ANALYSIS OF THE CERVICOVAGINAL MICROBIOME IN NEISSERIA GONORRHOEAE INFECTIONS AND ITS CONTRIBUTION TO DISEASE PRESENTATION

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ABSTRACT

Angela Lovett: Analysis of the Cervicovaginal Microbiome in Neisseria gonorrhoeae Infections and its Contribution to Disease Presentation
(Under the direction of Joseph A. Duncan)

N. gonorrhoeae is a strictly human pathogen responsible for 100 million infections annually. Infections are typically localized to the lower genital tract in women, but when left untreated, infections can ascend to the upper genital tract leading to a number of health complications including pelvic inflammatory disease and infertility. Despite a localized inflammatory response, individuals do not develop an effective adaptive immune response to the bacteria and remain susceptible to repeated infection. Surprisingly, a large proportion of *N. gonorrhoeae* infections are carried asymptomatically and women are more likely than men to carry asymptomatic infections. Women with bacterial vaginosis (BV), which is characterized by a shift in the cervicovaginal microbiome to a polymicrobial dysbiosis, are associated with an increased risk of acquiring and transmitting STIs. Studies examining the impact of the vaginal microbiome on basal inflammatory states reported that women with BV were associated with higher expression levels of pro-inflammatory cytokines when compared to BV-negative women. Taken together, these data suggest that variations in vaginal microbial diversity can influence disease susceptibly and/or presentation.

We conducted a pilot study to assess differences in the cervicovaginal microbial community of patients presenting to a sexually transmitted infections (STI) clinic with symptomatic *vs*. asymptomatic *N. gonorrhoeae* infections. Specimens collected from

asymptomatic individuals with *N. gonorrhoeae* infection and no co-infection with *Chlamydia trachomatis* and/or *Trichomonas vaginalis* carried *Lactobacillus*-dominant microbial communities more frequently than symptomatic patients without co-infection. When compared to asymptomatic individuals, symptomatic women had microbial communities characterized by more diverse and heterogenous bacterial taxa, typically associated with bacterial vaginosis (BV). We utilized a murine model of *N. gonorrhoeae* infection in which mice pre-colonized with *Lactobacillus crispatus* to test whether pre-existing *L. crispatus* was protective from *N. gonorrhoeae* colonization or whether *N. gonorrhoeae* infection could drive the loss of *L. crispatus* during infection. Vaginal infection with either *N. gonorrhoeae* strain 1291 or an isogenic mutant known to exhibit lower inflammatory had no impact on *Lactobacillus* burden recovered from the mice. These data taken together suggest that *Lactobacillus*-dominant vaginal microbial community may protect individuals from developing symptoms during lower genital tract infection with *N. gonorrhoeae.*

This body of work is dedicated to my village. To my family, friends and mentors that contributed to my growth along this journey.

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LIST OF ABBREVIATIONS

Abx	Antibiotics
BV	Bacterial vaginosis
BVAB1	Bacterial vaginosis associated bacterium-1
CD	Cluster of differentiation
CT	Community types
CFUs	Colony Forming Units
DGI	Disseminated gonococcal infection
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
GCSF	Granulocyte colony stimulating factor
HIV	Human immunodeficiency virus
Ig	immunoglobulin
IL	Interleukin
IFN	Interferon
LOS	Lipooligosccharide
mAbs	Monoclonal antibodies
MUSCLE	Multiple sequence comparison by log expectation
Ng	Neisseria gonorrhoeae, N. gonorrhoeae
NHS	Normal human serum
OD600	Optical Density 600
OMP	Outer membrane protein
OMV	Outer membrane vesicles
OTUs	Operational taxonomic units

PID	Pelvic inflammatory diseases
PMBC	Peripheral blood mononuclear cell
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
STD	sexually transmitted disease
STI	Sexually transmitted infection
Th	T-helper cell
TNF	Tumor necrosis factor
UV	Ultraviolet

CHAPTER 1: INTRODUCTION AND NATURAL HISTORY OF INFECTION[1]

Introduction

N. gonorrhoeae is a bacterial sexually transmitted pathogen that most commonly infects the lower genital tract, the cervix in women and anterior urethra in men. *N. gonorrhoeae* can also infect other mucosal surfaces, particularly the pharynx and rectum. Symptoms associated with disease including purulent urethral or cervical discharge and discomfort at the site of infection are due to the pathogen's ability to induce robust localized inflammation within the host. However, asymptomatic infections with *N. gonorrhoeae* are common and may serve as a significant reservoir of transmissible bacteria in the population. In women, untreated infections can ascend to the upper genital tract leading to a number of health complications including pelvic inflammatory disease and infertility. Rarely, infection with *N. gonorrhoeae* disseminates, leading to septic arthritis and skin manifestations. Antibiotic resistance in *N. gonorrhoeae* strains is on the rise worldwide and effective treatment options have become limited. Although individuals with gonococcal infections are known to produce anti-gonococcal humoral immune responses, it is clear that most of these responses are insufficient for providing protection from future infection. Better understanding of the immune response to natural infection with *N. gonorrhoeae* is vital for the prevention of disease transmission and the development of an effective gonococcal vaccine.

[1] This chapter previously appeared as an article in Frontier immunology. The original citation is as follows: Lovett, A., & Duncan, J. A. (2018). Human Immune Responses and the Natural History of Neisseria gonorrhoeae Infection. *Front Immunol, 9*, 3187. doi:10.3389/fimmu.2018.03187

Natural history of *Neisseria gonorrhoeae* infection

Our understanding of the natural history of *N. gonorrhoeae* infection is hampered by a lack of rigorous scientific studies of microbiologically defined *N. gonorrhoeae* infection from the pre-antibiotic era. Since the introduction of sulfa-based antibiotics and subsequently penicillin, antibiotic treatment for men with symptomatic *N. gonorrhoeae* infection, usually urethritis, has been the standard of care. In studies of men seeking care for gonococcal urethritis, subjects reported average incubation periods of ~6-8 days between presumed exposure and onset of symptoms (Schofield, 1982; Sherrard and Barlow, 1996). However, some individuals reported symptom onset as early as 1-2 days. These studies also indicated that men with symptomatic gonococcal urethritis were symptomatic on average for 7 days before seeking care, though that time ranged from one day to one year. Because current guidelines indicate men with symptomatic gonorrhea should be treated with antibiotics, there are no prospective studies describing natural clearance or natural progression of symptomatic infection. However, some information about the persistence of symptomatic *N. gonorrhoeae* infection can be drawn from treatment failures in therapeutic trials of antibiotics for gonococcal urethritis. Svinland *et al.* examined bacterial clearance after treatment with flumequine in 239 patients with uncomplicated gonorrhea (Svindland *et al.*, 1982). Although multiple dosing regimens with flumequine were effective at curing the vast majority of patients, there was a small number of patients who failed to clear the infection following treatment. *N. gonorrhoeae* infection was found to persist at the test of cure obtained after 14 days in 10 subjects. Six of those subjects harbored strains with high level flumequine resistance. Those six strains represented all high-level resistant strains found in the study and therefore represented a complete cohort of subjects who received ineffective antibiotic therapy in the study. The persistence of infection in all of these subjects suggests that a

large proportion of symptomatic *N. gonorrhoeae* infections that go without treatment are likely to persist at least 14 days. Treatment failures have also been reported in a number of other therapeutic trials that also support the hypothesis that *N. gonorrhoeae* can infect the lower genital tract and persist in the face of localized inflammatory response for at least 14 days (Hook *et al.*, 1984; Tanphaichitra *et al.*, 1986).

To characterize the average bacterial load during infection, Isbey *et al.* analyzed the urine and semen of men with symptomatic urethritis. The total number of *N. gonorrhoeae* recovered per urine sample (~$6*10^6$ CFU) and from semen (~$7*10^6$ CFU) suggest that *N. gonorrhoeae* is carried and/or excreted in large quantities during infection (Isbey, Alcorn *et al.* 1997). Experimental gonococcal infection with male volunteers mimics much of the clinical features of naturally acquired infections and has provided some insight on the natural history of early symptomatic infection. Infected subjects often develop dysuria and urethritis with the onset of symptoms ranging from ~1-6 days post inoculation and *N. gonorrhoeae* can be recovered from the urine in as little as 2hrs following inoculation. The quantity of bacteria recovered (~10^2-10^5 CFU/sample) does not appear to correlate with the severity of infection symptoms (Cohen *et al.*, 1994). Since the treatment of men with asymptomatic gonorrhea has not always been the standard for clinical management of *N. gonorrhoeae* infection, Handsfield and colleagues conducted a prospective study of the natural history of asymptomatic male infection in the early 1970's. Asymptomatic men were identified via positive *N. gonorrhoeae* urethral cultures from men requesting STI screening or men that were contacts of women positive for symptomatic gonorrhea at Seattle STD clinics. Of the 28 patients examined weekly, 18 remained asymptomatic until they were treated, which varied from 7-165 days. Of the remaining 10 subjects, 5 developed urethritis and the other 5 spontaneously cleared the infection (Handsfield *et*

al., 1974). Overall, these studies highlight the ability of *N. gonorrhoeae* to persist for prolonged periods in both symptomatic and asymptomatic men. Additionally, it is clear that asymptomatic infection can progress to symptomatic infection across a broad time spectrum. The determination of the natural rate of clearance of infection is complicated by the need to treat infected individuals with effective antibiotics.

Studies of the natural history of *N. gonorrhoeae* infection in women are also limited due to a standard of care that requires the use of antibiotics to treat known *N. gonorrhoeae* infection. In a case survey comparing cure rates of *N. gonorrhoeae* infection after treatment with sulfathiazole or penicillin, patients were followed for 3 months before being declared cured. The majority of the observed patients were female in this report. Sulfathiazole treatment had a 21% failure rate, with some patients found to have positive cultures 3 months or longer after initial therapy (Mauss, 1946). A study of women determined to be recently exposed (average of 11 days after exposure) to *N. gonorrhoeae* provided some insight into the acquisition and presentation of *N. gonorrhoeae* infection. 26 women were identified through contact tracing of partners of men with gonococcal urethritis. Of the 26 subjects, 19 were found to be infected with *N. gonorrhoeae*. Risk of infection after exposure in these women increased with number of exposures to the infection (sexual encounters with the infected contact): 6/12 women with one exposure were found to be infected while 6/7 women with two exposures and 7/7 women with more than two exposures were found to be infected. Of the 19 infected subjects, 9 subjects had clinically defined pelvic inflammatory disease or tenderness of the adnexa suggestive of inflammation in the upper reproductive tract. (Platt *et al.*, 1983). Because of the risk for ascending infection, study of the natural history of asymptomatic infection in women once identified is not considered ethically acceptable. However, Stupianksy *et al.* performed a study

using self-collected vaginal swabs samples collected in a prospective cohort designed to study sexual health in adolescent women. In this study, subjects underwent examination and clinic-based STD screening every 3 months, 4 times more frequently than recommended for asymptomatic adolescent women. In between screenings, the subjects self-collected cervicovaginal swabs and kept diaries of sexual activity and urogenital symptoms. *N. gonorrhoeae* DNA was identified in cervicovaginal samples collected prior to the identification of *N. gonorrhoeae* infection at a quarterly visit in 18 women. Although the quantity of *N. gonorrhoeae* DNA found in self collected swabs from individual women varied greatly, the mean bacterial load (~10^3-10^5 CFUs/ sample) was similar regardless of the length of time of infection. Additionally, women with *Chlamydia trachomatis* coinfections displayed higher mean bacterial load, though the difference in *N. gonorrhoeae* DNA levels between *C. trachomatis* co-infected and uninfected subjects was not statistically significant. Because the longest period between clinic-based STD screens in this study was 12 weeks, persistent infection longer than 12 weeks could not be identified in this group. Interestingly, vaginal discharge and dysuria were reported in the diaries of 3 of the 18 women. However, the presence of symptoms did not correlate with bacterial load (Stupiansky *et al.*, 2011). These studies suggest that both symptomatic and asymptomatic *N. gonorrhoeae* infections can persist in women for at least 12 weeks. Further, the frequency of natural clearance in the setting of prolonged infection was not reported for these 18 subjects, but it at least some infections persisted from initial onset up to the quarterly in-person visit. Taken together with evidence of persistent symptomatic and asymptomatic infections in men, this report suggests that *N. gonorrhoeae* is capable of evading or resisting host immune responses to infection.

Studies that provide insight into the natural history of *N. gonorrhoeae* infection suggest that prior infection with *N. gonorrhoeae* induces little protective immune responses to the pathogen. Platt's study of *N. gonorrhoeae* acquisition by women exposed to men with gonococcal urethritis demonstrated that 1 of 7 women exposed and uninfected had prior history of *N. gonorrhoeae* infection while 10 of 19 exposed and infected individuals had prior history of *N. gonorrhoeae*, which is consistent with prior infection not leading to protective immune response (Platt *et al.*, 1983). An epidemiologic study of *N. gonorrhoeae* infections in rural North Carolina found 14.8% of *N. gonorrhoeae*-infected individuals experienced a second infection during the study period. The *N. gonorrhoeae* strain recovered from those repeatedly infected individuals was more likely to be the same serovar as the strain recovered from their initial infection than an alternative serovar, suggesting that strain-specific immune responses were inadequate to provide protection from *N. gonorrhoeae* (Fox *et al.*, 1999). In the prospective cohort study of adolescent women that described the presence of *N. gonorrhoeae* DNA in self collected cervicovaginal swabs, no difference in the quantity of *N. gonorrhoeae* DNA was observed between individuals with a history of prior infection (Stupiansky *et al.*, 2011). Overall, data from clinical studies of *N. gonorrhoeae* infections suggest that immune clearance of infection and protection from repeated infection are largely inadequate, leading to prolonged symptopmatic and asymptomatic infections (Figure 1).

Humoral immune responses to *N. gonorrhoeae* infection (Table 1)

Individuals infected with *Neisseria gonorrhoeae* have been shown to produce anti-gonococcal antibodies in sera, seminal plasma and cervical secretions. Although increases in the different immunoglobulin classes are detected in the serum and secretions of infected individuals, the difference between infected and uninfected individuals have often been reported

as modest. Hedges *et al.* tested both serum and secretions of infected and uninfected male and female attendees of an STD clinic for antibodies against the MS11 strain and homologous infecting strains. When compared to uninfected patients, slight increases in serum IgA1 from female patients and serum IgG from male patients against the MS11 strain were observed. The level of serum IgA and IgG against infecting isolates did not drastically change over the 6-week observation period. Additionally, no difference in anti-gonococcal antibody levels was noted between individuals with no prior history of infection and previously infected individuals regardless of their current infection status (Hedges *et al.*, 1999). However, this study focused on antibody directed against fixed whole bacteria and not specific antigens. Antigen specific antibody responses have been reported for a number of gonococcal polysaccharide and protein antigens.

