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Bacterial interactions in the female genital tract

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2019

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citation for published version (APA)

Singer, M. (2019). *Bacterial interactions in the female genital tract: A triangle affair between pathogens, microbiota, and host.*

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DISCUSSION

GENERAL DISCUSSION

This thesis explores the interactions between bacterial STIs present in the female genital tract, the vaginal microbiota, and the response of – and consequences to – the host. A focus is put on clarifying the underlying biological causes of differences in the clinical presentation of bacterial STIs. In addition to this focus, we assess the impact of the vaginal microbiota on the outcome of *in vitro* fertilisation (IVF) treatments. In the future this information may allow us to modulate the vaginal microbiota in order to affect IVF treatment outcomes. In the discussion of this thesis we compare data resulting from the previous chapters with the state of art data. Moreover, possible consequences and opportunities of the aggregated chapter results are discussed, after which recommendations are made.

The three chapters making up **Part 1**: “Pathogens in the vaginal tract and their interaction with the immune system” examine cytokine expression, bacterial genetics, and host genetics as three immunological factors that influence the immune response to – and clinical presentation of – bacterial STIs. Through meta-analysis of the current knowledge regarding cytokine expression during infection with bacterial STIs, we showed TLRs and pro-inflammatory cytokines that have significant effect on the severity of infections with bacterial STIs in **Chapter 1**. In **Chapter 2** we show that the potential of bacterial STIs to stimulate or inhibit the TLR9 NF- κ B pathway through CpG DNA motifs present in the bacterial DNA is highly variable between pathogens, with *N. gonorrhoeae* and *T. pallidum* being clear outliers. Finally, **Chapter 3** shows that TLR9 and IL-10 are significantly associated with the severity of experimental infections with *H. ducreyi*.

Part 2: “The interaction between the vaginal microbiota and host reproductive health” comprises four chapters showing the interactions of the vaginal microbiota with pathogen infection processes, as well as with the host reproductive process. Dysbiotic microbiota compositions such as bacterial vaginosis are of particular interest in these chapters. **Chapter 4** shows, through review of the current literature, a difference in the interactions of *C. trachomatis* and *M. genitalium* with microbiota related factors such as pH, H₂O₂, and tryptophan. Additionally, it highlights that knowledge of *M. genitalium* and its interactions with the host and microbiota lags behind the better studied *C. trachomatis*. In **Chapter 5** a review and meta-analysis shows that dysbiotic microbiota profiles negatively affect early pregnancy development stages. Subsequently, the effect of the vaginal microbiota

on the outcome of IVF treatments is examined in **Chapters 6** and **7**. These two chapters provide an algorithm that can determine the likelihood of an IVF treatment to fail based on the microbiota composition at the start of the treatment. Finally, in **Chapter 8** we showed that 16s rRNA sequencing and IS-pro analysis can both be used to determine the vaginal microbiota profile in vaginal swabs, while IS-pro analysis was more capable of determining the microbiota profile from urine samples.

Part 1: Pathogens in the vaginal tract and their interaction with the immune system

The effect of variety in expression of cytokines after infection with bacterial STIs

The connection between cytokines expression variations and the impact of this expression on the severity of clinical infections is a well-established fact. From mental disorders like depression, to infections with for example *Streptococcus*, these variations have been linked to the severity of disease (1-4). In **chapter 1** we found that for *C. trachomatis* in particular, an extensive knowledgebase allowed us to make several strong associations between cytokine expression and complications such as tubal factor infertility. Similar associations could be made for *N. gonorrhoeae* and *T. pallidum*. As an alternative to the host mediated differences found in this chapter, differences in bacterial antigenic factors can also lead to increases and decreases in immune response of the host. The most common antigenic factors that are known to cause these differences in immune response are the virulence factors of bacteria, which often lead to an increase in symptom severity in the host (5). Nonetheless, every part of the bacteria that comes into contact with the immune system has the potential to produce a disproportionate immune response instead of the commonly expected and desired immune response.

