Bacterial interactions in the female genital tract

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GENERAL INTRODUCTION

Every person on earth enjoys the presence of millions upon millions of bacteria that are present on, or even in, his or her body. These living creatures not only interact with the person they are found on, but also with each other. This interaction can take the form of infection, symbiotic relationship, or commensal colonization of the host. A shift in environmental characteristics, can equally shift the nature of this relationship from beneficial to pathogenic.

This thesis will focus on the female genital tract and the interactions occurring therein. The first bacteria to come to mind that interact with the host at this anatomical site, are the commonly known sexually transmitted infections (STI) like Chlamydia and Gonorrhoea. However, there are a number of less commonly known STIs that proof to be unique and interesting subjects of study into host-pathogen interaction. A selection of these STIs are subject of study in this thesis. Much more commonly present in the female genital tract are the bacteria comprising the vaginal microbiome. These bacteria are consistently in contact with the host, often living in a state that is mutually beneficial.

The host itself is also a focus in this thesis. The host immune response affects bacteria in the female genital tract most significantly. Because of this, we look into the genetic basis of immune related genes and their effect on infections at this anatomical location. The expression of cytokines is another subject of interest. There are a number of mitigating factors that might come into play between the transcription of genetics and the actual expression of immune related genes, which could result in different expression than is expected.

Providing better insight into the various interactions between the pathogens, the microbiota, and the host will not only lead to a better understanding of how things work on a molecular level, it is also a requirement for the development of new treatments and therapies. Considering the function of the female genital tract, more knowledge on the subject of vaginal microbiota and pathogen interaction with the host may lead to new breakthroughs in treatments for subfertility, and work as a supporting force for the creation of healthy pregnancies.

CONTENTS

1. Inducing inflammation: the innate immune system

When a pathogenic bacterium enters a healthy human host, a healthy immune system interacts with this invasion in a manner that is both effective and not too harmful to the host body. This interaction consists of two parts: the innate immune system and the adaptive immune system. The first phase of the immune response is the responsibility of the innate immune system, which includes proper recognition of the invading pathogen. This recognition is the function of the four groups of so called Pattern Recognition Receptors (PRR): Toll like receptors (TLR), NOD like receptors (NOD), RIG-I-like receptors, and C-type lectin receptors (1-5), which are present in and on the host cell. All these PRR groups contain a number of PRRs which recognise specific pathogen associated molecular patterns (PAMPs) unique to that specific PRR. The PRRs that are most significant for the detection of bacteria related to the female genital tract and their respective PAMP targets are listed in Table 1. PRRs can also bind to damage associated molecular patterns (DAMP), however, these are not related to the infections covered in this thesis.

The main difference between TLRs and NODs is the location of their expression (6). TLRs are transmembrane proteins, and are generally expressed on the surface of the cell. An exception to this localization is the presence of three TLRs in endosomes of the cell, with TLR9 being the most significant TLR expressed in the endosome membrane for the detection of bacteria studied in this thesis. In contrast, NODs are found in the cytoplasm of the cell. Here the NODs act as a scaffold for the creation of an inflammasome at the time of binding with their respective PAMPs.

Table 1: Pattern recognition receptors most significantly related to bacterial detection and their main ligands.

PRR	Main ligand
TLR1	Bacterial lipoproteins
TLR2	Bacterial peptidoglycans
TLR4	Lipopolysaccharide
TLR5	Bacterial flagellin
TLR9	Unmethylated CpG DNA
NOD1	Peptidoglycans
NOD2	Peptidoglycans