Polysaccharides are a major source of microbial antigens from pathogenic Neisseria species, as well as many other bacteria. Unlike *N. meningitidis*, *N. gonorrhoeae* does not produce a polysaccharide capsule, which is a major immunogenic, saccharide-based antigen for *N. meningitidis*. However, both *N. gonorrhoeae* and *N. meningitidis* produce lipooligosaccharide which makes up roughly 50% of the mass of the outer membrane of the bacteria. LOS from *N. gonorrhoeae* and *N. meningitidis* share a common core oligosaccharide along with lipid A structure (Mandrell *et al.*, 1988). The structure of the oligosaccharide of LOS is determined by the expression of multiple phase variable saccharide transferases (Shafer *et al.*, 2002). Thus, even within a single strain there can be substantial LOS heterogeneity. The ability of these variations in structure to lead to heterogeneity in antigenic epitopes that can be recognized by antibodies was demonstrated clearly by the characterization of *N. gonorrhoeae* LOS from 20 different strains with a panel of murine monoclonal antibodies (Mandrell *et al.*, 1986). Despite

these variations in LOS structure, almost all *N. gonorrhoeae* strains examined by Gulati and colleagues maintain expression of the LOS structure recognized by monoclonal antibody 2C7 (Gulati *et al.*, 1996). Antibodies to LOS have been detected in both normal human sera and in sera from *N. gonorrhoeae* infected individuals. Sera collected from a small subset of male subjects in a gonococcal pilus-based vaccine trial were used to characterize antigen-specific antibody responses during acute gonococcal infection. Those subjects who received the placebo vaccination reported no previous history of STIs, had a negative culture at the start of the trial, and developed gonococcal urethritis during the trial period. Sera were collected bi-weekly over the course of 8 weeks. This study found 4 of 13 subjects carried pre-existing LOS antibodies that presumably arose from cross reactivity with polysaccharides from other commensal bacteria species. Of the remaining 9 subjects who did not have preexisting antibodies recognizing *N. gonorrhoeae* LOS, 6 developed LOS antibodies after developing *N. gonorrhoeae* infection (Hicks *et al.*, 1987). In another study, sera collected from patients with complicated gonococcal infections (8 DGI, 4 PID and 1 epididymitis) was used to characterize antibody responses to Ng outer membrane proteins. Sera obtained from a majority of patients (9/13) exhibited antibody responses to LOS (Hook *et al.*, 1984). Antibodies against LOS were also studied by researchers using an experimental human male urethral inoculation trial to test whether recent gonococcal urethritis led to protection from reinfection. Some immunoreactivity towards *N. gonorrhoeae* LOS was detected in serum from 21 of 24 subjects prior to inoculation with *N. gonorrhoeae* for the first time. Of the 14 subjects in the study who were rechallenged, 6 of 14 demonstrated an increase in anti-LOS antibody demonstrated by immunoblot and 9 of 14 had at least a twofold increase in antibody titer measured by ELISA (Schmidt *et al.*, 2001). Consistent with baseline observations of subjects in these studies, the presence of *N. gonorrhoeae* LOS-directed

antibodies was detected from pooled sera taken from eight volunteers with no history of gonococcal infection (Yamasaki *et al.*, 2005). The predominant antibodies that bound LOS in this study were of the IgG class. Affinity-purified anti-oligosaccharide IgG isolated from normal human serum was found to contain bactericidal activity towards a serum-sensitive strain of *N. gonorrhoeae*. In a separate study, Yamasaki and colleagues found that *N. gonorrhoeae* LOS-binding IgG2 from NHS recognized at least 3 different oligosaccharides (Yamasaki *et al.*, 2010). Thus, numerous studies indicate that the majority of, but not all, humans mount antibody responses to a variety of saccharide epitopes found in the lipooligosaccharide of *N. gonorrhoeae* in response to mucosal infection with the bacteria.

A number of studies have surveyed whether different gonococcal protein antigens induce humoral immune responses during infection. Sera isolated from 68 patients with uncomplicated gonococcal infections and 35 women with pelvic inflammatory disease (PID) were used to characterize antibody responses to gonococcal pili (Miettinen *et al.*, 1989). The mean antibody levels of IgG, IgA, and IgM against *N. gonorrhoeae* pilus was significantly higher in men with urethritis (~2-fold increase) and women with cervicitis (~4-fold increase) when compared to sera from uninfected patients. Anti-pilus antibodies were also significantly higher in sera from women with confirmed gonococcal PID when compared to sera from patients with non-gonococcal PID. In addition to testing for anti-LOS antibodies, sera isolated from male volunteers participating in the pilus vaccine trial who acquired gonorrhea and who reported no previous history of gonococcal infection were analyzed for the presence on anti-gonococcal protein antibodies using immunoblot analysis. Pre-existing antibodies were detected in 12 of 13 subjects by western blot analysis using pre-infection serum. Not only were preexisting antibodies against LOS detected, but antibodies against a number of abundant outer-membrane proteins

were also observed, including Protein I (PorB), Protein III (Rmp), Pili, and Lip. The authors suggested that the preexisting antibodies to LOS and *N. gonorrhoeae* proteins may be a result of cross reactivity to antigens from nasal carriage of N. meningitis. After acquisition of *N. gonorrhoeae* infection, 9 of 13 subjects developed IgG serum antibody responses with antibodies against all 6 major outer-membrane antigens analyzed in this study (LOS as well as Proteins I, II, III, Pili, and Lip). In the previously mentioned study by Hook and colleagues in which sera collected from 8 patients with DGI, 4 with PID and 1, Protein I antibodies were found in sera collected from all 13 patients with complicated gonococcal infections. Antibodies against both PorB and Rmp were detected in the sera of a large portion of commercial sex workers in Nairobi, Kenya, though only the presence of Rmp antibodies was associated with increased susceptibility to infection.

In another study, immune responses to gonococcal transferrin-binding proteins were analyzed by ELISA using sera and secretions collected from patients attending STD clinics who had confirmed positive *N. gonorrhoeae* cultures. Sera from healthy volunteers were used as controls. Antibodies to both transferrin binding proteins, TbpA and TbpB, were detected in sera from men and women. However, only IgA and IgM concentrations against TbpB in women was significantly higher than the levels of anti-TBP antibodies in uninfected control sera. One male subject's serum antibody levels to TbpA and TbpB were followed for 6 months. A slight increase in IgG, IgM, and IgA was observed 1 month after infection and the levels returned to baseline for the following 5 months of the study(Price *et al.*, 2004). In the previously mentioned two-phase study utilizing urethral challenge and rechallenge with *N. gonorrhoeae* in male volunteers with no previous history of gonococcal infection, pre-infection sera from each volunteer contained IgG to at least one of the major gonococcal OMPs. A majority (18/24) of the volunteers had pre-

existing anti-pilus antibodies. Immunoblots conducted with pre- and post-infection sera were reported from a subset of these subjects. Increases in sera antibodies to outer-membrane proteins, particularly IgG, was detected following infection in most subjects however the pattern of recognized proteins varied from subject to subject. Overall, these studies also support the finding that as with lipooligosaccharide, pre-existing antibodies to gonococcal protein antigens are common in humans and antibodies to some antigens do appear to increase in response to infection.

While there is clear in vitro evidence that some antibodies directed towards *N. gonorrhoeae* antigens can promote either complement-mediated killing or opsonophagocytic killing of the bacteria, whether these antibodies can prevent human infection or play a role in immunologic clearance of the pathogen remains an open question. In a study of experimental human infection followed by rechallenge with *N. gonorrhoeae*, 7 of 8 subjects who experienced at least a 2-fold increase in anti-LOS antibody resisted reinfection while only 1 of the 6 subjects who had less than a 2-fold increase in anti LOS were susceptible to reinfection (Schmidt *et al.*, 2001). Human IgG and monoclonal antibodies that recognize *N. gonorrhoeae* LOS epitopes are bactericidal (Apicella *et al.*, 1986; Yamasaki *et al.*, 2005). Passive immunization with an antibody directed against the common 2c7 epitopes of *N. gonorrhoeae* appears protective in the mouse model (Gulati *et al.*, 2015). In a prospective study of a cohort of commercial sex workers in Kenya, a strong association between the number of *N. gonorrhoeae* Opa protein-directed antibodies and reduced relative risk of gonococcal salpingitis was observed (Plummer *et al.*, 1994). However, in the same study, detectable antibody to Rmp (protein III) was associated with increased risk of gonococcal infection during the study as well as increased rate of gonococcal salpingitis (Plummer *et al.*, 1993). Although antibodies against Rmp are detected during

infection, the presence of these antibodies may contribute to serum resistance and disseminated gonococcal infection. GC isolated from patients with DGI have been shown to be resistant to killing from both NHS and in some cases immune sera. Early studies aimed at identifying the mediators responsible for blocking the bactericidal activity of NHS serum identified IgG as a major source of blocking antibody (McCutchan *et al.*, 1978; Rice and Kasper, 1982). IgG reactive with gonococcal outer-membrane proteins was more effective at inhibiting bactericidal activity when compared to normal IgG and depletion of OMP-IgG restored the bactericidal activity of serum. Further examination into specific antigens that elicit blocking antibody responses found that IgG-Rmp contributed to the majority of the blocking activity observed in vitro (Rice *et al.*, 1986). Price and colleagues were able to demonstrate that preincubating GC with IgG-Rmp isolated from immune sera decreased the binding of bactericidal antibodies from NHS to GC and inhibited killing by NHS (Joiner *et al.*, 1985; Rice *et al.*, 1986). Analysis of the Rmp protein identified two epitopes that elicited bactericidal antibodies and another non-bactericidal epitope that could be bound by mAbs SM50 and SM51. The location of this blocking epitope was identified using a series of small, synthesized peptides that spanned the Rmp protein in conjunction with the mAbs SM50/51. The blocking epitope was almost identical for both mAbs, with SM50 having the highest reactivity with amino acid residues 26-33 and SM51 with amino acids 24-33 (Virji and Heckels, 1989). Although the blocking epitopes are similar for both mAbs, only SM50 was shown to have the ability to block the bactericidal activity of mAbs against LOS and pili (Virji *et al.*, 1987; Virji and Heckels, 1989; de la Paz *et al.*, 1995) . Another study examining the blocking epitope of Rmp demonstrated that the blocking ability of Rmp antibodies could be attributed to residues in the disulfide loop portion (amino acids 47-64) of the protein. Gonococcal strains lacking Rmp or carrying Rmp mutated at

the disulfide loop were shown to have decreased blocking activity in the presence of immune sera (Rice *et al.*, 1994). In a recent study, Gulati and colleagues demonstrated that passive immunization with an anti-Rmp monoclonal antibody abrogated the protective effects of the 2c7 monoclonal antibody causing both increased bacterial burden and duration of infection in a female mouse that received 2c7 (Gulati *et al.*, 2013; Gulati *et al.*, 2015). Although the Rmp protein is conserved and immunogenic, the production of anti-Rmp antibodies can inhibit the development of protective humoral responses. Overall, despite clear evidence that humans develop anti-gonococcal antibodies in response to infection, repeat infections with *N. gonorrhoeae* are common. These data indicate that the humoral immune responses induced by *N. gonorrhoeae* infection are complex and in most cases are insufficient to provide protection from infection.

Cell mediated immune responses to *N. gonorrhoeae*

In addition to humoral immune responses to *N. gonorrhoeae*, there is also evidence that humans with *N. gonorrhoeae* develop adaptive cellular immune responses specific to *N. gonorrhoeae* antigens during infection. Early studies of *N. gonorrhoeae* cellular immunity were conducted by measuring proliferation in response to antigen stimulation as demonstrated by radioactive adenine incorporation into DNA in cultured primary lymphocytes. Mauss and colleagues studied proliferation of lymphocytes obtained from patients with *N. gonorrhoeae* infection as well as a set of uninfected male controls in response to a variety of *N. gonorrhoeae* antigen preparations. For the best characterized antigen preparation, 3 of 6 female subjects and 4/4 male subjects with *N. gonorrhoeae* infection had a positive proliferative response, while 1 of 7 uninfected men had a positive response (Mauss, 1946). Two similar but larger and better controlled studies confirmed these findings. Kraus and colleagues studied lymphocyte

proliferation in response to gonococcal antigens in men with gonorrhea and control male subjects with no reported history of gonorrhea (Kraus *et al.*, 1970). Lymphocyte proliferation was higher in lymphocytes from men with two or more gonococcal infections while lymphocytes from those subjects with their initial case of gonorrhea did not exhibit antigen induced proliferation. Similarly, Wyle and colleagues found that 21 women and 29 men with culture-confirmed *N. gonorrhoeae* infection were found to have significantly higher proliferative indexes in response to *N. gonorrhoeae* antigen than lymphocytes from uninfected men and women who denied prior history of *N. gonorrhoeae* infection (Wyle *et al.*, 1977). Proliferative responses observed in lymphocytes from *N. gonorrhoeae* infected individuals fell below the level of detection in most individuals within 5 weeks of treatment (Esquenazi and Streitfeld, 1973). The specific *N. gonorrhoeae* antigens that are recognized by cellular immune responses in humans are poorly studied. One study demonstrated that the abundant membrane protein PorB could provide antigenic stimulation to lymphocytes from 20 and 24 of 30 *N. gonorrhoeae* infected subjects (Simpson *et al.*, 1999). In that study, a lack of PorB-induced proliferation was reported for lymphocytes from uninfected control subjects, suggesting PorB-directed lymphocytes that might arise from cross-reactive bacterial antigens are not highly represented in humans. In a separate study, lymphocytes from 8 of 8 healthy individuals demonstrated proliferative responses to treatment with *N. gonorrhoeae* IgA protease (Tsirpouchtsidis *et al.*, 2002). It is possible these findings result from cross-reactive responses in lymphocytes that recognize homologous IgA proteases from commensal oral pharyngeal bacteria or even *N. meningitidis*. Genomic analysis of 4 gonococcal strains identified 23 conserved proteins with predicted T and B cell epitopes that could serve as universal antigens (Jain *et al.*, 2016). However, the gonococcal antigens that elicit

natural immune responses during infection remains largely unstudied, or at least unreported in the medical literature, at this time.

Lymphocytes can elicit a variety of functional responses that are aimed at clearing pathogens from the host upon recognition of pathogen-derived antigens. Differential functions of T lymphocytes are mediated by differential expression of a combination of cell surface proteins and secreted mediators (cytokines and chemokines). Lymphocytes responsible for coordinating immunologic responses (known as T helper cells) are positive for the CD4 antigen (CD4+) and are generally sub-classified into 4 broad groups that each have a predominant secreted cytokine: 1) Th1, which secrete interferon-gamma (IFNγ); Th2, which secrete interleukin-4 (IL-4); Th17, which secrete interleukin-17 (IL-17); and Treg, which secrete interleukin-10 (IL-10). Mucosal infections typically do not induce profound differences in systemic or circulating levels of cytokines. However, sera isolated from patients with gonococcal infection were found to have modestly higher circulating levels of IL-17, IFNγ, and IL-23, when compared to sera from healthy controls (Gagliardi *et al.*, 2011). An inverse correlation between serum levels of IL-17 and serum levels of IFN-γ was observed suggesting that Th1 responses are blunted as Th17 responses are generated during *N. gonorrhoeae* infection. In a study of cervical immunologic factors in women undergoing STI screening at primary care clinics in Durbin, South Africa, IL-17 was found to elevated in cervical secretions of women with *N. gonorrhoeae* infection when compared to women without evidence of bacterial STI (Masson *et al.*, 2015). IL-17 and other inflammatory cytokines (IL-1α, IL-1β, IL-12p70, TNF-α, RANTES, G-CSF, Flt3L, IL-2, IL-5, IL-15 and IL-17) were also elevated in cervical lavage of *N. gonorrhoeae*-infected women when compared to women with no detectable STI (Masson *et al.*, 2014). In a study of women seeking care in a Nairobi STI clinic for acute abdominal pain or vaginal discharge, cervical IL-10, a

cytokine produced by both regulatory T cells (Treg) and other immunoregulatory cells, was detected in 19 of 59 women with no detected bacterial STI and in 19 of 36 women with *N. gonorrhoeae* infection. Overall, published studies suggest that *N. gonorrhoeae* induces a complex lymphocyte response that is largely driven by an Il-17 or Th17 pro inflammatory response.

While there are a number of studies in which the level of cytokines in serum or site-specific fluids from *N. gonorrhoeae*-infected individuals were measured, few reported studies of the CD4+ T cell functional responses to *N. gonorrhoeae* in humans have been published. IgA protease-directed CD4 cells from healthy individuals without known *N. gonorrhoeae* infection have been shown to produce IFNγ using both elispot and intracellular cytokine staining. This finding is consistent with the ability of some gonococcal antigens to elicit Th1 type CD4 responses. In that study, cultured PBMC produced IFNγ, TNF-α, IL-4 and IL-10 in response to IgA protease antigen while only producing IFNγ and TNF-α in response to tetanus toxoid (Tsirpouchtsidis *et al.*, 2002). These data suggest that the commensal bacteria colonization that resulted in cross reactive immune response to *N. gonorrhoeae* IgA protease elicited a polyfunctional immune response different in character from that induced by tetanus toxoid vaccination. In another ex vivo study of PBMC responses to *N. gonorrhoeae*, cultured PBMCs from 2 anonymous donors (and unknown *N. gonorrhoeae* infection history) were exposed to *N. gonorrhoeae* and CD4+ T cells were found to upregulate CD25, a T cell surface marker that is elevated in T regulatory cells and in activated CD4+ T cells. Culture supernatants from these *N. gonorrhoeae*-treated PBMCs contained cytokines associated with Th1 (IFNγ), Th2 (IL-4), and Treg (IL-10) responses as well as the chemotactic factors IL-8 and MCP (Rarick *et al.*, 2006). Though the cellular source of these cytokines is unknown, these findings are consistent with the

observation that *N. gonorrhoeae* infection induces a pleotropic immunologic response at the site of infection.