Initial contact of antigens with the innate immune system commonly occurs through interaction of the bacteria with the pattern recognition receptors (PRR) such as Toll like receptors. In **chapter 2** we examine bacterial unmethylated CpG DNA, which acts as the ligand to the PRR TLR9. Previous research has already shown that specific CpG DNA motifs can affect the host immune response (6-8). For example, the *Herpes simplex virus* DNA was shown to contain a high amount of immune-stimulatory CpG DNA motifs, which elicits more pro-inflammatory cytokine production after binding with TLR9 (8). The lowest CpG index we found was -77.1 for *N. gonorrhoeae*, showing a strong inhibitory potential for TLR9 activation by *N. gonorrhoeae* and making it the only included pathogen to reach an inhibitory index value. Interestingly, a study by Sanders *et al.* has shown a CpG index of -106.8 for *N. meningitides*, indicating that

a such a low CpG index is genus-specific (7). The highest CpG index found for an STI was 17.7 for *T. pallidum*, the bacteria which causes syphilis. The height of the index score brings *T. pallidum* close to the CpG index of *E. coli* at 21.1, which was shown *in vitro* to stimulate the production of relatively high amounts of TLR9 related cytokines compared to other common bacteria (9). CpG DNA is not the only antigenic factor with the potential to affect the innate immune response, as variations in a number of other bacterial antigenic factors have been shown to affect the expression of cytokines through other PRR pathways.

One other example of an antigenic factor that can affect the innate immune response is Lipid A, which is the specific section of lipopolysaccharide (LPS) that interacts with TLR4 (10). Lipid A shows a high diversity, with characteristics such as its location in the LPS structure and molecular build being highly variable depending on the bacterial species and strain (11-13). An example of this variability is shown in figure 1, where immunogenic differences between Lipid A molecules found in *Escherichia coli* and *Helicobacter pylori* are highlighted. This variability has been shown to affect host TLR4 in recognition of LPS as its target (11). Subsequently, the ability to properly recognise LPS through TLR4 has been shown to directly impact the clinical presentation of infection, leading to differences in symptom severity in patients (11, 12, 14, 15). A similar effect, in which differences in bacterial peptidoglycans affect the stimulation of TLR2, exists as well (16, 17). It is important to note the various redundancies in the host immune system. Bacterial antigenic factors impacting the stimulation of one PRR do not affect the interactions of other bacterial antigens with the respective PRR for that antigen. Therefore the inhibition of one PRR pathway does not necessarily translate into an increased or decreased immune response. Nevertheless, this thesis has added to the knowledge of how bacterial STI genetics can affect the expression of cytokines through TLR9 activation to affect the host immune response to - and clinical presentation of- bacterial STIs.