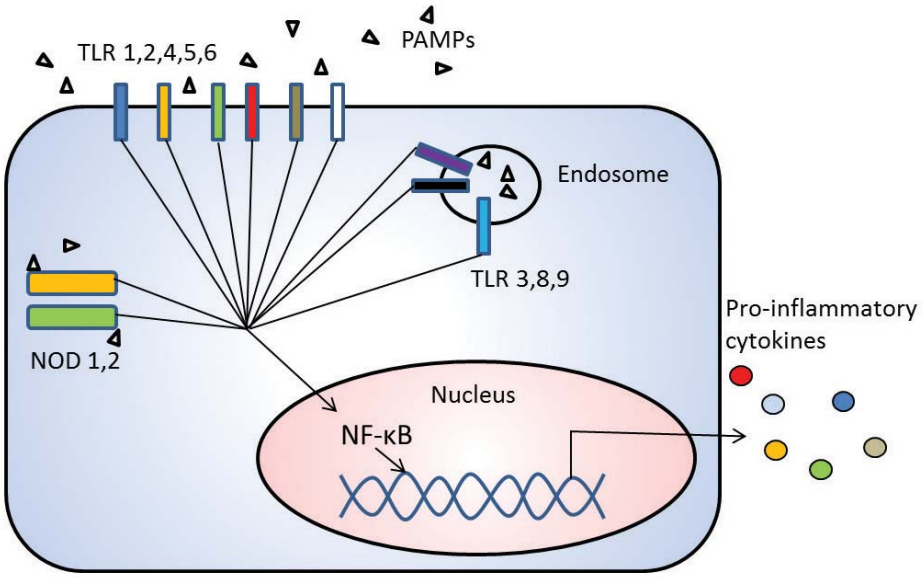

Figure 1: Distribution and localization of pathogen recognition receptors. TLRs and NODs are stimulated by their specific ligands, after which they initiate complex pathways which all end at the transcription factor NF-κB. NF-κB transcribes DNA for production and secretion of pro-inflammatory cytokines. PAMPs: Pathogen associated molecular patterns.

One major similarity between TLRs and NODs is the eventual outcome of pattern recognition. When a receptor is stimulated, a molecular signalling pathway is activated which ends at the transcription factor NF-κB (7). Among other effects this transcription factor enables expression of a number of pro-inflammatory cytokines, leading to the early signs of immune response (inflammation and attraction of innate immune cells). Additionally, it leads to the initial steps of activating the adaptive immune response for further response to the infection.

2. The variety in immune response to vaginal pathogens

There is a wide variety of bacterial STIs that can plague the female reproductive system. Symptoms for these diseases are often directly linked to the host immune response aimed at the pathogen, and tissue damage resulting from the infection. This can vary from light inflammation and itchiness, to infertility and miscarriage. In this thesis we cover a part of the host interaction with common bacterial STIs: *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum*, and *Haemophilus ducreyi*. Although these are all bacterial STIs, the host immune responses to the different pathogens differ strongly.

C. trachomatis is the cause of Chlamydia, and is the most common bacterial STI in the world. In 2012 it was shown that every year there are approximately 130 million new cases of infection with *C. trachomatis*, and despite efforts to reduce this number Chlamydia remains highly prevalent (8). *C. trachomatis* is an intracellular bacterium, and the localization of *C. trachomatis* in the endosome of the cell means that for a large part of the infection only a selection of PRRs can make contact with the pathogen. The significant role of TLR9 in chlamydial infections has been shown in previous studies, corroborating this claim (9). Additionally, the surface of *C. trachomatis* cells is largely made up of lipopolysaccharides (LPS) which stimulate the TLR4 receptor. Therefore TLR4 has a central role in the immune response to *C. trachomatis* (10).

Similarly to *C. trachomatis*, *N. gonorrhoeae* is highly prevalent around the world, with approximately 78 million new cases of infection every year (8). Infection with *N. gonorrhoeae* causes gonorrhoea, a well-known sexually transmitted disease. *N. gonorrhoeae* shows an interesting interaction with TLR9, with which it interacts after phagocytosis of the bacteria. The lysis of the bacteria allows binding of the unmethylated CpG DNA to TLR9. Due to the makeup of the unmethylated CpG DNA of *N. gonorrhoeae*, TLR9 is less efficient in the recognition of *N. gonorrhoeae* (9). It is likely that this is part of the defence of the bacteria to suppress the immune response of the host, as *N. gonorrhoeae* is found in the extracellular space, and is therefore vulnerable to immune activity. Indeed, a number of studies have shown that *N. gonorrhoeae* actively suppresses signalling related to inflammatory signalling in the immune response (11, 12).