The role of cytotoxic T lymphocytes (CD8+ T cells), as well as cytotoxic innate immune cells like Natural Killer cells (NK cells), in gonococcal infection are unknown. Gonococcal PorB has been shown to induce robust IL-4 production in CD8+ T cells from *N. gonorrhoeae*-infected individuals (Simpson *et al.*, 1999). However, contradictory effects of *N. gonorrhoeae* infection on CD8+ T cell function in humans have been reported. In a longitudinal study of female commercial sex workers, Kaul and colleagues found that CD8+ T cell functional responses to HIV (in HIV-infected subjects) and CMV (in HIV-infected and -uninfected individuals) were reduced during episodes of incident *N. gonorrhoeae* infection when compared to lymphocytes obtained when subjects were not infected with *N. gonorrhoeae* (Kaul *et al.*, 2002). In contrast, a longitudinal study of HIV acquisition in a cohort of HIV-negative female commercial sex workers demonstrated that early HIV-directed CD8+ T cell responses were more robust in subjects who were infected with *N. gonorrhoeae* at the time of HIV acquisition than in *N. gonorrhoeae*-uninfected individuals (Sheung *et al.*, 2008). Additionally, asymptomatic anorectal *N. gonorrhoeae* infections in men who have sex with men while taking pre-exposure prophylaxis to prevent HIV were found to be associated with increased activation marker in circulating CD8+ T cells (Vieira *et al.*, 2017). The adaptive immune response in cytotoxic T cell populations to *N. gonorrhoeae* infection remains largely unexplored at this time.

Cytokines from the IL-17 family are important for the recruitment of neutrophils and to induce localized antimicrobial responses. Studies in a mouse model of *N. gonorrhoeae* infections suggest that the magnitude of Th17 responses to infection are more robust than Th1/Th2 responses (Liu *et al.*, 2012). These findings are consistent with the neutrophilic inflammatory

response commonly associated with *N. gonorrhoeae* infection in humans and the inverse relationship between systemic IL-17 and IFNγ levels reported in *N. gonorrhoeae*-infected individuals (Gagliardi *et al.*, 2011). The relative resistance of *N. gonorrhoeae* to neutrophil-mediated killing may render this Th17-skewed immune response less effective at clearing the pathogen than a more robust Th1/Th2-supported immune response might (Witt *et al.*, 1976; Criss *et al.*, 2009). Further, the ability of *N. gonorrhoeae* to induce IL-10 secretion and promote or stimulate Treg cells may also reduce humoral and cellular immune responses to infection (Liu *et al.*, 2014). In support of this hypothesis, manipulation of the mouse immune system to block the induction of Treg-related activity or to drive more robust Th1/Th2 responses leads to enhanced clearance of *N. gonorrhoeae* in model infection. Further, stimulation of Th1 responses through intravaginal IL-12 administration during infection in the mouse vaginal infection model resulted in enhanced clearance upon rechallenge when compared to mice initially infected without supplemental IL-12 (Liu *et al.*, 2013). Because studies of lymphocyte proliferative response demonstrated that prior natural infection was associated with significant increase in proliferative response in subjects with current *N. gonorrhoeae* infection when compared to subjects without previous infection, it appears the cell mediated immunity being measured was inadequate to provide robust protection from repeat infection. Unfortunately, there are no studies to date that measure cellular immune responses to *N. gonorrhoeae* either in naïve or infected individuals in which the subjects were subsequently prospectively followed to determine whether these cellular immune responses correlate with any degree of protection from re-infection.

Discussion

Despite public health efforts, gonorrhea still remains one of the most common sexually transmitted bacterial infections responsible for ~100 million infections per year. A large

proportion of infections are carried asymptomatically contributing to not only its transmission but the transmission of other STIs. With resistance in *N. gonorrhoeae* strains on the rise and treatment options becoming more limited, the need for an effective vaccine is ever present. The development of a gonococcal vaccine has been the focus of the field for some time, but potential vaccines have had little to no success. The vaccine candidates that have advances to clinical trials have been ineffective. The lack of a protective immune response observed in humans, coupled with the complex nature of antigen-specific responses and limited animal infection models, have all confounded past vaccine development efforts. Large scale efforts with meningococcal OMV vaccines have been effective at reducing the incidence of meningitis, and data emerging from these studies suggest that the vaccine may also be affording at least temporary protection from *N. gonorrhoeae* infections. The mechanism by which this vaccine may be protective against *N. gonorrhoeae* is not fully understood and further studies that evaluate the cellular and humoral responses that develop after vaccination are needed. Studies using the murine model of infection have highlighted antigens that induce beneficial immune responses in the host and may also be of interest for vaccine development. This review of published literature of human immunologic responses to *N. gonorrhoeae* largely supports the widely accepted supposition that *N. gonorrhoeae* manages to infect and re-infect humans using some combination of immunologic evasion and resistance to host mediators of clearance. Though there are some reports that support the possibility that natural clearance or protection from infection may develop in response to gonococcal infection in a small portion of individuals, comprehensive studies that combine sophisticated immunologic analysis and prospective monitoring of subjects are needed to understand both natural and the possibility of vaccine mediated immunity to *N. gonorrhoeae* infection.

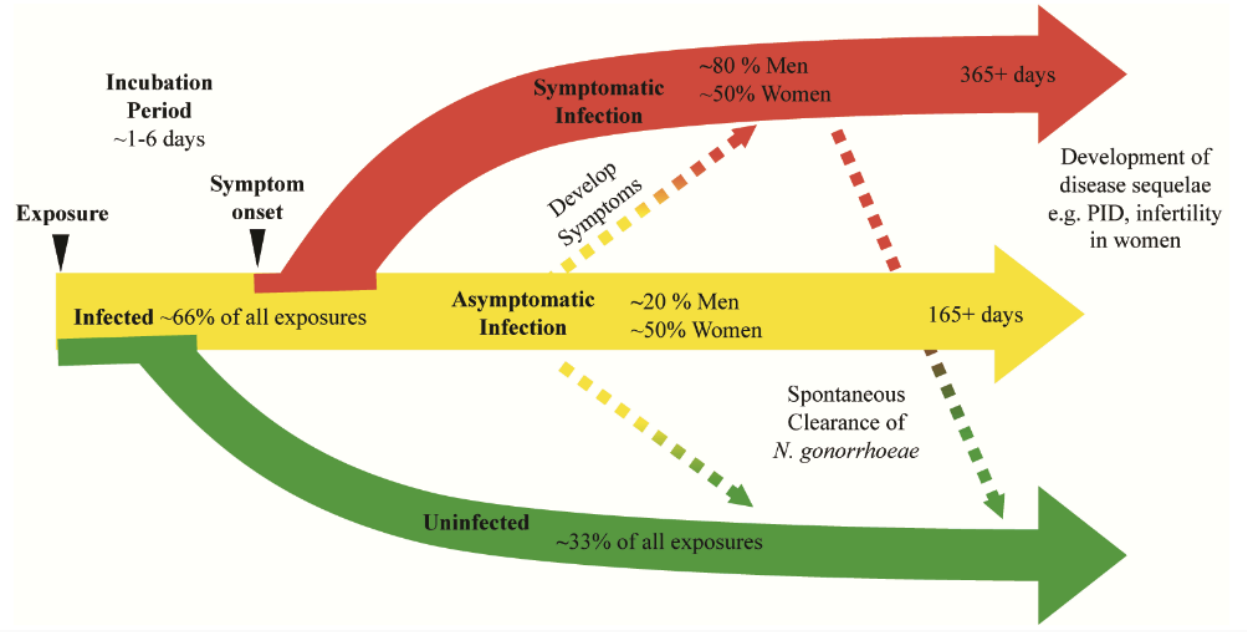

Figure 1. 1 | *N. gonorrhoeae* exposure can lead to bacterial clearance or prolonged infection with or without symptoms. A diagram of the natural history of *N. gonorrhoeae* infection after exposure demonstrates as many as 33% of exposed individuals will not develop infection. Infected individuals can have symptoms or remain asymptomatic. Asymptomatic infection can eventually progress to symptomatic infection, with studies indicating this progression may occur in as many as 25% of asymptomatic infections. Symptomatic infection can persist at least 14 days and infection has been documented to last as long as 1 year. Asymptomatic infection has been documented to persist as long as 165 days with as many as 25% of asymptomatic infections clearing over that time frame. The use of antibiotic therapy in all studies generating these data limit our knowledge of the actual rates of bacterial clearance and length of time chronic infection can persist.

Table 1.1 Anti-gonococcal immunoglobulins identified in humans

Antigen:	LOS	Pili	Protein I (PorB)	Protein II (Opa)	Protein III (Rmp)	Lip	Tbp	Whole Bacteria
Pre-existing Antibody (or present in healthy controls)	+	+	+	+	+	+	+	+
Post-infection Antibody	++	++	+	+	+	+	+	+
Immunoblot/ELISA	I/E	E	I	I	I	I	E	E

Table 1.2 Anti-gonococcal cellular immune responses identified in humans

Antigen:	Protein I (PorB)	IgA protease	Whole Bacteria
Lymphocyte Proliferation:			
uninfected, pre-infected	-	+	+
infected	+	NT	++
Stimulated Cytokine Release: (cell type)			
IL-4	+ (CD4 & CD8 T cell)	+ (PBMC)	+ (PBMC)
IL-10	-	+ (PBMC)	+ (PBMC)
IFNγ	-	+ (CD4 T cell, PBMC)	+ (PBMC)
TNF-α	-	+ (PBMC)	NT

NT, not tested; CD4 or CD8 T cell, cytokine detected in T cell population by intra-cellular cytokine staining; PBMC, cytokine detected in Peripheral Blood Mononuclear Cell culture supernatant by ELISA

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CHAPTER 2: ANALYSIS OF THE CERVICOVAGINAL MICROBIOME IN N. GONORRHOEAE INFECTED WOMEN[2]

Introduction

Previous studies have shown that women with BV are at increased risk of acquiring and transmitting STIs, including *N. gonorrhoeae* (Bautista *et al.*, 2017; Borgdorff *et al.*, 2014; Gallo *et al.*, 2012; Wiesenfeld, Hillier, Krohn, Landers, & Sweet, 2003). Studies examining the impact of vaginal microbiomes on basal inflammatory states found that women with BV or high diversity genital tract microbial communities were associated with higher expression levels of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12 and IL-8) when compared to BV negative women (Masson *et al.*, 2014). This indicates that the variations in vaginal microbial diversity that are common in women with BV could influence inflammatory responses that characterize symptomatic *N. gonorrhoeae* infection. Symptomatic *N. gonorrhoeae* infection most commonly leads to localized host inflammation at the site of infection, urethritis in males and cervicitis in females. *N. gonorrhoeae* itself is resistant to many host antimicrobial responses which may contribute to its ability to cause infection under these conditions of localized inflammation. Surprisingly, a large proportion of *N. gonorrhoeae* infections are carried asymptomatically (Handsfield, Lipman, Harnisch, Tronca, & Holmes, 1974; Platt, Rice, & McCormack, 1983; Sandstrom *et al.*, 1984). Upwards of 50% of lower genital tract infections in females are

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asymptomatic (Kent *et al.*, 2005; Lovett & Duncan, 2018). Untreated asymptomatic infections can ascend to the upper genital tract leading to health complications, including pelvic inflammatory disease and infertility in women (Reekie *et al.*, 2018; Reekie *et al.*, 2019; Wiesenfeld, Hillier, Meyn, Amortegui, & Sweet, 2012). Asymptomatic genital *N. gonorrhoeae* poses a risk of onward transmission to sex partners and ascending infection. The mechanisms that lead to the development of asymptomatic and symptomatic gonorrhea infection are unknown. During an infection, *N. gonorrhoeae* must compete with the natural microbial community at the mucosal surface to establish infection (Aroutcheva *et al.*, 2001). Cervicovaginal microbial communities play an important role in sexual and reproductive outcomes, including protection from pathogens, as the composition of the cervicovaginal microbiota has been shown to modify susceptibility to several sexually transmitted pathogens (Anahtar *et al.*, 2015; Atashili, Poole, Ndumbe, Adimora, & Smith, 2008; Coleman *et al.*, 2007; Masson *et al.*, 2014; Masson *et al.*, 2015; McClelland *et al.*, 2018; Sha *et al.*, 2005). We sought to understand whether differences in microbial composition of the genital tract were associated with symptomatic or asymptomatic presentation of *N. gonorrhoeae* infection.

Cohort characteristics of *N. gonorrhoeae* infected women

In this pilot study, we used remnant nucleic acid material from cervical swabs collected for *N. gonorrhoeae* and *C. trachomatis* diagnostic testing from a convenience cohort of 19 females deemed to be *N. gonorrhoeae* positive by Aptima. *N. gonorrhoeae* and *C. trachomatis* clinical diagnostic testing. Of these, ten individuals reported symptoms to the provider (defined as symptomatic) and nine did not (defined as asymptomatic) (Table 2.1). The reported symptoms at the time of cervical swab sampling included vaginal discharge (9/10, 90.0%), genital irritation (1/10, 10.0%), and dysuria (2/10, 20%). There was a trend for younger females to report

symptoms (p=0.090). Of the 19 specimens, 17 (89.5%) were collected from women who identified as having African American race. Among these, the proportion of symptomatic and asymptomatic individuals was comparable (p=0.211). The presence or absence of *C. trachomatis*, another STI pathogen, was assessed by NAAT testing in conjunction with clinical *N. gonorrhoeae* testing and no difference in *C. trachomatis* prevalence was observed between asymptomatic and symptomatic presentation (p=0.590).

Since other STI pathogens could also be responsible for causing lower genital tract symptoms (e.g. *Trichomonas vaginalis* or *Mycoplasma genitalium*), we used a commercial microbial qPCR array to test for the presence of other STI pathogens (Table 2.2). Specimens from two women did not provide evaluable results due to insufficiently recovered DNA material. Among the 17 specimens that gave analyzable results on this array, 6 of 7 (85.7%) asymptomatic specimens and 8 of 10 (80.0%) symptomatic specimens had detectable *Neisseria* species DNA (Table 2.2). Because these specimens all tested positive for *N. gonorrhoeae* using the APTIMA Combo 2, these results indicate that the sensitivity of the microbial qPCR array may be lower for detecting *N. gonorrhoeae* in this specimen type than the APTIMA Combo 2. *C. trachomatis* was detected in 3 of 3 specimens that were positive by APTIMA Combo 2 test and had evaluable qPCR results in the qPCR array (Table 2). *T. vaginalis* was detected in 4 of 10 (40.0%) evaluable symptomatic individuals and 2 of 7 (28.6%) asymptomatic individuals (Table 2.2). *M. genitalium* was not detected by qPCR array in any of the specimens (Table 2.2). When accounting for both clinical *C. trachomatis* testing by APTIMA Combo 2 and real-time PCR array results, the proportion of STI coinfection with *C. trachomatis* or *T. vaginalis* was not significantly different between symptomatic (5/10, 50.0%) and asymptomatic (5/9, 55.6%) individuals (p=0.625). Because coinfection with other STI pathogens was not associated with symptomatic presentation

we next sought to assess whether the non-STI vaginal microbial community was associated with the presence or absence of symptoms.