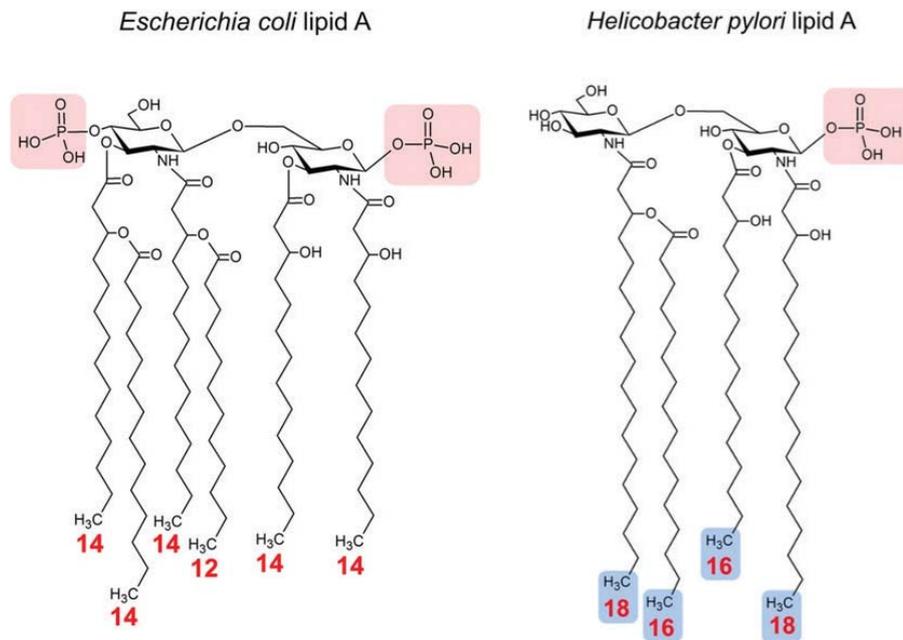


Figure 1: Two different Lipid A structures from *E. coli* (left) and *H. pylori* (Right). Immunogenic phosphate groups are highlighted in pink. The red numbers below each chain refer to the number of carbon atoms of the respective chain. The light blue highlights refer to structural differences found in the *H. pylori* acyl chains compared to the chains in the *E. coli*. Image adapted from Steimle et al.(12).

The impact of host genetics on the immune reaction to bacterial STIs

In concordance with the immune response expression affected by bacterial factors, host genetic factors also play a major role in the expression of cytokines during the initial immune response. In **chapter 3** we show to what extent host genetics affect the severity of infections with the lesser known STI *H. ducreyi*. Single nucleotide polymorphisms (SNP) in the genes coding for TLR9 and IL10 were related to pustule formation during the disease. This chapter is the first to show a relation between host genetics and *H. ducreyi* severity. The discovered relations are in line with relations that have been made previously for other bacterial STIs.

Notably, the much more extensively studied *C. trachomatis* has a large number of host genetic relations with both susceptibility and severity of the infection. Currently, sixty-nine different genetic relations have been made to the STI chlamydia. These relations include a wide variety of PRRs, cytokines, chemokine receptors, and other genes related to the innate immune response of the host. The relations with *TLR2*, *TLR4*, *IL10*, and *TNFA* have

the most studies devoted to them (18-22). These relations partially overlap with those found for *H. ducreyi* infections, where *TLR9* and *IL10* are the strongest contributors to the immune response and disease presentation. The host genetics related to the susceptibility and severity of *C. trachomatis* infections have also been shown to affect the development and presentation of later complications of chlamydia. There is an opportunity for future studies to examine whether *H. ducreyi* and recurrence of infection or tissue scarring after a patient has suffered from chancroids are similarly affected by host genetic factors.

Another well studied STI with regards to host genetic relations is gonorrhoea, which is caused by *N. gonorrhoeae*. There are nine genes reported to be related to susceptibility and/or severity of the disease. Studies confirm that during *N. gonorrhoeae* infection, as with *C. trachomatis* and *H. ducreyi*, PRRs and inflammatory cytokines are the most relevant to the outcome of the disease. Most host genetic relations between *N. gonorrhoeae* infections and susceptibility or severity of the disease have been reported for *TLR1*, *TLR2*, *TLR4*, and *TLR6* (23, 24). Regrettably, many other bacterial STIs that are not as well-known as *C. trachomatis* or *N. gonorrhoeae* do not have the same amount of knowledge available with regards to host genetic variation in relation to susceptibility to and severity of the infection. Our study in **chapter 3** reduced this gap of knowledge for *H. ducreyi* infections.

The methodology of **chapter 3** is relatively unique, and provided us with a valuable angle of approach for host genetic analysis. Severity of *H. ducreyi* infections is known to vary between individual patients. By experimentally infecting volunteers, we took away the possibility for the difference in severity to be caused by bacterial load or strain of the bacterium, leaving only host genetics as a potential influencing factor. By analysing selected inflammation related genes we showed the first relations between host genetics and *H. ducreyi* infection. Furthermore, the results of the study are found for subdermal experimental infections in the arm. These results can likely be carried over to infections in or near the genitals due to the similar functional effect of the genes in which the SNPs were found. Nevertheless, potential differences of expression of these genes in the genital tract should still be accounted for in future studies.