T. pallidum causes syphilis. Although untreated syphilis can lead to severe neurological symptoms and even death, the overall clinical burden caused by syphilis has reduced significantly

due to a severe drop in cases. Currently, the prevalence in Europe is approximately 0.005% (13). *T. pallidum* is an interesting pathogen with regards to pathogen detection in the host. The bacterium is Gram-negative, which usually means that there is LPS in the membrane of the bacterial cell for TLR4 to recognise. However, in practice *T. pallidum* does not appear to interact with TLR4. Rather it interacts with TLR2, which is a receptor that mainly targets Gram-positive bacteria through recognition of the associated glycolipids and lipoteichoic acid (14, 15). *T. pallidum* has specific membrane lipoproteins that interact with TLR2, which is uncommon for gram-negative bacteria (16).

H. ducreyi causes the lesser known chancroid. This is typically an ulcer on or around the genitals which, if left untreated, can begin to necrotise. Unlike the previous three well studied pathogens, the exact interactions between *H. ducreyi* and host PRRs is not entirely understood. The ulcer-based nature of the symptoms and previous immunological studies downstream from the PRRs do emphasize how impactful the innate immune system is in creating a proper response to infection with *H. ducreyi* (17, 18).

The variety between individuals in immune responses to infections is not only caused by the invading bacteria. Host genetics have a significant impact on the immune response as well. The most notable way host genetics can have this effect is through polymorphisms, which are host specific. Single nucleotide polymorphisms (SNPs) are point mutations that are present in a significant part of the population. A point mutation means that a single base pair in the host DNA is affected by a nucleotide change. For example, where in most of the Dutch population people have a C nucleotide at a given base pair location, a person with a SNP at this location may have an A nucleotide instead. When this genetic data is translated, it may end up affecting the expression, shape, and/or function of the related genes. A schematic overview of this process can be seen in Figure 2. This may in turn affect the ability of the host to respond to infections. As an example of this, a combination of SNPs, called a haplotype, in the gene coding for TLR9 showed effects on susceptibility and development of late complications related to *C. trachomatis* infections (9).

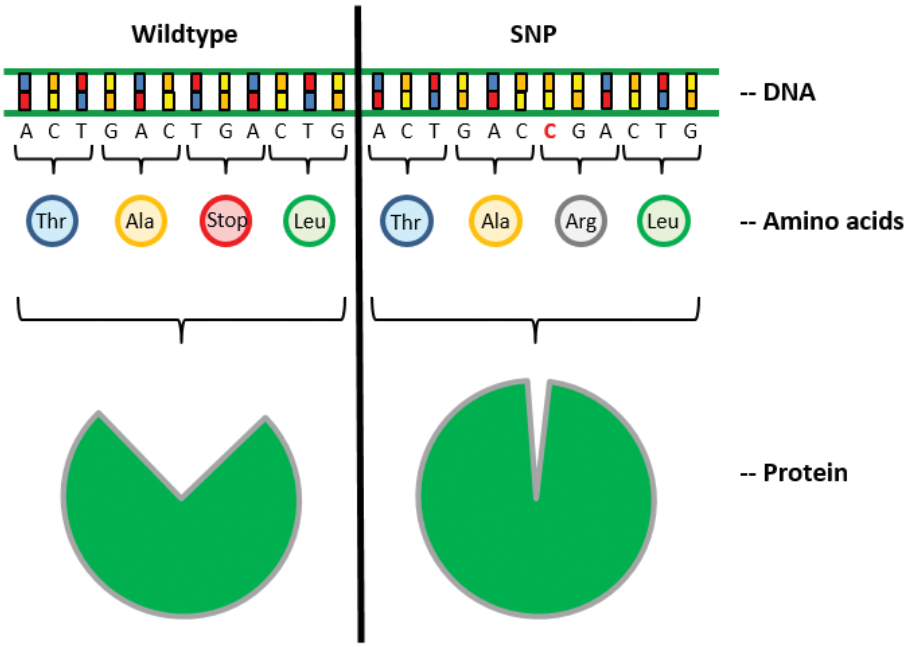

Figure 2: The effect of a Single Nucleotide Polymorphism (SNP) on the functionality of a protein. At the top of this figure two strands of DNA are shown. One with a wildtype DNA composition, and one where a single nucleotide has been altered in the form of a SNP. This SNP is marked as the red C nucleotide. In this case, the SNP causes a difference in the amino acid sequenced derived from the DNA, as the wildtype stop codon is replaced by an arginine amino acid. A difference like this can lead to a vastly different protein structure, influencing the function of the protein greatly. It should be noted that an altered protein structure does not always lead to reduced effectiveness.