***Neisseria spp*. abundance represents a small proportion of bacterial communities in both symptomatic and asymptomatic patients.**

To characterize these genital microbial communities, the remnant nucleic acid specimens from the cervical swabs were analyzed using 16S amplicon deep sequencing. A total 157,006 paired-end reads were obtained. After demultiplexing and eliminating low-quality reads, 100,216 reads were retained for downstream analyses of α-diversity and β-diversity (mean number of reads per sample = 5,274; range = 2,470–9,528). The individual microbial communities of *N. gonorrhoeae*-infected patients were compared to those that presented with and without reported symptoms. Because other STI pathogens might be associated with different microbial community profiles on their own, we also compared specimens from individuals without coinfecting *C. trachomatis* or *T. vaginalis* (by clinical test and/or real-time PCR) separately from those with coinfection. We first examined whether the relative burden of *N. gonorrhoeae* was associated with symptomatic presentation. Interestingly, in these *N. gonorrhoeae*-infected individuals, *Neisseria*-assigned 16S ribosomal DNA reads made up 0.24% of all reads and thus were a minor component of the bacterial community (Figure 2.1). In this limited set of specimens, the point estimate of the relative of abundance of 16S ribosomal DNA reads from *Neisseria spp*. was highest in symptomatic individuals without *C. trachomatis* or *T. vaginalis* coinfection, but no significant difference in relative abundance between any group was observed (Figure 2.1). Although there was not a significant association between *N. gonorrhoeae* abundance relative to other microbial species by 16S analysis and symptoms, the trend between higher levels of *N. gonorrhoeae* and the presence of symptoms in individuals without other STI

pathogens suggests that further study is needed to definitively determine whether an association exists.

Microbial community diversity is different between symptomatic and asymptomatic *N. gonorrhoeae* infection

The overall alpha diversity did not differ when observed taxa, Chao1 and phylogenetic diversity were compared between individuals with symptomatic and asymptomatic *N. gonorrhoeae* infection and between individuals with and without other STI (Figure 2.2). However, the number of dominant taxa comprising the majority of the microbial community (i.e., 90% of all detected taxa) was significantly lower in individuals with asymptomatic *N. gonorrhoeae* infection without STI coinfection *vs*. both individuals with symptomatic *N. gonorrhoeae* and with STI co-infection. Differences between symptomatic and asymptomatic patients, but not between patients with and without *C. trachomatis* and/or *T. vaginalis* coinfection, were reflected in beta diversity analyses, with statistically significant ANOSIM tests and clear separation on PCoA plots by two different methods: weighted Unifrac ANOSIM R = 0.20, p-value = 0.032, Figure 2.3) and unweighted Unifrac ANOSIM R = 0.24 p-value = 0.011, Figure 2.3).

Asymptomatic patients without an STI coinfection are more frequently characterized by low diversity, *Lactobacillus*-dominant genital communities

Differences in alpha and beta diversities with respect to symptoms were evident for each patient when inspecting the taxa plots (Figure 2.4). The four asymptomatic patients with only *N. gonorrhoeae* infection and no detected coinfection with either *C. trachomatis* or *T. vaginalis* were dominated by *Lactobacillus* taxa (Figure 2.4). *Lactobacillus*-predominance was observed less frequently in specimens from women with symptomatic *N. gonorrhoeae* infection regardless

of the presence of additional STI (2/10, 20.0% symptomatics *vs*. 6/9, 66.7% asymptomatics, p=0.040, Figure 2.4). Across all specimens, the distribution of the fraction of reads assigned to the *Lactobacillus* genus was: 92% among asymptomatic individuals with no coinfections, 35% among asymptomatic individuals with coinfections, 22% among symptomatic individuals with no coinfections, and 11% among symptomatic individuals with coinfection (Figure 2.4). Using BLAST and Multiple Sequence Comparison by Log Expectation (MUSCLE) (Edgar, 2004), we investigated the 16S sequences of each taxa that was assigned *Lactobacillus* taxonomy and identified *L. iners* as the likeliest species. This led us to define *Lactobacillus*-dominant samples (n=8) as community-type 3 (CT3), using standard definitions of vaginal microbial structure (Anahtar *et al.*, 2015; Gosmann *et al.*, 2017; Ravel *et al.*, 2011) (Figure 2.4).

The presence of specific *Lactobacillus* species was further assessed by presence/absence real-time PCR (Figure 2.4). The *Lactobacillus* species most commonly detected were *L. iners*, *L. crispatus*, *L. jensenii*, and *L. gasseri*. In line with our microbiome analyses and BLAST homology searches, all asymptomatic women were positive for *L. iners* and in 85% and 75% of symptomatic women with and without co-infection, respectively. Although *L. crispatus* was not the predominant *Lactobacillus* species in any of the specimens using 16S sequencing, *L. crispatus* was detected by real-time PCR only in *N. gonorrhoeae*-infected individuals with asymptomatic presentation (Figure 2.5).

Symptomatic *N. gonorrhoeae* and STI coinfection are associated with a diverse cervicovaginal microbial community composed of bacterial vaginosis-associated bacteria

Having established that 8 of 19 samples (42.1%) were *L. iners*-dominated (CT3) and more commonly associated with asymptomatic *N. gonorrhoeae* infection, we investigated the remaining 11 samples, which were dominated by a diverse group of non-lactobacilli (*Prevotella*,

a *Lachnospiraceae* genus, *Sneathia*, or *Mycoplasma*) in more detail. We used BLAST and MUSCLE alignments to further characterize the composition of the non-*Lactobacillus* communities dominated by *Lachnospiraceae* and *Mycoplasma*. By applying BLAST on representative reads assigned to each genus of interest, we found that the likeliest species for *Lachnospiraceae* and *Mycoplasma* OTUs were Bacterial Vaginosis-Associated Bacterium-1 (BVAB1)/"*Candidatus* Lachnocurva vaginae" and *M. hominis*, respectively (Supplementary Table 1). Samples dominated by *Prevotella* (n=6), *Sneathia* (n=1), BVAB1 (n=3), and *M. hominis* (n=1) were classified as CT4/molecular BV. The *Mycoplasma*-dominant sample was included in the CT4 and molecular BV classifications. The CT4/molecular BV samples were more frequently found in symptomatic patients (8/10, 80% of symptomatics *vs*. 3/9, 33.3% of asymptomatics, p=0.040) (Figure 4A). Among STI-coinfected individuals, 7/10 (70.0%) carried CT4 microbial communities compared to patients without coinfections (4/9, 44.4%), though this difference did not attain statistical significance (p=0.255) (Figure 2.6).

The prevalence of common bacterial vaginosis-associated bacteria was also assessed by microbial DNA real-time PCR array (Figure 2.6). Of the BV-associated species included on the array, *Gardnerella vaginalis*, was present in all samples. Other species commonly associated with BV, e.g. *Atopobium vaginae*, certain *Prevotella spp.*, *and Sneathia sanguinegens,* were also highly prevalent among this cohort of women, regardless of symptoms or STI coinfection status. Asymptomatic women infected only with *N. gonorrhoeae* (n=4) carried the following species less frequently than symptomatic women infected only with *N. gonorrhoeae* (n=5): *M. hominis* (25% *vs*. 80%), *Prevotella buccalis* (25% vs. 80%), *and Ureaplasma urealyticum* (0% vs. 100%). Similarly, asymptomatic females without coinfections also carried BV-associated bacteria less frequently when compared to those with symptoms or STI coinfections (Figure 2.6).

Because of the nature of the study design, there is no information on whether the studied individuals have clinically defined bacterial vaginosis. However, the association between BV associated microbes and cervicovaginal community type suggests that further studies of the association of BV with symptomatic *N. gonorrhoeae* infection are needed.

Discussion

A large body of evidence links vaginal dysbiosis, such as clinical bacterial vaginosis (BV), to the risk of acquisition of several sexually-transmitted infections (STIs), including gonorrhea (Atashili *et al.*, 2008; Bautista *et al.*, 2017; Brotman, 2011; H. L. Martin *et al.*, 1999; Tamarelle *et al.*, 2019). Despite the clear association between BV and STI acquisition risk, treatment of asymptomatic BV was not found to reduce the incidence of *N. gonorrhoeae* or *C. trachomatis* infection incidence, raising the question whether suboptimal vaginal environment is a modifiable biological cause of gonorrhea risk (PMID: 26611782). However, *Lactobacillus*-based live probiotic therapy of vaginal dysbiosis has been recently shown to reduce not only BV (Aroutcheva *et al.*, 2001; Witkin & Linhares, 2017), but also bacterial STI incidence (van de Wijgert & Verwijs, 2020). To the best of our knowledge, this report is the first study to examine the association between the cervicovaginal microbiota composition and clinical disease presentation of *N. gonorrhoeae* infections (self-reported vaginal discharge, dysuria and/or genital irritation). Using 16S ribosomal RNA gene deep sequencing approaches on patient samples confirmed to be infected with *Neisseria gonorrhoeae* by APTIMA clinical testing, we show that the cervicovaginal microbiome is predictive of gonorrhea clinical presentation in females attending an STI clinic in the United States. These findings were confirmed by real-time polymerase chain reaction assays specific for several *Lactobacillus* species and BV-associated bacteria deployed in parallel on the same clinical samples.

Specimens collected from asymptomatic individuals with *N. gonorrhoeae* infection and no coinfection with *Chlamydia trachomatis* and/or *Trichomonas vaginalis* carried *Lactobacillus*-dominant microbial communities more frequently than symptomatic patients without coinfection. Notably, this *Lactobacillus* dominance was due to *L. iners* and these microbiotas were classified as community type 3 (CT3), according to established definitions in the field (Anahtar *et al.*, 2015; Gosmann *et al.*, 2017; Ravel *et al.*, 2011). Previous studies have established that *L. iners*-dominated vaginal microbiotas compared to *L.crispatus*-dominated vaginal microbiotas may place patients more at risk of STI infection, like chlamydia or HIV (Gosmann *et al.*, 2017; Tamarelle *et al.*, 2019; van der Veer, Bruisten, van der Helm, de Vries, & van Houdt, 2017). Interestingly, none of the females in our study had cervicovaginal microbiomes dominated by *L. crispatus*. Although our study did not examine the microbial communities from women that did not have *N. gonorrhoeae* for comparison to *N. gonorrhoeae* infected individuals, the findings are consistent with a potential protective effect of *L. crispatus*, as it has been shown in prospective studies of HIV acquisition (Gosmann *et al.*, 2017). This is supported by *in vitro* studies with clinically-isolated and lab strains of *L. crispatus* have been shown to inhibit the growth of *N. gonorrhoeae in vitro* (Vielfort, Sjolinder, Roos, Jonsson, & Aro, 2008), possibly through the effects of lactic acid acidification of the growth environment (Graver & Wade, 2011). While *L. crispatus* produces both isomers of lactic acid, *L. iners* and human cells only make l(+) lactic acid (Spurbeck & Arvidson, 2010; Tachedjian, Aldunate, Bradshaw, & Cone, 2017). Accumulating evidence also suggests that d(−) lactic acid may impart greater protection against STI pathogens than l(+) lactic acid, potentially via effects on human host cells rather than pathogen cells (Edwards *et al.*, 2019; Nunn *et al.*, 2015). In humans, *L. crispatus*-dominant vaginal microbiota is associated with reduced risk of acquisition of other STI, like HIV

(Borgdorff *et al.*, 2014; Gosmann *et al.*, 2017; Tamarelle *et al.*, 2019; van der Veer *et al.*, 2017). This offers a partial explanation why no women in our cohort had genital microbiomes dominated by *L. crispatus*. Though there was no *L. crispatus*-dominant microbial communities in our studies, the presence of *L. crispatus* was detected by real-time in a minority of *N. gonorrhoeae*-infected women, but was exclusively detected in asymptomatic individuals.

N. gonorrhoeae-infected patients who reported symptoms were found to have genital microbiomes composed of a mixture of various bacterial anaerobes, such as *Prevotella*, *Sneathia*, *Mycoplasma hominis* and Bacterial Vaginosis-Associated Bacterium-1 (BVAB1)/"*Candidatus Lachnocurva vaginae*" (Holm *et al.*, 2020). These women with genital microbiomes composed of anaerobes were deemed to have molecular bacterial vaginosis (BV), as defined by established classifications in the field based on diversity and relative abundance of bacterial taxa (Anahtar *et al.*, 2015; Gosmann *et al.*, 2017; Ravel *et al.*, 2011). This included the *Mycoplasma*-dominant sample because of three main reasons that definitions of molecular BV take into account: like *Prevotella* and *Sneathia*, it can overgrow in cases of BV (Fredricks, Fiedler, & Marrazzo, 2005; Onderdonk, Delaney, & Fichorova, 2016), its prevalence in BV patients is three times higher compared to healthy women (Rumyantseva, Khayrullina, Guschin, & Donders, 2019), and it is associated with severe genital mucosal inflammation (D. H. Martin *et al.*, 2013).

A possible explanation for the association of symptoms in *N. gonorrhoeae* infection and BV-associated microbial communities relates to the known increase of inflammation and inflammatory mediators in women with BV. Several studies have shown that females with clinical BV or low *Lactobacillus* abundance and high diversity of anaerobes also harbor higher concentrations of pro-inflammatory cytokines in their genital tract (Anahtar *et al.*, 2015) and

higher levels of the pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12 and IL-8) when compared to BV negative women (Kyongo *et al.*, 2015). Further, symptoms of abnormal vaginal discharge were also found to associate with elevated levels of IL-1beta, IP-10, IL-8, and GCSF, linking inflammatory cytokines to vaginal symptoms, particularly vaginal discharge (Kyongo *et al.*, 2015). Notably, the more the vaginal microbiota shifts towards dysbiosis, the more marked the inflammation (Anahtar *et al.*, 2015; Cohen *et al.*, 2010; Lennard *et al.*, 2018), independently of concurrent STIs, including HIV and gonorrhea (Anahtar *et al.*, 2015). Our findings indicate that females who present to the clinic and report lower genital tract symptoms and confirmed *N. gonorrhoeae* infection are also likely to have BV. Further studies defining the relationship between genital tract microbiomes and the and pro-inflammatory immune responses in symptomatic presentation of *N. gonorrhoeae* infection are needed to elucidate whether *Lactobacilli* or BV-defining microbial communities serve as a biomarker for symptoms in *N. gonorrhoeae* infections or directly impact symptoms.

Tables

Table 2.1| Study population characteristics at baseline. The age, race, and self-reported symptoms of 19 women positive for *N. gonorrhoeae* infection by clinical test who presented at a local STI clinic are provided.

Baseline characteristics	**Symptomatic**	**Asymptomatic**	**P-value**
	n = 10	**n = 9**	
Age, mean (range)	21.1 (15-37)	26.6 (16-40)	0.09
	Black (10)	Black (7)	n/a
Race (n)	Latino (0)	Latino (1)	
	White (0)	White (1)	
Symptoms (%)	Vaginal Discharge (90.0)	None (100.0)	n/a
	Genital irritation (10.0)		
	Dysuria (20.0)		

Table 2.2 | Results of clinical Aptima (NAAT) test results and microbial DNA quantitative real-time PCR (qPCR) array. Each column across the categories reflects one individual participant.