The increasing interest in using host genetics in medical practice means that there is significant support for medical application of host genetic knowledge and relations with infectious diseases. Already, a test using host genetics for prediction of later complications of *C.*

trachomatis is in development (25). The similarity of related host genes between bacterial STIs may be used as basis for experiments examining other STIs. However, all current studies point towards bacteria specific interactions being crucial for relations to form. In light of our findings in **chapter 2** of this thesis, *N. gonorrhoeae* was shown to have an especially low stimulation potential for TLR9. Such bacterium specific characteristics are one reason that overlap of genetic relations between bacterial STIs should not be assumed. Another reason is the varying level of impact host genetics may have. For *C. trachomatis* it has been described, through the *in vitro* study of infections in cells of twins, that approximately 40% of the variation between disease severity is caused by host genetic variation (26). Another study using this twin study design noted that for respiratory syncytial virus infection the host genetics accounted for 16% of the infection severity (27). Although this infection is not very comparable to *C. trachomatis*, and is only included here due to lack of similar study designs for more relatable diseases, it shows that the role of host genetics in severity of infections can be highly variable and should therefore not be easily assumed.

In the previous paragraphs the associations between host genetics and STIs have been discussed from a viewpoint primarily focussed on the meaning for the host immune system. Interestingly, the host genetic relations can also tell us something about specific aspects of the pathogenesis of the studied bacteria. In the case of *H. ducreyi*, the relation between the infection and TLR9, even though other PRRs were also investigated, shows that there may be characteristics of *H. ducreyi* that actively work against recognition by other PRRs in the host. This potential evasion of detection by the innate immune system is especially notable in the interaction between *H. ducreyi* and TLR4. *H. ducreyi* is a gram negative bacteria and therefore consist of a significant amount of LPS. Nevertheless, mutations affecting the functionality of TLR4 did not show a significant influence on the course of the infection. The fact that TLR4 does mediate infections of the genetically closely related *H. influenza* could indicate that the composition of the LPS molecule is not the primary reason for the lack of TLR4 activation (28-30). Further research may find that *H. ducreyi* carries a species specific mechanism for minimizing interactions with TLR4.

Through the work in the first part of this thesis we have shown the extent of current knowledge into cytokine expression during bacterial STIs, the differences in inflammatory potential between the DNA of bacterial STIs, and have for the first time shown an association between host genetic variations and the outcome of *H. ducreyi* infections. For *C. trachomatis* and

N. gonorrhoeae our results support further translational research. For lesser known and studied bacterial STIs the results of this part of the thesis especially support further research concerning host genetic variation and the relation the host genetics have with the outcome of the bacterial STIs.

Part 2: The interaction between the vaginal microbiota and host reproductive health

The extent of the microbiota in the female genital tract

In **chapters 4 to 8** of this thesis the vaginal microbiota is studied as a proxy marker for effects that the female genital tract microbiota can have on pregnancy and IVF outcomes. Indeed, most of the knowledge we have about the microbiota in the female genital tract comes from previous research into the vaginal microbiome. It is often posited that microbiota found further up the female genital tract, such as the endometrial microbiota, can more accurately portray the effects on the reproductive process because these microbiota are in closer contact with the reproductive process (31, 32). Unfortunately, whereas compositions of bacteria in the vaginal microbiota are well studied with regards to composition and potential impact on host health, research into other microbiota in the female genital tract is generally lacking. Contamination during sampling is an important reason that studies into these microbiota are less reliable. For instance, during the process for sampling the endometrial microbiota the tools for sampling are passed through the vagina/cervix where high bacterial loads are present. For this reason, sampling is most often done through the use of catheters that are also used for placement of embryos during IVF treatment (33). Even then, contamination is an issue in these studies. Additionally, studies such as the one performed in **chapter 7** of this thesis show that the use of the vaginal microbiota as a proxy measurement is sufficient for determining reproductive effects, lowering the need for more complicated sampling methods.

