture medium was cytotoxic whereas at lower concentrations it only reduced viral entry. Thus, if Vaginolysin was responsible for loss of cell viability, it is possible that at low concentrations the abiotic cell culture caused TZM-bl cell-blebbing that altered the cell membrane in such a way as to decrease viral entry (Cassidy & O'Riordan, 2013; Gurcel et al., 2006). Future studies are required to confirm whether the inhibition of HIV infection by ATCC (14018) abiotic culture is due to Vaginolysin. The impact of commercial Vaginolysin and Vaginolysin inhibiting antibodies on pseudovirus entry and physiological changes to the cell membrane integrity in the presence of *G. vaginalis* abiotic culture medium should be tested. Furthermore, additional studies should also look at whether different strains of *G. vaginalis* also result in HIV entry inhibition. Inhibition of HIV infection by *G. vaginalis*, whether due to the action of Vaginolysin or a novel inhibitor, would be valuable to the design of future HIV prevention strategies.

Some women remain BV-negative despite the presence of *G. vaginalis* in the FGT and a recent study suggested that BV does not increase HIV acquisition (Heffron et al., 2017). It is thus

tempting to speculate that the global distribution of strains of *G. vaginalis* that produce Vaginolysin (Yeoman et al., 2010) and/or sialidase (Janulaitiene et al., 2017), determines the relationship between HIV infection and BV. Further studies that compare the prevalence of different *G. vaginalis* strains, with a focus on sialidase and vaginolysin production, will help to understand the relationship between *G. vaginalis*, BV and HIV acquisition.

In conclusion, contrary to what we expected, low volumes of abiotic culture medium used to grow *G.vaginalis* inhibited HIV infection instead of enhancing it. This effect was not due to changes in pH or culture medium. However, the effect was abrogated once the abiotic culture was heated. This suggests that the ATTC 14018 *G. vaginalis* strain produces a proteinaceous compound that inhibits HIV infection. This could represent a natural inhibitor specific to *G. vaginalis* strains.

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