	***N. gonorrhoeae* only**									***N. gonorrhoeae* and *C. trachomatis*/*T. vaginalis***									
Clinical test	**Asymptomatic**				**Symptomatic**					**Asymptomatic**					**Symptomatic**				
Aptima GC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aptima CT	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	+	-
qPCR																			
N. gonorrhoeae	+	+	+	+	+	+	-	+	+	+	+	n/a	n/a	-	+	+	-	+	+
C. trachomatis	-	-	-	-	-	-	-	-	-	-	+	n/a	n/a	-	-	+	-	+	-
M. genitalium	-	-	-	-	-	-	-	-	-	-	-	n/a	n/a	-	-	-	-	-	-
T. vaginalis	-	-	-	-	-	-	-	-	-	+	-	n/a	n/a	+	+	+	+	-	+

+ detected; - not detected; n/a insufficient sample for qPCR; GC, *N. gonorrhoeae;* CT, *C. trachomatis.*

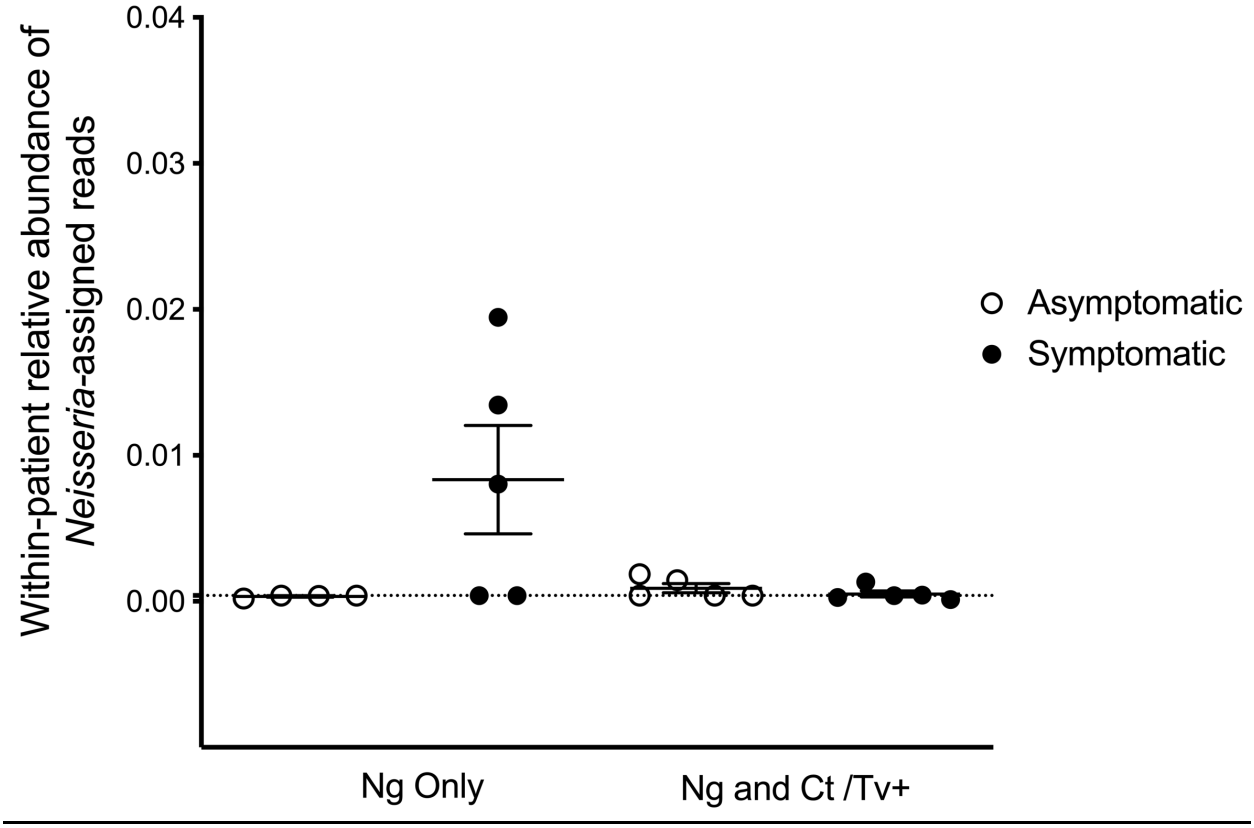
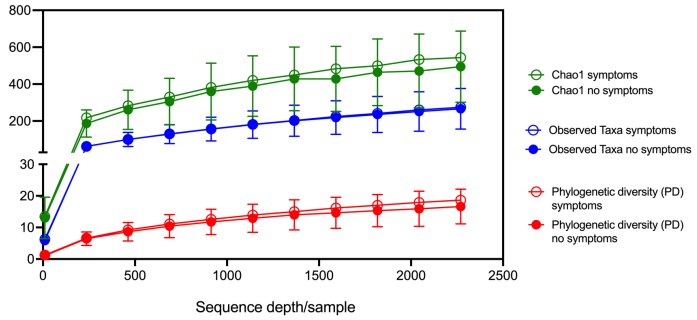
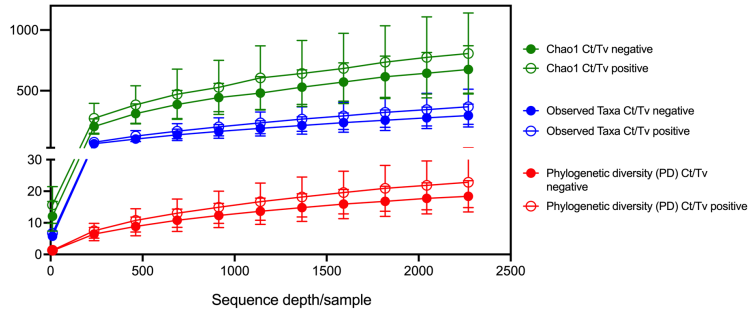


Figure 2.1 | *Neisseria* genus comprises a small proportion of the cervicovaginal microbial community by 16S ribosomal RNA (rRNA) microbiome analysis. Relative abundances (displayed as fraction of total 16s rRNA sequencing reads) of the *Neisseria* genus among symptomatic and asymptomatic patients with and without STI coinfection. Ng, *N. gonorrhoeae*; Ct, *Chlamydia trachomatis;* Tv, *Trichomonas vaginalis*.

A. Alpha diversity by presence of reported symptoms



B. Alpha diversity by *C. trachomatis* or *T. vaginalis* co-infection



C.

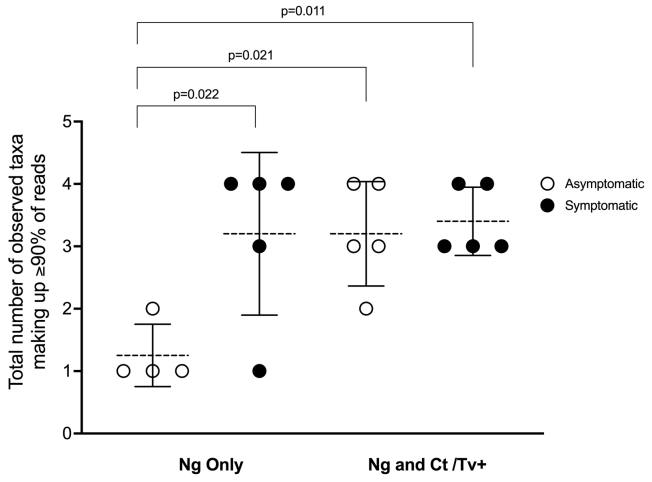


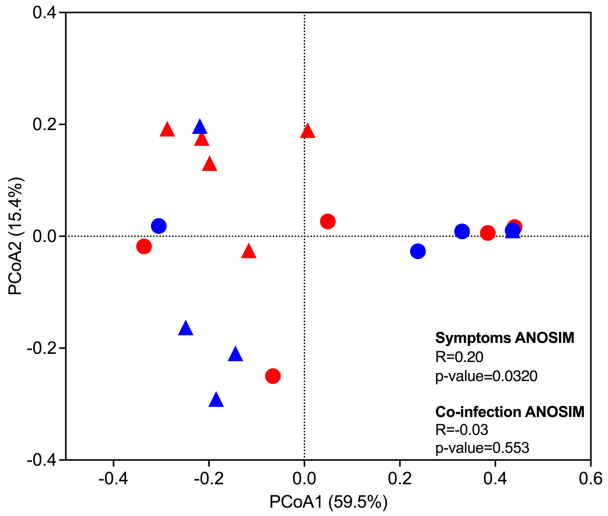
Figure 2.2 | Asymptomatic females infected only with *N. gonorrhoeae* have lower diversity cervicovaginal microbial communities.

(A) Rarefaction plots of alpha diversity metrics, by reported symptoms

(B) Rarefaction plots of alpha diversity metrics, by *C. trachoma*tis and/or *T. vaginalis* coinfection

(C) Number of taxa comprising 90% of the relative abundance among symptomatic and asymptomatic individuals with and without coinfection.

A. Weighted Unifrac



B. Unweighted Unifrac

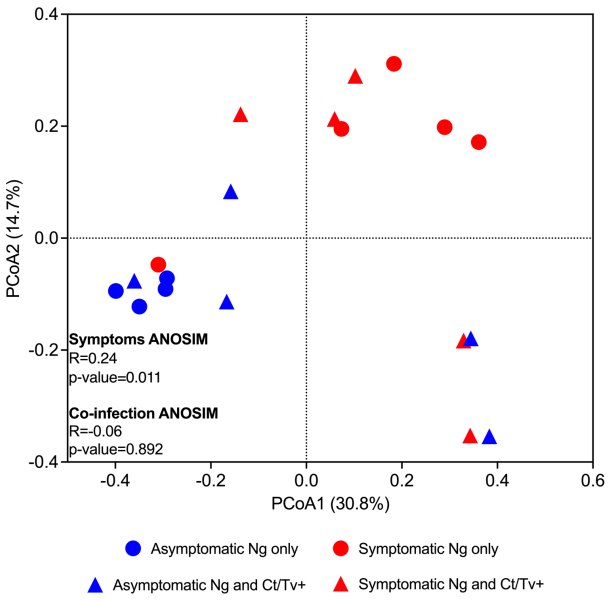
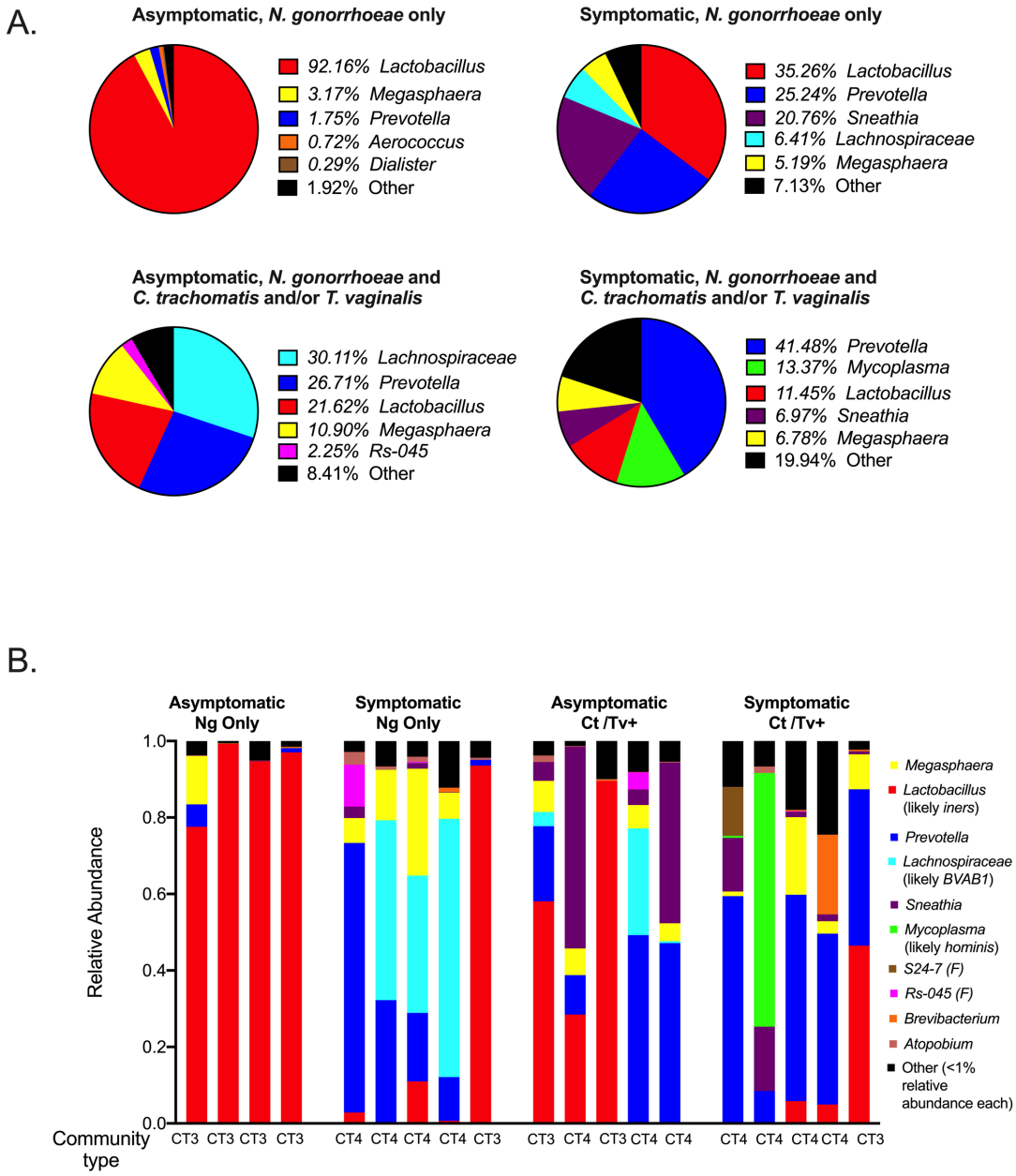


Figure 2.3 |Beta diversity analyses, using weighted and unweighted Unifrac measures, show differences in microbial composition between symptomatic and asymptomatic patients, but not between patients with and without *C. trachomatis* and/or *T. vaginalis* coinfection. ANOSIM tests were used to assess the strength of the clustering patterns and statistical significance.

(A) Principal Coordinates Analysis (PCoA) plot of weighted Unifrac.

(B) Principal Coordinates Analysis (PCoA) plot of unweighted Unifrac.

Figure 2.4 | Community



composition of symptomatic and asymptomatic individuals with and without *C. trachomatis* and/or *T. vaginalis* coinfection.

(A) Taxa summary plots displaying the relative abundances of the top ten taxa (genera or family-level taxa, as applicable) observed among the 19 study participants across all 16S rRNA sequencing reads in the dataset. These top 10 taxa comprised ³99% of all reads in the entire dataset. We used BLAST and MUSCLE alignments to determine the likely species of communities dominated by *Lactobacillus*, *Lachnospiraceae* and *Mycoplasma* and found them to be *L. iners*, Bacterial Vaginosis-Associated Bacterium-1 (BVAB1)/"*Candidatus* Lachnocurva vaginae" and *M. hominis*, respectively. The microbial community type (CT) designated for each participant, using standard definitions in the field, is provided.

(B) The mean relative abundance of the top 5 most prevalent bacterial genera identified within each patient group via 16s rRNA sequencing.

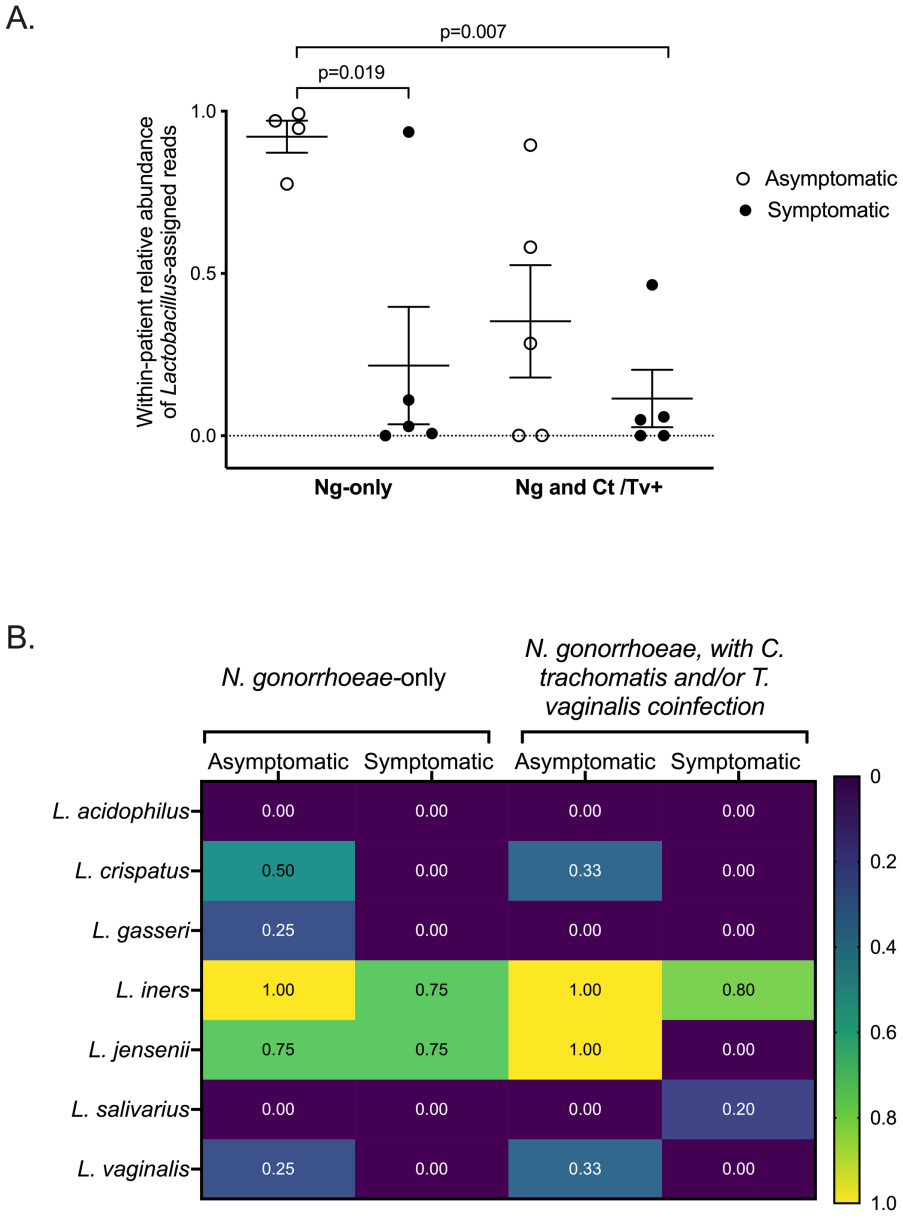


Figure 2.5 | *Lactobacillus* composition of symptomatic and asymptomatic individuals with and without *C. trachomatis* and/or *T. vaginalis* coinfection

(A) Within-patient relative abundances of *Lactobacillus*-assigned reads. The fraction of reads assigned to *Lactobacillus* among all sequence reads comprising an individual's microbiome is shown. Participants have been grouped by symptom and coinfection status. No statistically significant difference was observed between groups using one way ANOVA with Tukey's correction for multiple comparisons.