Other SNPs can result in differences in, for example, recognition of invading bacteria, cytokine expression, and thus the host's ability to clear infections. Besides currently known genetic factors which are often related to the most relevant PRR pathways of the specific bacteria, there are likely to be more genetic factors to be discovered in the future, especially for a less studied pathogens such as *H. ducreyi*, which is further studied within this thesis. In addition, genetic differences between bacteria can also affect the interaction between PRRs and bacteria. A study of bacterial genetic sequences related to the interaction with TLR9 is therefore also included in this thesis.

3. Profiling the vaginal microbiome

Besides individual bacteria contributing to infection, large amounts of bacteria are present on and in the host. These are often not harmful, and even beneficial to the host in some way. The complete collection of bacteria inhabiting an anatomical site is called the microbiome.

The recognition of the microbiome and the way it is characterized has been the subject of much change, especially with the advent of molecular characterization techniques. Initial full microbiome characterization attempts used a mixture of culturing and 16s ribosomal RNA analysis (39;40). This was used mostly to examine the diversity of the bacteria making up the microbiome, and to create a general overview of the phylogenetic characteristics. This technique proved lacking for characterization of vaginal microbiomes when it was found that bacteria making up the microbiomes that have high impact on the vaginal health were often difficult to cultivate, and some even proved entirely uncultivatable (41;42).

The introduction of PCR allowed for the rapid amplification of the 16s gene and, when combined with hybridization techniques, made microbiome characterization attempts faster and easier (43). However, before a PCR could be done the bacteria needed to be cultured, meaning the drawbacks of cultivation were still there. The advent of metagenomics sought to solve this problem by genetically characterizing the vaginal microbiota without first culturing it (44). In this approach, the 16s rRNA of the bacteria is sequenced using next generation sequencing techniques such as pyrosequencing or Illumina (Solexa), sequencing directly on a microbiome sample. More recently, a high throughput method of analyzing human microbiota was developed called the IS-pro. This technique combines differentiation accuracy with taxonomic classification using phylum specific PCR primers and fluorescent labels, making it more suitable for clinical settings than other next-generation sequencing techniques (45). Besides IS-pro, sequencing is also being developed further. So called 3rd generation sequencing techniques, are currently in their infancy, primarily being used for specialized cases (19)4. However, these show enormous potential in increasing the amount that can be sequenced regarding both reads and samples, and lowering the time this takes significantly. A timeline showing the usage of the techniques is shown in Figure 3.

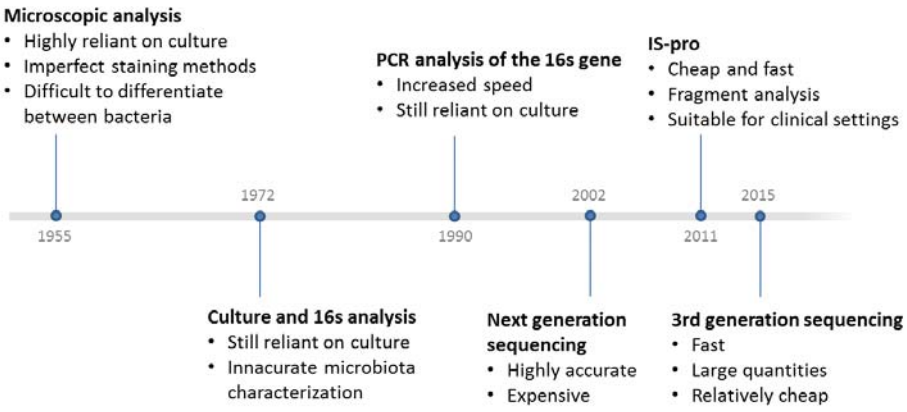

Figure 3: Timeline depicting the onset of molecular characterization tools for microbiome.