As is described in a study by Tao *et al*, proper sampling and analysis of the endometrial microbiota is often hindered by potential contamination by the vaginal microbiota (33). Regardless, there is significant interest from the scientific community in research into the microbiota in the upper female genital tract. One reason for this is that, unlike what was found for microbiota in urine in **chapter 8**, significant differences have been found in the composition of these microbiota when compared to the vaginal microbiota (31-37). Previously, it was commonly thought that the microbiota found in the upper female genital tract was a diluted run off from the microbiota in the vagina (38). This was proven to be

incorrect, as the microbiota in the upper female genital tract can be divided even further. Unique compositions have been found on the endometrium, in the uterus in general, and even in the fallopian tubes (32, 34-36). The presence of these differing microbiota suggests a complex situation regarding interactions between microbiota and host, as multiple significantly different microbiota appear capable of impacting the host reproductive health at the same time. This situation creates opportunity for further research to elucidate the relative impact of each microbiota on the host reproductive health. Regardless, the vaginal microbiota will likely continue to be studied as a proxy for microbiota in the female genital tract for the foreseeable future.

The role of the vaginal microbiota in infection with bacterial STIs

The vaginal microbiota has an effect on the susceptibility to a number of STIs, both bacterial and viral (39-42). An example of this interaction featuring the vaginal microbiome and *C. trachomatis* or *M. genitalium* is described in **chapter 4** of this thesis. **Chapter 4** also reviews the current knowledge on the microbiota interacting with bacterial STIs, and how it is focussed primarily on the best studied STIs. We showed that there appears to be a common theme in the negative effect on the host when there is a dysbiotic vaginal microbiota. That said, specific interactions between pathogens and bacteria making up the microbiota are often pathogen specific (43-48). An example of one such specific interaction is the presence of tryptophan producing *Prevotella* being beneficial specifically for *C. trachomatis*. Addressing the gap in knowledge for less studied STIs may lead to discovery of interesting bacterium specific interactions with the host.

The knowledge of interactions between the vaginal microbiota and bacterial STIs can be used to further our understanding of the pathogenesis of these STIs. A recent study has comprehensively charted the immune response to dysbiotic microbiota in the vaginal tract (49). In this study, the markers IL1ra and IL2 were found to be specifically increased during symptomatic bacterial vaginosis (BV), while the biomarker expression profile of intermediate microbiota was characterised by increases in expression of IL1 α , IL1 β , IL8, MIG, MIP1 α , and RANTES. This information opens up opportunities to specifically study bacterial STIs that have host genetic associations to disease progression, in patients that have microbiota compositions that increase the expression of these biomarkers. One such example can be derived from **chapter 3** of this thesis. None of the factors that were associated to dysbiosis in the study of Campisciano *et al.* were shown to have an effect on the severity

of *H. ducreyi* infections (49). Through the lack of these associations we can speculate that the immunological factors related to the vaginal microbiota do not significantly affect *H. ducreyi* during infection.

As associations between the vaginal microbiome and susceptibility and severity are present for most major STIs there may be potential to use this knowledge for intervention. A number of strategies for modulation of the vaginal microbiome have gained in popularity in the past decade (50-52). Notably, there are a number of studies that describe successful long term change of the vaginal microbiome composition from dysbiotic states to healthy states (53-58). The modulation of the microbiome is achieved by treating the patient with the standard treatment for BV, which is Metronidazol, and following this up with a *Lactobacillus*-based probiotic. Table 1 gives a more detailed overviewed of the treatment protocols in the studies. It would be interesting to examine a potential relation between the modulation of the vaginal microbiota through for instance probiotics, with a positive effect on the susceptibility to – or severity of – STIs. Microbiome modulation strategies are further expanded upon in a later part of this discussion.

Table 1: Overview of studies showing a long term modulation of the vaginal microbiome using *Lactobacillus* based probiotics.