(B) Percentage of individuals with detectable levels of *Lactobacillus spp.* detected via microbial DNA real-time PCR array.

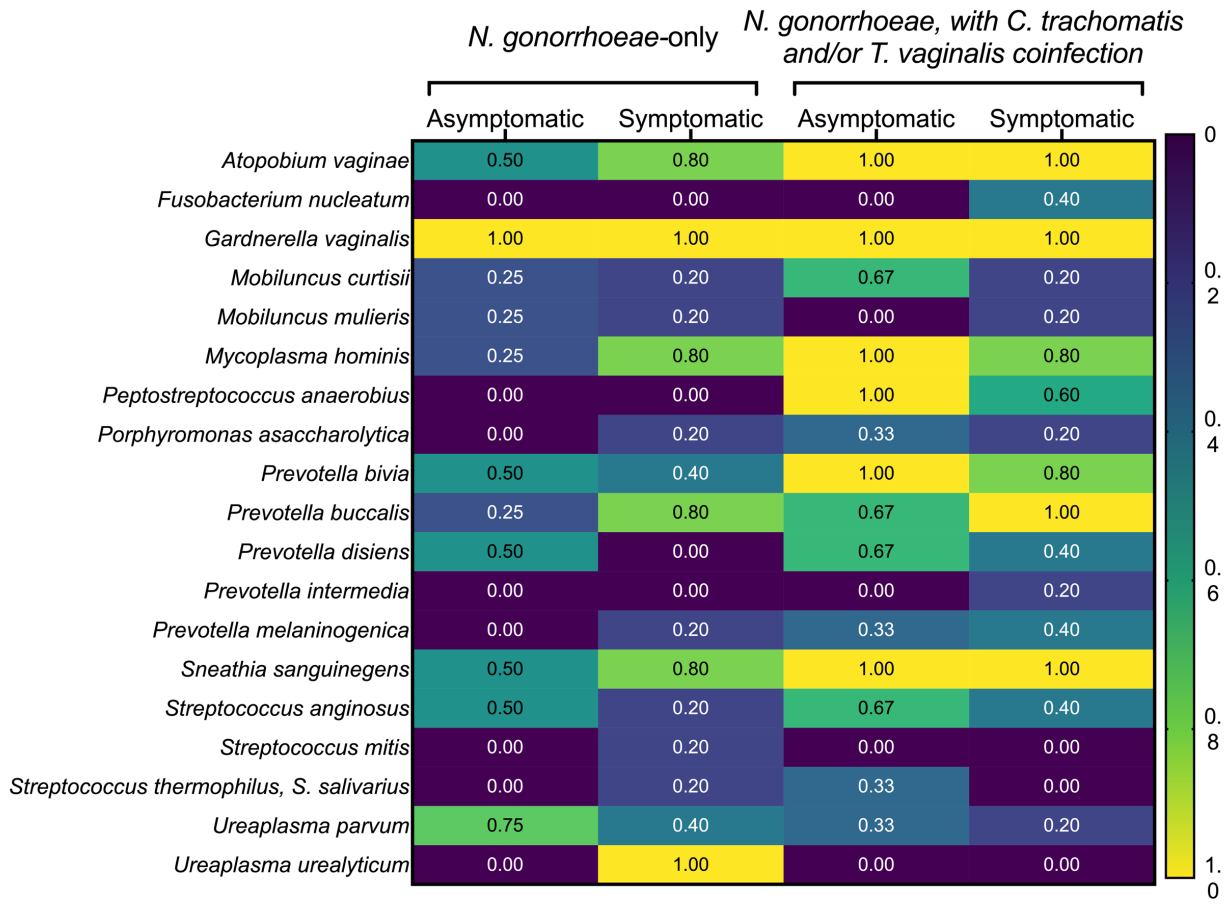


Figure 2.6 | BV-associated bacteria were found to be more prevalent among symptomatic *N. gonorrhoeae* infected individuals and in those coinfected with *C. trachomatis* and/or *T. vaginalis* coinfection. Percentage of individuals with detectable levels of BV-associated bacteria by real-time microbial DNA array.

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CHAPTER 3: USING THE MURINE MODEL OF *N. GONORRHOEAE* INFECTION TO UNDERSTAND THE IMPACT OF THE MICROBIOME ON PATHOGENESIS[3]

Introduction

Following the analysis of the cervicovaginal microbiome of *Neisseria gonorrhoeae*-infected women with and without symptoms, it was not clear whether symptomatic infection drove the reduced *Lactobacillus* abundance or whether pre-existing low *Lactobacillus* abundance mediated symptomatic infection. From our observations, we generated two hypotheses to further examine these questions. The first hypothesis we tested was whether the level of inflammation observed during symptomatic infection was capable of shifting the microbial diversity of the genital tract. The potential of *N. gonorrhoeae* to induce inflammation can be altered depending on what genes it expresses. Using two different strains of *N. gonorrhoeae*, one of which has reduced immune potential, allows us to test the hypothesis of whether differences in the microbiome can be observed post infection with both immunostimulatory and non-immunostimulatory *N. gonorrhoeae* infection models (hypothesis diagram). Humans are the natural host of *N. gonorrhoeae* and would have been the ideal for testing our hypotheses but the clinical samples previously collected were limited in sample size and don't allow longitudinal examination of the microbiome. An estradiol-treated female mouse model established previously by the Jerse lab has been used in a number of studies as a surrogate host for *N. gonorrhoeae*

[3] Portions of this chapter are currently submitted for review in Frontiers Microbiology and is available online as a preprint. The citation is as follows: Lovett, A, Seña A. C. Macintyre A. N., Sempowski G. D., Duncan J. A., and Waltmann A. Cervicovaginal microbiota predicts *Neisseria gonorrhoeae* clinical presentation. medRxiv 2021.10.07.21264698 (preprint)

genital infection (Jerse *et al.*, 2011). To test this hypothesis, the murine model of infection was utilized in combination with two strains of *N. gonorrhoeae* with different immune stimulating properties. Our lab has previously shown that a mutant strain of *N. gonorrhoeae* (Δ*msbB*) that makes pentaacylated LOS rather than hexaacylated LOS colonizes the mouse vagina similar to the wild type strain but displays reduced vaginal inflammation (Zhou *et al.*, 2014), mimicking asymptomatic *N. gonorrhoeae* infection in humans (Figure 3.1).

Adapting the murine model to reduce antibiotic effects on natural microbiome

The established murine model of infection entails using estradiol-treated mice in combination with antibiotics to promote infection. Mice are injected subcutaneously with estradiol (0.5 mg, days -2,0,2) and administered streptomycin sulfate, vancomycin hydrochloride, and trimethoprim sulfate throughout the infection period. The extent to which this antibiotic regimen given to the animals would affect their natural microbiome is unclear. Since we were interested in studying the effects of *N. gonorrhoeae* on the natural microbiome, we began by modeling *N. gonorrhoeae* infection without the antibiotic regimen. 4-6 week old BALB/c mice were inoculated with *N. gonorrhoeae* and vaginal swabs were collected daily to quantify CFUs and monitor genital tract colonization. Removal of the antibiotic regimen resulted in low genital tract colonization overall (~25%) and a shorter period of colonization (~2 days) when compared to mice given the full antibiotic regimen (Figure 3.2). From this, we concluded that the use of no antibiotics would not be feasible for collecting the samples we needed to study the vaginal microbiome in mice. To improve the number of mice colonized and length of time colonization while limiting the effects of antibiotics on the natural microbiome, we included only streptomycin sulfate from the previously established model during infection in our next set of experiments. Mice treated with estradiol and streptomycin had colonization levels comparable to

mice treated with the full antibiotic regimen. Confident in our ability to infect mice at a sufficient level while administering antibiotic regimen of solely streptomycin, we then set out to analyze the microbiome from mice infected with different immunostimulatory potentials.

Microbiome analysis limited by low DNA isolated from individual mouse swabs

Preparation of the 16s rRNA amplicon library requires at least 120ng of total DNA. Following DNA extraction individual mouse swabs were quantified to make sure they met the minimum threshold; however, the majority of samples fell below the limit (<50ng) (Figure 3.3). To determine if total DNA isolated from mouse samples also correlated with the amount of 16s DNA present in the samples, the amount of 16s rRNA DNA was quantified via quantitative PCR with 16s rRNA bacterial primers followed by UV spectrometry. No correlation was found between total DNA isolated from individual mouse swabs and the amount of 16s rRNA DNA present DNA (Figure 3.4). To determine if we could characterize the microbiome of mice that met the 120ng threshold, we processed those individual mouse swab samples through the Novogene 16s high throughput sequencing pipeline. Following DNA extraction, PCR amplification and library preparation, 16s amplicons were sequenced using Illumina paired-end platform to generate 250bp paired-end raw reads. In order to analyze the species diversity in each sample, sequencing reads were grouped by 97% DNA sequence similarity into OTUs (Operational Taxonomic Units). The top 10 OTUs were selected among the samples and used to from the relative abundance/taxa summary plot (Figure 3.5). Among the mice, *Staphylococcus* was the predominant genus present in their vagina and *Pseudomonas* was also found in each sample.

Pooling of individual mouse samples to overcome low biomass limitation

Over the limitations in DNA from individual mouse samples, we adopted a DNA pooling strategy. DNA from groups of mice infected with the same strain of *N. gonorrhoeae* were pooled together to meet library preparation thresholds for microbiome analysis. (Figure 3.6). To compare the effects of the wild type and *ΔmsbB* mutant strains of *N. gonorrhoeae* on microbial diversity of the genital tract, 4- to 6-week-old female mice BALB/c mice were infected with wild type 1291 or 1291 *ΔmsbB*. Prior to infection, bacterial strains were grown overnight (16 h), harvested and transferred into phosphate-buffered saline for murine inoculation. Bacterial suspensions were quantified by measuring the optical density at 600 nm (OD_{600}). Mice were inoculated with ~10^6 Colony forming units (CFU) of the indicated strains of *N. gonorrhoeae*. To quantify the actual dose of CFU delivered, serial dilutions of the bacterial suspensions were plated and counted.

Over the course of infection vaginal swabs were collected daily and suspended in PBS to quantify bacterial burden and prepare DNA extractions. DNA extracted from mouse vaginal swabs were extracted and quantified using UV and Quant-IT PicoGreen fluorescent assay. Samples collected one Day -1, Day and Day 3 (refer to schematic) of infection from mice treated with PBS or infected with 1291 WT or 1291 *msbB* strains were processed through the Novogene 16s high throughput sequencing pipeline. (Pooling Figure without lac mice). Prior to *N. gonorrhoeae* infection, we expected the microbiomes of the pools to be relatively similar across the different infection groups. The microbiomes were predominantly Staphylococcus. There was a low abundance of *N. gonorrhoeae* reads in the specimens prior to infection suggesting a DNA contamination issue, perhaps due to the possible exposure to environmental *N. gonorrhoeae*

DNA in the laboratory. DNA preparations from some mouse pools had comparable levels of Staphylococcus or Enterococcus present in their microbiome when compared to mouse pools from day 3. Other specimens resembled the negative control suggesting the quantity of microbial DNA in those specimens was lower than environmental contamination. Pooling mouse swabs together prior to DNA extraction did improve the biomass quantities to a level sufficient to continue with library preparation and sequencing but there are still technical issues that prevent meaningful interpretation of these data relative to our hypothesis (Figure 3.7).

Neisseria gonorrhoeae infection does not alter the lactobacillus abundance in a murine model of infection

From previous work done by others and from analysis of our data, we know that the diversity of the vaginal microbiome varies between humans and mice (Gosmann *et al.*, 2017; Vrbanac *et al.*, 2018). From analysis of our human data, we observed an association between asymptomatic infection and lactobacillus-dominant vaginal microbiomes. Mice do not carry lactobacillus naturally so before we could investigate whether lactobacillus abundance could be affected by *N. gonorrhoeae* infection in our mouse model, we had to produce an artificial lactobacillus microbiome in the murine model prior to infection with *N. gonorrhoeae* (Figure 3.8).Mice were pre-colonized with *L. crispatus* by vaginal inoculation on day -1 with an estimated 108 CFUs of *L. crispatus* prepared in PBS. Mice were subsequently infected with N. gonorrhoeae (1291 or 1291-ΔmsbB) by vaginal inoculation with an estimated 106 CFUs of the indicated N. gonorrhoeae strain in PBS on day 0. Vaginal swabs were collected daily from each mouse for 10 days following inoculation with N. gonorrhoeae. The quantity of *L. crispatus* was determined by plating of serial dilutions of the vaginal swabs on De Man, Rogosa and Sharpe (MRS) agar. The quantity *N. gonorrhoeae* was determined by plating of serial dilutions of the

vaginal swabs on Gonococcal Base (GCB) agar supplemented with Vancomycin, Colistin, Nystatin, Trimethoprim (VCNT) *Lactobacillus* colonization was maintained throughout the infection in all mice, regardless of *N. gonorrhoeae* infection status ((Figure 3.9). No difference was observed in *Lactobacillus* burden between uninfected mice and mice infected with either strain of *N. gonorrhoeae*. To test whether the presence of *L. crispatus* differentially impacted the ability of "symptomatic" or "asymptomatic" infection inducing *N. gonorrhoeae* strains to establish and/or maintain an infection in mice, we quantified the percentage of mice infected with wild type or the Δ*msbB* strain over the course of the 10-day infection period. The presence of *L. crispatus* did not have a significant effect on the ability of the mice to clear wild-type *N. gonorrhoeae* infection (wild type survival) or type *N. gonorrhoeae* infection with the mutant strain (Figure 3.9). Although there are caveats between mouse model and human our data suggest that the presence of *L. crispatus* is not impacted by *N. gonorrhoeae* infection and that prior colonization with L. crispatus does not have a differential effect on *N. gonorrhoeae* infection regardless of the capacity of the *N. gonorrhoeae* to induce localized inflammation in the female mouse model of lower genital tract infection.