4. Characteristics of the vaginal microbiome

The vaginal microbiome plays an important part in the host immune system. Consisting of commensal bacteria living in a symbiotic fashion with the host, it provides a mechanical barrier shielding the vaginal epithelial layer from potential pathogens. A vaginal microbiome in a healthy state has a single anaerobic *Lactobacillus* as the dominant presence (20). *Lactobacilli* have the capacity to acidify the vaginal environment through production of lactic acid, which works against any opportunistic pathogens seeking to infect the vagina. *Lactobacilli* also generally produce H_2O_2, and bacteriocin substances which aid in the protection against invading pathogens and make sure that *Lactobacilli* remain the dominant presence (20-23). The most common kinds of *Lactobacillus spp.* are *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii.* The microbiome compositions dominated by these *Lactobacilli* have been labeled by Ravel *et al.* as Community State Type (CST) I, II, III, and V, respectively (24-27). CST IV is defined as a diverse microbial profile where no *Lactobacillus* has a significantly dominant presence over the other present bacteria. CST IV is also linked to Bacterial Vaginosis (BV) (26). Besides these most commonly found microbiome profiles, there are a number of different *Lactobacilli* that can also be found in the healthy vaginal microbiome, primarily including: *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. casei*, *L. delbrueckii*, *L. salivarius*, and *L. vaginalis* (26). It is important to note however, that *Lactobacilli* are not the only bacteria that can be found in a healthy vaginal microbiome, as low amounts of anaerobic bacteria such as *Prevotella*, *Megasphaera*, *Gardnerella vaginalis*, and *Atopobium vaginae* can colonize the vagina without leading to an unhealthy state (22, 26).

During pregnancy the vaginal milieu changes. An increased level of oestrogen during gestation results in an increased production of vaginal glycogen, which is an environment that benefits *Lactobacillus spp.*. Unsurprisingly, this environment leads to an increase in microbiome profiles featuring *L.vaginalis, L.crispatus, L.gasseri, L. iners,* and *L. jensenii* in pregnant women (28-31). Any change in the vaginal microbiome that occurs during pregnancy almost exclusively goes from domination by one *Lactobacillus* species to another (31, 32). Additionally, non-*Lactobacillus* species hold significantly lower presence in pregnant women (24, 28, 31). The increased abundance of *Lactobacillus spp.* during pregnancy may be one way the female body attempts to prevent non-indigenous bacteria in the vagina from colonizing and to prevent bacterial vaginosis (BV), infections, or preterm birth (24).

Besides pregnancy, the sexual phase of a woman's life is also a factor in the composition of the vaginal microbiome. Before a woman is sexually active, the vaginal microbiome is more stable and less prone to infection and overgrowth (33, 34). This is characterized by the low amounts of BV detected in women before they become sexually active (33). Once women are of reproductive age, the menstrual cycle does not appear to cause significant change to the vaginal microbiome, although other studies emphasize that this effect is not entirely certain yet(22, 35). Post-menopausal women do have a significantly changed vaginal microbiome, as dryness and tissue atrophy are cause for a decrease in *Lactobacilli* (36).

Ethnicity is also a distinctive factor for vaginal microbiome profiles. Figure 4 shows a comparison of multiple studies that used next-generation sequencing to characterize the vaginal microbiome and label CST diversity in hosts of various ethnicities. It clearly shows varying levels of CST abundance, and even CST presence in differing populations. Hispanic, African, and African American women were found to have less abundance of the dominant *Lactobacillus spp.* and a higher average pH level compared to Asian and Caucasian women (26, 37, 41). Perhaps as a consequence of these vaginal microbiome characteristics, ethnicity is linked to pre-term birth, the prevalence of vaginal inflammation, prevalence of STIs, and fertility problems such as miscarriage in several studies (39, 41, 42). The differences in the vaginal microbiome between ethnicities also exist in pregnant women, raising concern for populations with a relatively large abundance of BV linked microbiome profiles, as this would imply a higher risk for adverse pregnancy outcomes (43, 44).