We tested whether the level of *L. crispatus* colonization was impacted by either wild type or hypo-inflammatory *N. gonorrhoeae* infection in a murine model. However, no change in *L. crispatus* colonization was observed in mice infected with either n. gonorrhoeae strain. We also utilized this murine model to examine whether *L. crispatus*, which was only found in asymptomatic N. gonorrhoeae-infected humans, might reduce bacterial burden of wild type N. *gonorrhoeae* or support the expansion of hypo-inflammatory *N. gonorrhoeae* that could theoretically be associated with asymptomatic infections. We found no impact of *L. crispatus* pre-colonization on N. gonorrhoeae burden from either strain. Because the murine model of *N.*

gonorrhoeae infection may not fully reflect pathogenesis of *N. gonorrhoeae* infection in humans, future studies examining the microbiome longitudinally during infection are needed to provide insight into whether *N. gonorrhoeae* can influence the genital microbial composition of the host or whether pre-existing microbial composition can influence disease presentation.

Discussion

From our previous analysis of the cervicovaginal microbiome of *N. gonorrhoeae* infected women we observed associations between symptomatic infection and a reduced lactobacillus abundance. Due to the limitations of the study, it was still not clear whether symptomatic infection drove the reduced *Lactobacillus* abundance we observed or whether pre-existing low *Lactobacillus* abundance mediated symptomatic infection. Ideally, these questions could be answered in a longitudinal study in women, but for both ethical and logistical reasons make that study quite challenging. Using the next best option, the female murine model of infection, we set out to determine whether differences observed in the immune potential of *N. gonorrhoeae* modulated the natural microbiome during infection. Adapting the murine model to study the natural microbiome came with a number of technical challenges and in our data was insufficient to draw any conclusions on *N. gonorrhoeae's* potential to alter the natural microbial community in the mouse vagina. To attempt to overcome the challenges of studying the natural microbiome, we decided to introduce a lactobacillus containing microbiome in the murine to allow us to directly access impact of *N. gonorrhoeae* infection on lactobacillus abundance. From our analysis of the artificial microbiome in mice, we found that *N. gonorrhoeae* infection does not increase or decrease the abundance of lactobacillus recovered from the vagina. The current mouse model may not reflect what we observed in the human vagina. It does not take into account the *Lactobacillus* predominance of a vaginal microbiome. *L. Iners* was the predominant

lac species observed in our *N. gonorrhoeae* infected women, our model using *L .crispatu*s also doesn't take into account the capacity of *N. gonorrhoeae* to alter abundance of different species of lactobacillus.

We have yet to test an alternative hypothesis that the level of inflammation observed during infection is driven by the microbiome of the genital tract. If the inflammation observed during infection is dependent on the preexisting microbiome when *N. gonorrhoeae* encounters a pro inflammatory microbiome, it promotes more inflammation at the site of infection and can lead to the development of symptomatic infection. However, if *N. gonorrhoeae* was to encounter a microbiome that was predisposed to be anti-inflammatory, this would reduce the amount of inflammation observed during infection and lead to the development of an asymptomatic infection (Figure 3.10). Data from human analysis suggests that lactobacillus predominant microbial communities could suppress immune response or diverse anaerobic microbial communities could promote inflammatory responses during infection. There is evidence lactobacillus can suppress inflammation in other disease models (Kim *et al.*, 2019; Li *et al.*, 2020). Experiments analyzing inflammation kinetics using the artificial microbiome models could provide insight into whether or not lactobacillus can modulate *N. gonorrhoeae* induced immune responses. Pre-cololnization with either Lactobacillus or BV associated bacterial species prior to infection, followed by the quantification of neutrophil influx or cytokine secretions would allow us to determine whether microbial communities alter *N. gonorrhoeae* induced immune responses and presumably symptoms.

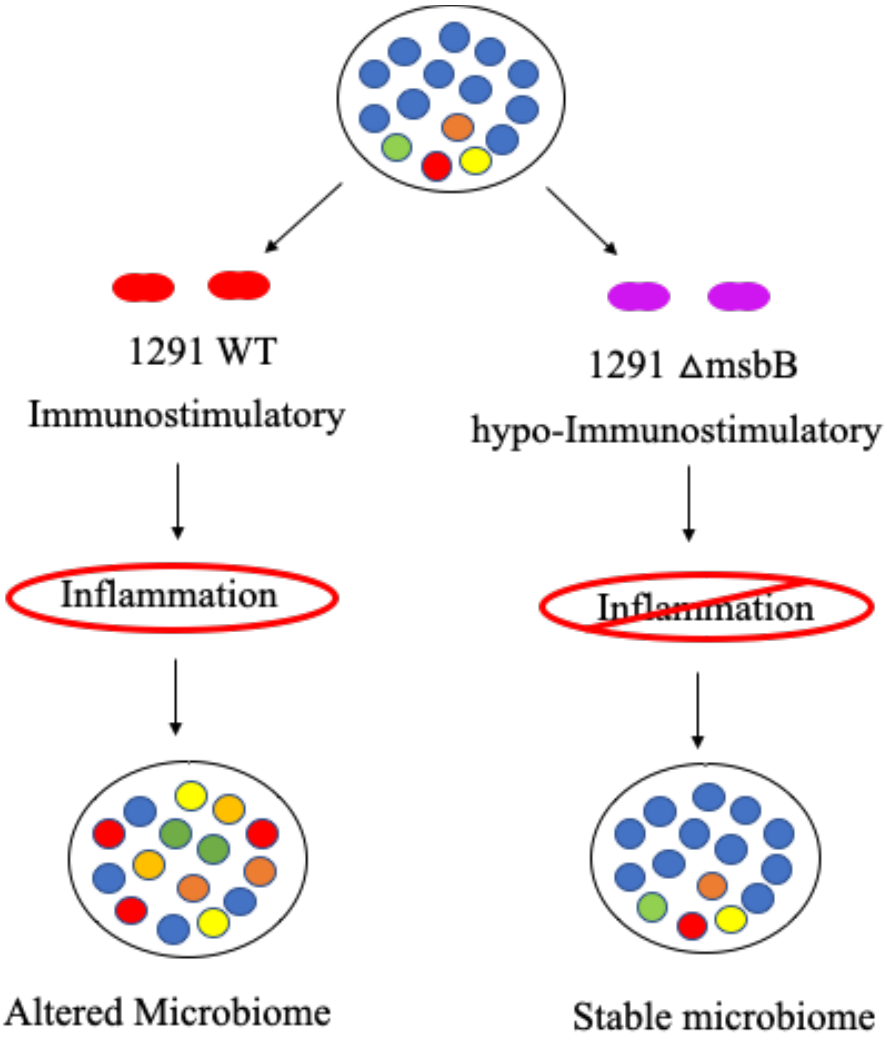

Figure 3.1 | A model of *N. gonorrhoeae* infections with immunostimulatory and hypo-immunostimulatory strains highlighting the potential of each to alter the cerrvicovaginal microbiome. In the inflammatory model (1291 WT) inflammation induced during infection increases the diversity of the microbiome. In or non-inflammatory model (1291 *msbB*), inflammation is reduced and the diversity of the microbiome remains stable following infection.

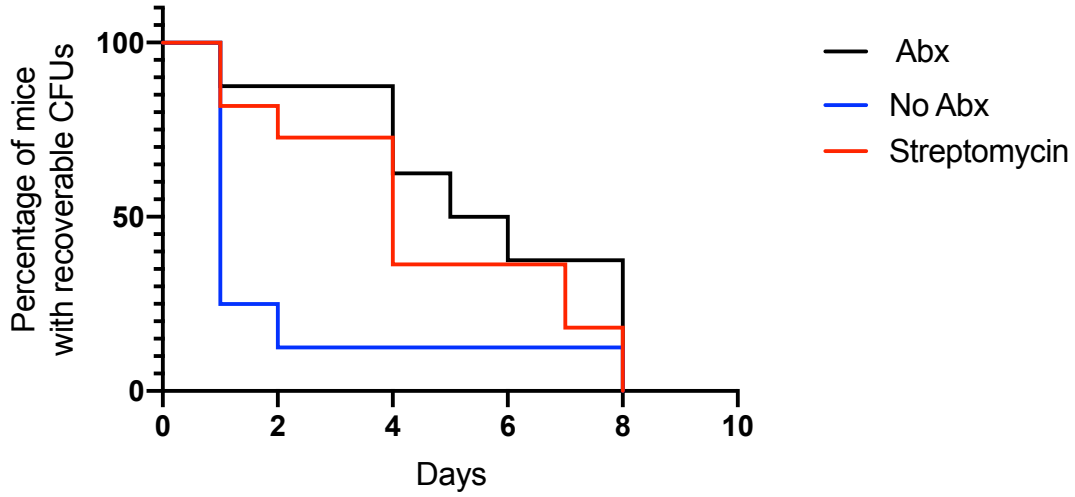

Figure 3.2 | Streptomycin sulfate infection model is sufficient to study microbiome with reduced antibiotic effects. The average time to clearance of *N. gonorrhoeae* strain 1291 in infected mice treated with antibiotics (streptomycin sulfate, vancomycin hydrochloride, trimethoprim sulfate), Streptomycin Sulfate alone, or no antibiotics (No Abx) .

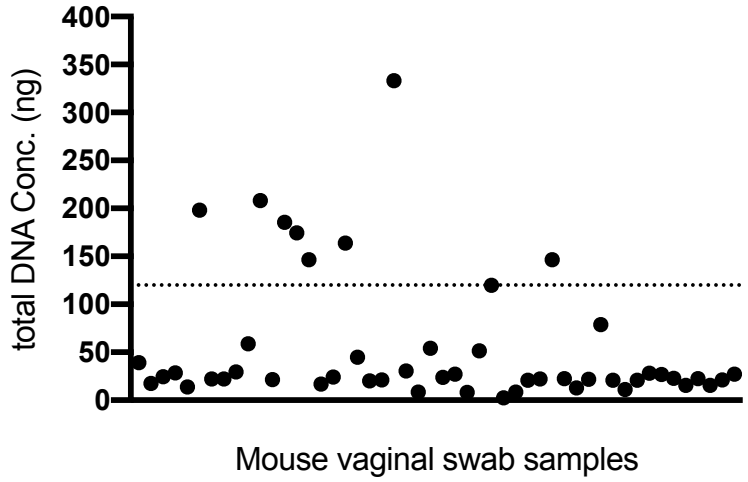

Figure 3.3 | DNA isolated from mouse vaginal swabs is limited. The total DNA isolated from mouse swabs for microbiome analysis.

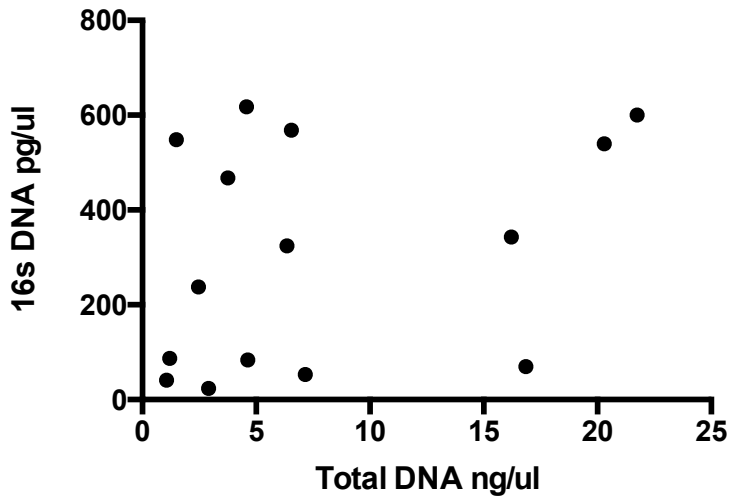

Figure 3.4 | No correlation between total DNA isolated from mouse vaginal swabs and 16s DNA The total DNA isolated from mouse swabs plotted against 16s DNA concentration from the same sample.

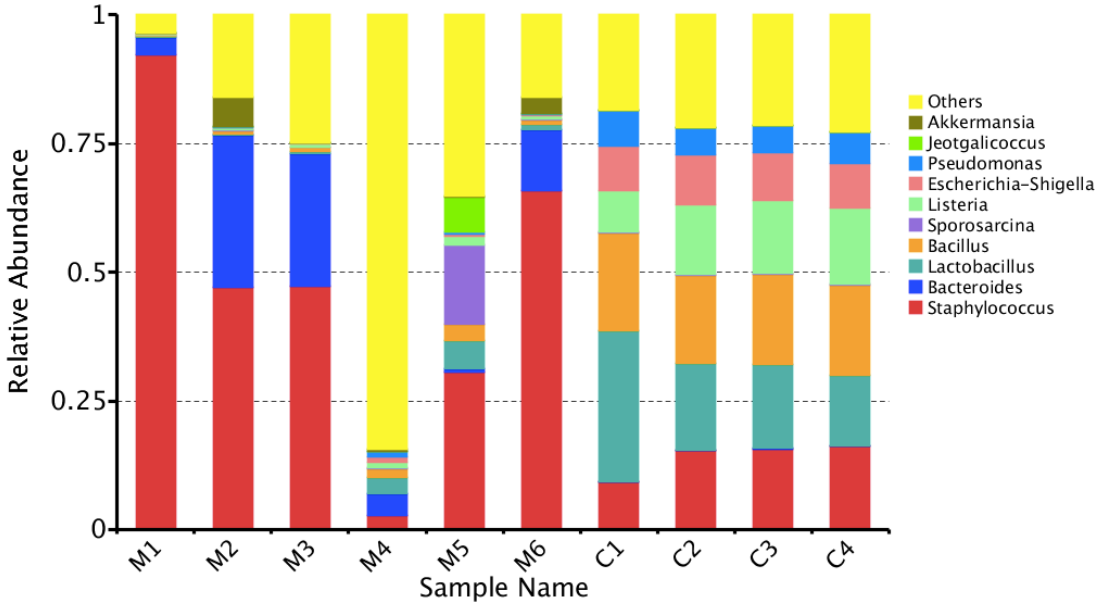

Figure 3.5 | Microbial composition of mouse vaginal swabs and microbial community standards. Taxa summary plot displaying *the* mean relative abundance of the top 10 bacterial genera identified among the six mouse swabs and four microbial community standards via 16s rRNA sequencing.

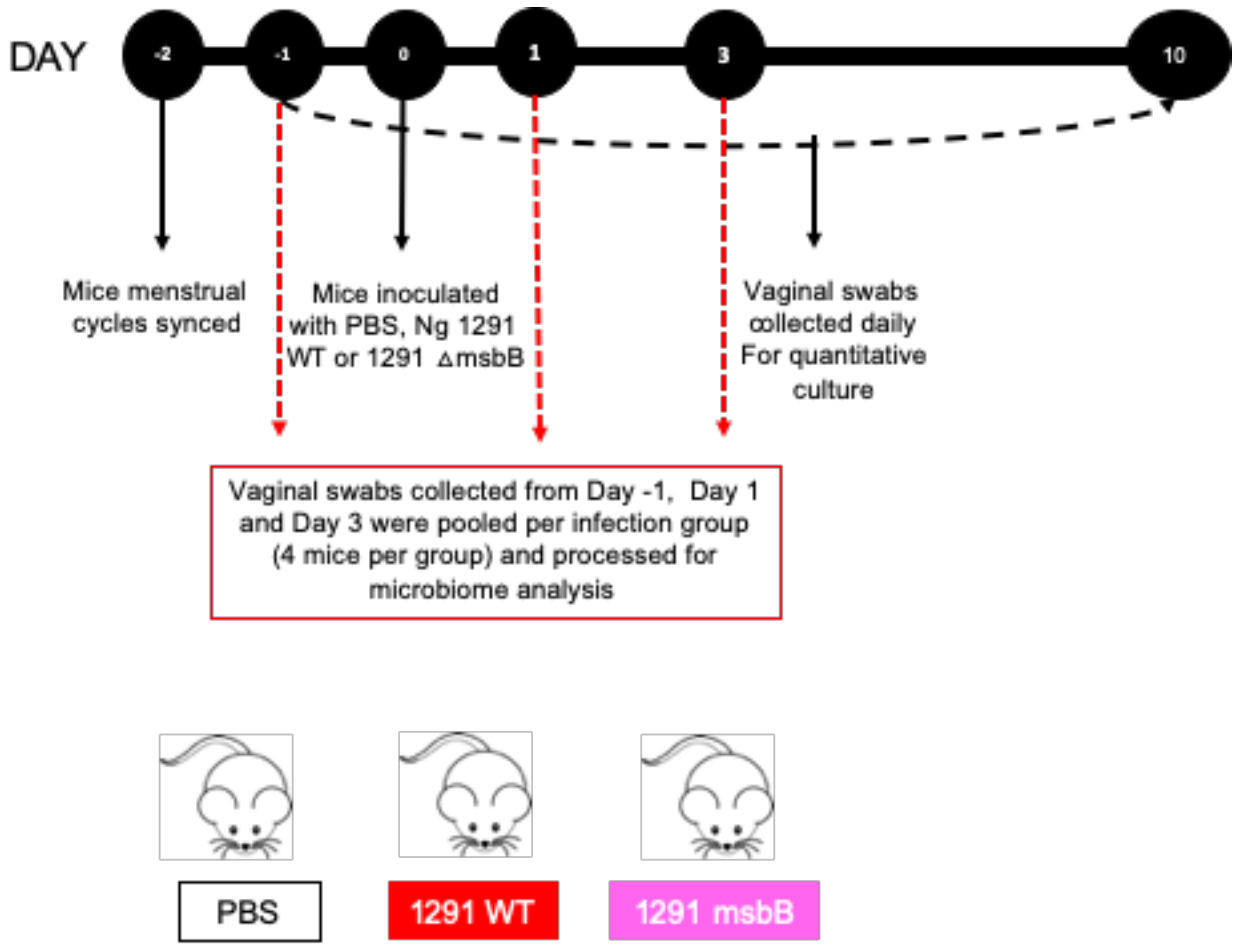

Figure 3.6 | Schematic of the infection protocol for microbiome analysis. Estrous cycles for mice are synced two days prior to inoculation with either PBS, *Ng* 1291 WT or *Ng* 1291 Δ*msbB*. Vaginal swabs are collected daily throughout the course of infection. For purposes of microbiome analysis, vaginal sabs from day -1 ,day 1 and day 3 were pooled to meet 16s rRNA gene sequencing library preparation DNA concentration limits.

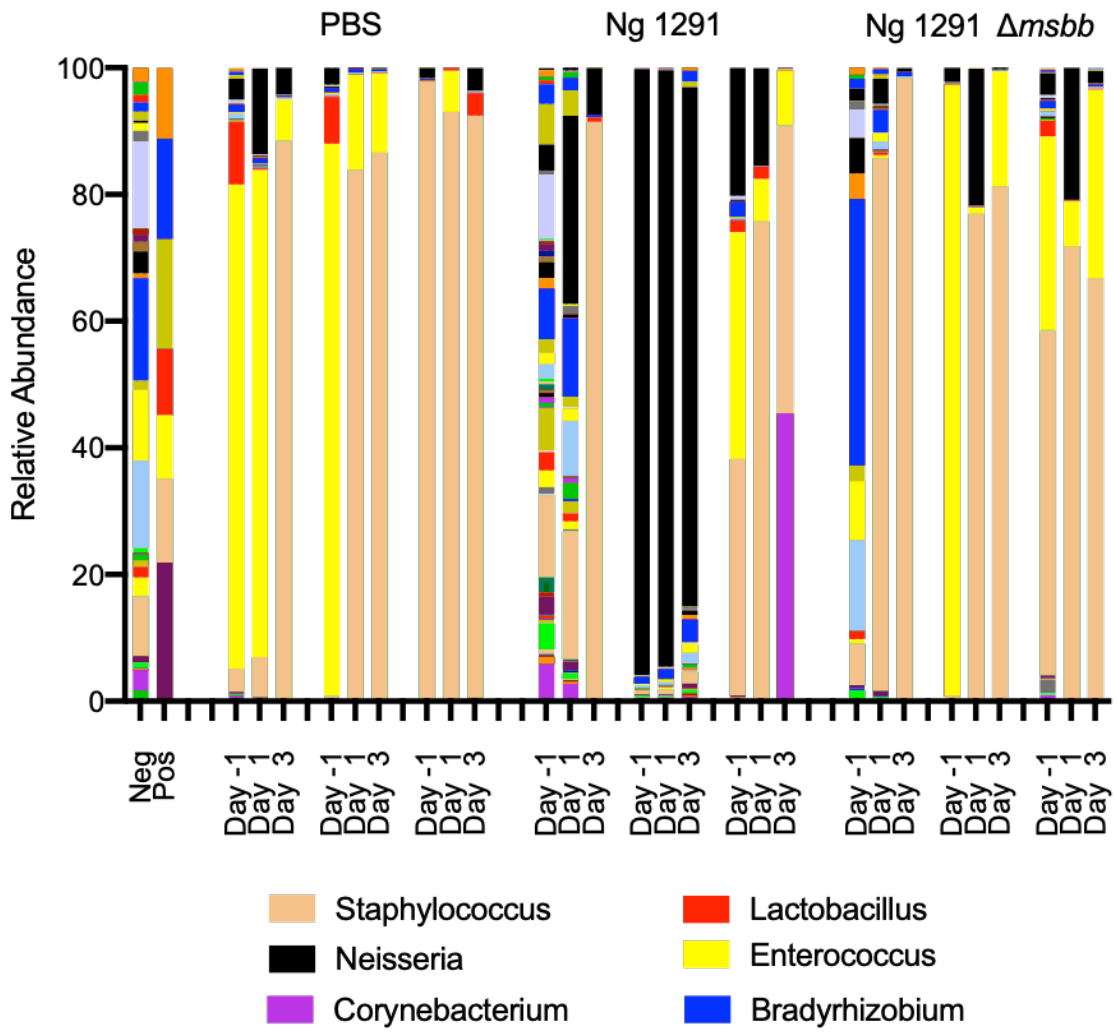

Figure 3.7 | Microbial composition of mouse vaginal swabs following infection with either PBS, *Ng* 1291 WT or *Ng* 1291 ΔmsbB. Taxa summary plot displaying *the* mean relative abundance of bacterial genera identified via 16s rRNA sequencing among mouse swab pools from Day -1, Day1 and Day 3 from three separate experiments. A microbial community standard (Pos) and PBS swab sampled (Neg) were included from sequencing controls.

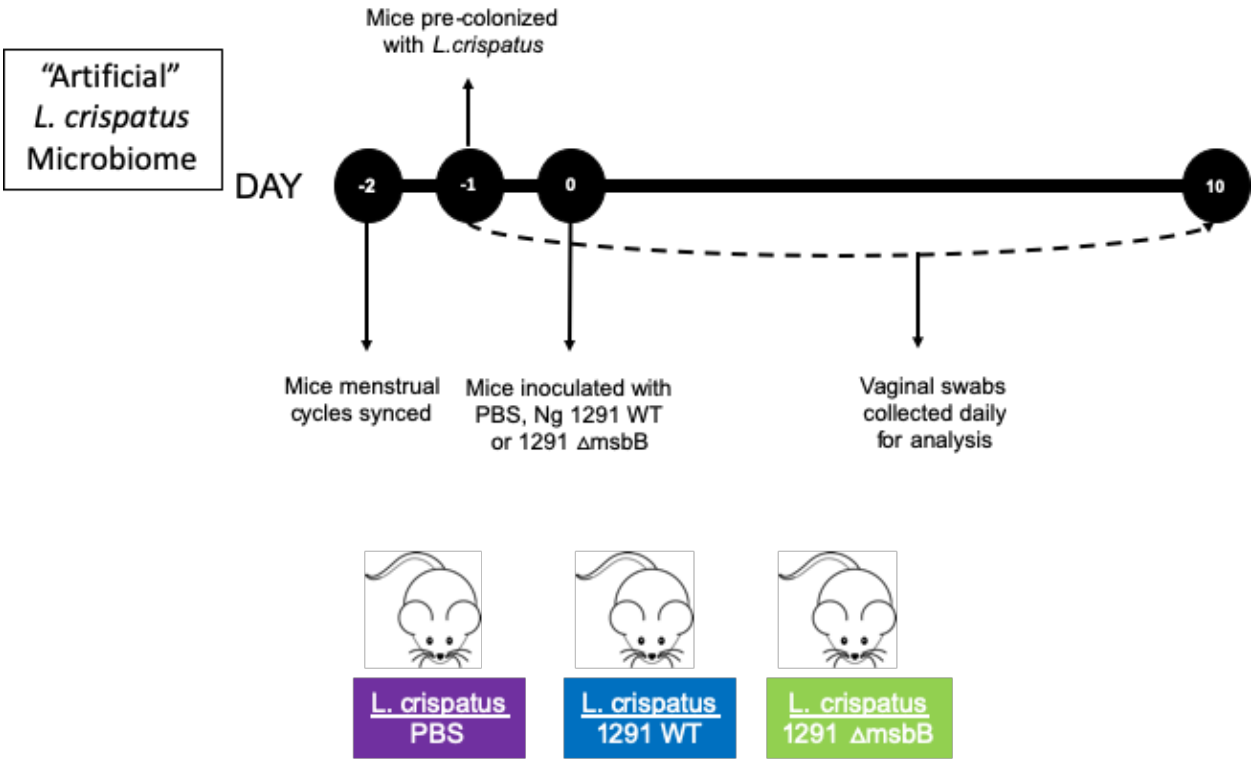

Figure 3.8 | Schematic of the infection protocol for the artificial microbiome analysis. Mice are inoculated with *L. crispatus* a day prior to inoculation with either PBS, *Ng* 1291 WT or *Ng* 1291 ΔmsbB. Vaginal swabs are collected daily throughout the course of infection. For purposes of microbiome analysis, vaginal sabs from day -1 ,day 1 and day 3 were pooled to meet 16s rRNA gene sequencing library preparation DNA concentration limits.

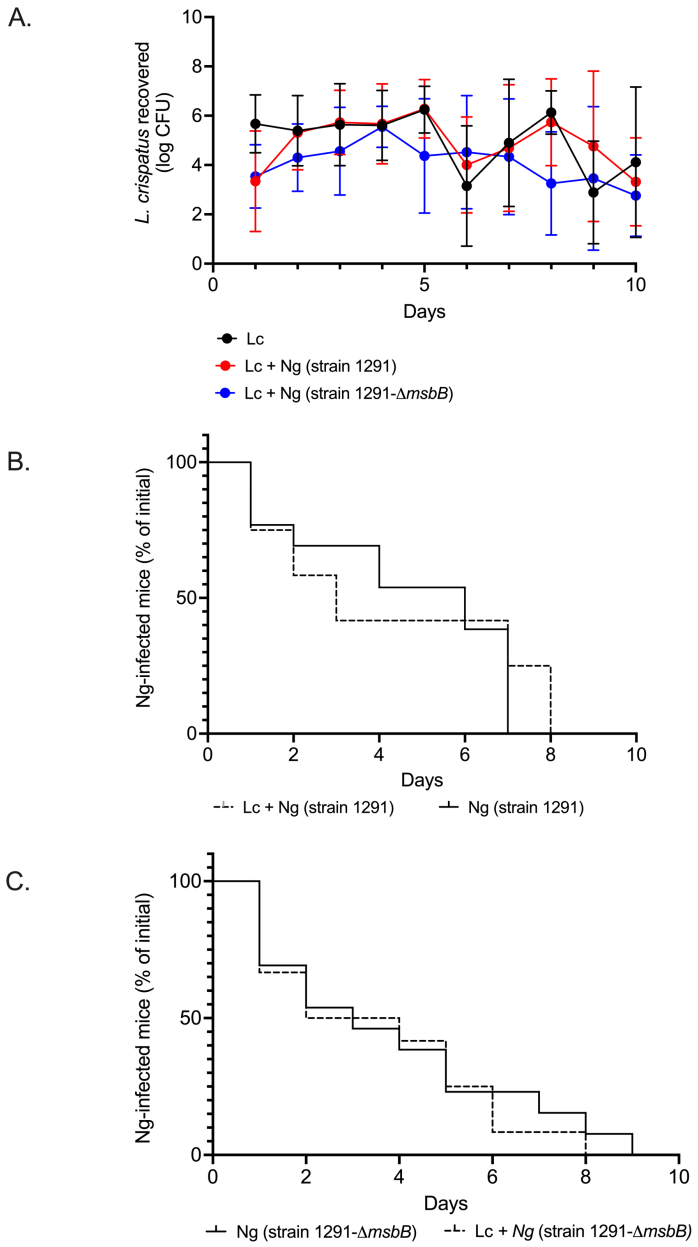

Figure 3.9 | The abundance of *Lactobacillus* is not affected by *N. gonorrhoeae* infection and does not impact *N. gonorrhoeae* clearance in the murine model.
Groups of 4-5 mice were precolonized with *L. crispatus* and then inoculated with either N. gonorrhoeae strains 1291 (wild-type, wt) or its isogenic strain lacking MsbB (1291-Δ*msbB*), results represent cobined results from three independent experiments (n=3)

(A) The average *L. crispatus* colony forming units (CFUs) recovered from daily vaginal swabs.

(B) The average time to clearance of *N. gonorrhoeae* strain 1291 in infected, estradiol-treated mice .

(C) The average time to clearance of *N. gonorrhoeae* strain 1291 Δ*msbB* in infected, estradiol-treated mice.

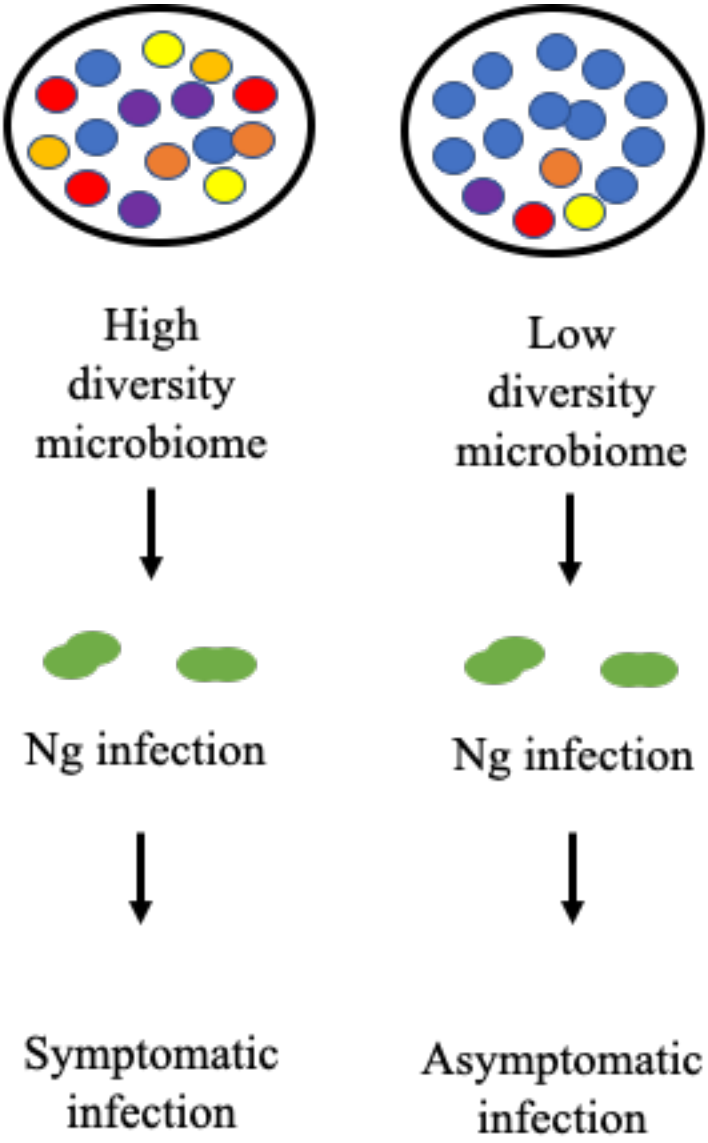

Figure 3.10 | A model of *N. gonorrhoeae* infections in individuals with either high diversity or low diversity microbiomes highlighting the potential to induce symptoms. Data collected thus far supports the hypothesis that microbial diversity of the genital tract can influence the development of symptoms.

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