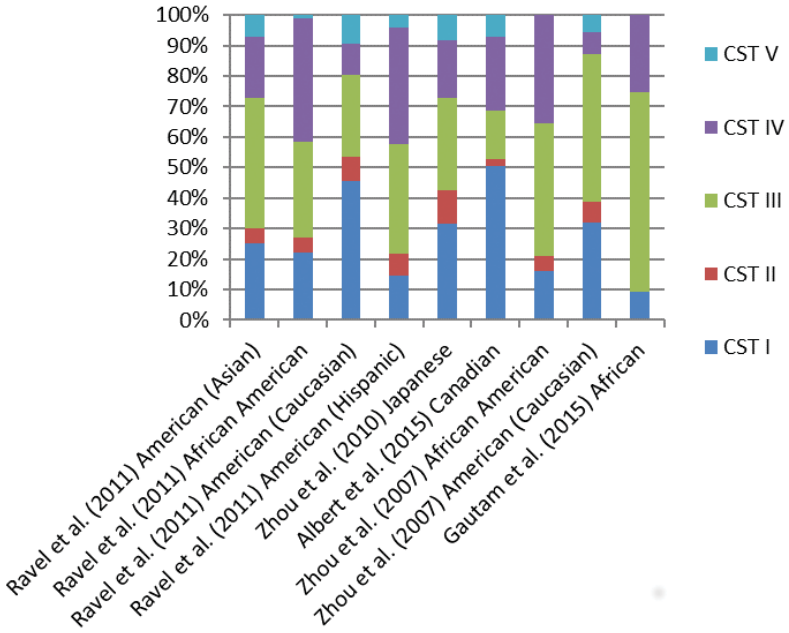

Figure 4: Comparison of dominant vaginal microbiome species found in studies using Next-generation sequencing to characterize the vaginal microbiome and label CST diversity in hosts of various ethnicities (26, 37-40).

5. The vaginal microbiome in relation to reproductive health

A healthy vaginal microbiome can become unbalanced due to a number of reasons. STIs, smoking, and vaginal hygiene are some reasons for a decrease of *Lactobacillus spp.* (35, 45). This allows opportunistic bacteria that are not normally part of the vaginal flora to colonize the vagina (46, 47). Additionally, bacteria that are part of the normal vaginal flora in low amounts are able to overgrow the healthy vaginal flora, often leading to BV (48).

There are a number of recent studies showing that BV increases risk of pregnancy loss, preterm delivery, and miscarriage, as well as lowering the rate of conception significantly (32, 41, 49-52). The risk for these pregnancy complications goes up significantly. For instance, pregnancy loss was two and a half times as likely to occur in women with BV compared to women with a healthy microbiome (42). When the BV state is persistent in the patient, or if there is a higher than average amount of BV related bacteria present, there is an even higher potential for adverse pregnancy outcomes (42, 53-55). Additionally, women undergoing their first pregnancy suffer a higher risk for pregnancy loss in the second trimester when the vaginal microbiome is shown to contain relatively low amounts of *Lactobacillus spp.* or

no *Lactobacillus spp.* at all (56). It is likely that the lack of *Lactobacilli* creates an opportunity for other opportunistic bacteria to colonize the vagina, which often leads to BV. It is currently unclear if assisted reproductive therapy (*e.g.* IVF) is affected by the composition of the vaginal microbiota. This thesis aims to clarify the role of the microbiome composition in successful IVF treatments.

6. The vaginal microbiome and bacterial STIs

Even though the healthy vaginal microbiome has a number of mechanical and molecular mechanisms to protect its dominance over the microbiome and protect the host, bacterial pathogens still manage to infect the host. When the microbiome composition is in dysbiosis, this becomes easier.

During dysbiosis of the vaginal microbiome the host is between two and three times as likely to be infected by *C. trachomatis* (57-59). The reduced or even absent mechanical barrier functionality of the microbiome would be a logical reason for the increased infections, as *C. trachomatis* is more likely to reach the targeted epithelial layer. However, there is an additional molecular reason for the increased susceptibility. *C. trachomatis* is reliant on tryptophan for its growth, which it can only obtain from outside sources. During dysbiosis *Prevotella spp.* are often considerably more abundant than in a healthy microbiome. *Prevotella spp.* produces tryptophan which *C. trachomatis* can use, facilitating an infection (60).

A dysbiotic microbiome also increases susceptibility for *N. gonorrhoeae* infections. The most recent studies find an approximate 1.5 to 2 times increase in susceptibility for this infection when the vaginal microbiome is in a dysbiotic state (58, 59). It is currently theorized that the lack of mechanical barrier function provided by the microbiome is the main contributor to the increased susceptibility to *N. gonorrhoeae*. *C. trachomatis* and *N. gonorrhoeae* currently represent the best understood bacteria with regards to their interaction with the microbiome of the host, primarily due to their relatively high prevalence and clinical impact. Unfortunately, for other bacterial STIs a large gap in the knowledge on this subject still exists.

It is well established that BV makes the host more susceptible to common STIs, however evidence shows that this action might go both ways. Studies show that STI infected women are subsequently also more likely to develop dysbiosis as a result (59). However, it is not yet

entirely clear whether this is because of pathogen - host- microbiome interaction, or whether there is a common set of risk factors for STIs and development of dysbiosis.

Regardless of the mechanisms behind it, it is clear that BV poses a clinical threat to those who have developed it through an increased susceptibility to STIs. This raises the question whether treatment of BV should be considered as a preventative measure for STI susceptibility. Certainly, in recent years the attention to modulation of the vaginal microbiome has increased rapidly. Approaches vary between standard antibiotic treatments to lactic acid releasing mechanisms (61-63). However, a conclusive longitudinal study highlighting the effectiveness of this strategy does not yet exist.

7. Aims and outline of this thesis

7.1 Aims

Bacteria in and around the host can have an enormous impact on its health. Within the female genital tract, such impact is often related to reproductive health in particular. Studies in this thesis examine the multifaceted subject of bacterial interactions within the female genital tract. This includes the impact of host and bacterial genetics and expression on the immune response, the commensal microbiota composition and its effect on assisted reproduction, and a description of interactions between known pathogens and microbiota in the host.

7. 2 Outline

This thesis is divided into two sections:

- Part 1: Pathogens in the vaginal tract and their interaction with the immune system
- Part 2: The interaction between the vaginal microbiota and host reproductive health

Part 1:

The expression of cytokines is a direct result of the interaction of bacteria with the recognition pathways of the innate immune system. However this expression differs between people based on various factors. What effect the differences in cytokine level expressions have on bacterial infections is discussed in **Chapter 1** of this thesis. A meta-analysis of the

available literature gives a clear image of the impact of host specific differences on infections with common STIs.

Chapter 2 provides an *in silico* examination of bacterial genetic sequences. The aim was to clarify the potential stimulation or inhibition of TLR9 signaling through the unmethylated CpG DNA makeup of various bacteria present in the female genital tract.

H. ducreyi is an STI that is less commonly known. Because of this, not much is known about its interaction with the host during infection. **Chapter 3** sheds light on the effect of host genetic polymorphisms on the immune response during *H. ducreyi* infection. In turn this increases knowledge of *H. ducreyi* pathogenesis as a whole.

Part 2:
The simultaneous interaction of pathogens, commensal microbiota, and the host immune response is, due to its inherent complexity, lacking in study. In **Chapter 4** an effort is made to clarify current knowledge related to this triangular integration for the otherwise well described pathogens of *C. trachomatis* and *M. genitalium*.

Chapter 5 quantifies and qualifies the current knowledge related to the vaginal microbiota and its relation with IVF outcomes through systematic review and meta-analysis of available scientific literature.

The relation between the vaginal microbiome and success of IVF treatment has been hinted at. **Chapter 6** details the protocol used for the study performed in **Chapter 7**, which aims to determine microbiome compositions that provide a positive or negative effect on IVF outcomes. Furthermore, it examines the potential to use this knowledge as a predictive rule for IVF outcome.

Recent literature suggests that a urinary microbiome exists which is distinct from the vaginal microbiome. **Chapter 8** examines to what degree urine shows a similarity with vaginal samples in its microbiome profiles on a per person basis. Additionally, a discussion is had on the viability of the distinct urinary microbiome as a concept.

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