Study	Year	Treatment of control group	Treatment of case group	Term of modulation	Ref
Recine <i>et al.</i>	2016	Metronidazole 500 mg orally twice a day for 7 days	Metronidazole 500 mg orally twice a day for 7 days followed by <i>L. rhamnosus</i> BMX 54 vaginal tablets	9 months	(53)
Anukam <i>et al.</i>	2006	0.75% metronidazole vaginal gel for 5 days	Two gelatin capsules containing <i>L. rhamnosus</i> GR-1 and <i>L. reuteri</i> RC-14 for 5 days	1 month	(54)
Bradshaw <i>et al.</i>	2012	Metronidazole 400 mg orally with or without 2% vaginal clindamycin-cream for 7 days	Metronidazole 400 mg orally with a <i>L. acidophilus</i> vaginal-probiotic containing oestriol for 12 days	6 months	(55)
Martinez <i>et al.</i>	2009	Single dose of tinidazole 2g	Single dose of tinidazole 2g and 2 capsules of <i>L. rhamnosus</i> GR-1 and <i>L. reuteri</i> RC-14 for 4 weeks	28 days	(56)
Marcone <i>et al.</i>	2008	Metronidazole 500 mg orally twice a day for 7 days	Metronidazole 500 mg orally twice a day for 7 days followed by one <i>L. rhamnosus</i> BMX 54 vaginal tablet per week for two months	3 months	(57)
Marcone <i>et al.</i>	2010	Metronidazole 500 mg orally twice a day for 7 days	Metronidazole 500 mg orally twice a day for 7 days followed by one <i>L. rhamnosus</i> BMX 54 vaginal capsule per week for six months	12 months	(58)

How the reproductive process affects the vaginal microbiota

As touched on in **chapter 4** of this thesis, the vaginal microbiota is constantly influenced by the hormonal levels of the female host (59). Most notably estradiol and progesterone. This influence is so outspoken that the use of hormonal contraceptives can noticeably change the vaginal microbiota composition towards a more *Lactobacillus* dominated state (60). Interestingly, the strong increase of these hormones during pregnancy can also lead to a lower diversity, and a generally higher abundance of *Lactobacillus* bacteria (61). Reference values for estradiol and progesterone are more than 100 times higher in pregnant women than in non-pregnant women (62). The height of these values appears to lead to a defensive effect in women whose immune system is less effective due to pregnancy.

The move away from a dysbiotic microbiome composition makes the host less susceptible to STIs. Nevertheless, a direct association between pregnancy and a decreased susceptibility

to bacterial STIs has yet to be made. Conversely, the use of hormonal contraception has directly been associated to reduced susceptibility to STDs (63). One factor that can affect a potential association between hormonal levels and bacterial STIs in pregnant women, is that during pregnancy the host immune system is significantly downregulated. This downregulated immune system leads to an impaired ability to combat infections in the host. Kourtis *et al.* produced a list of infections that women are either more susceptible to during pregnancy or which produce a more severe infection during pregnancy (64). Notably Kourtis *et al.*'s study did not include bacterial STIs. Nevertheless, they found that an increase in severity of infection was far more common than an increase in susceptibility to the infections. There is a clear opportunity for a study investigating the associations between hormonal levels in pregnant women and susceptibility to bacterial STIs, and we recommend researchers to take special note to control for various aspects of risk behaviour and other confounders.

How the vaginal microbiota affects the reproductive process

The effect that microbiota in the female genital tract has on pregnancies is a subject that has received much attention from clinical professionals. Because of this attention the effects microbiota have on pregnancies have been well documented. Most notable are the effects of BV on the outcome of pregnancies (65-67). BV has been strongly related to pre-term birth, maternal infectious morbidity, and miscarriage (65, 66). These characteristics are well described for normal pregnancies, but recently additional effort has been made to find the associations between microbiota compositions and assisted reproductive technology. **Chapter 5** discusses the current knowledge related to the vaginal microbiota and its relation with IVF outcomes. Showing that there is a commonly found negative effect on IVF outcome when affected by BV. Furthermore, the results presented in **chapter 7** show that specific microbiota compositions influence the outcome of IVF in such a way, that a prediction can be made of the outcome of the IVF treatment based on the microbiota compositions.

The main differences between normal pregnancies and IVF induced pregnancies are the strictly defined periods of hormonal variations during IVF. These strict periods have made the microbiota easier to study during the IVF process. Interestingly, a negative association has been made between the contamination of the IVF catheter tip and outcome of IVF treatments (68, 69). This contamination of the catheter tip occurs during the passage of the tip past the vagina and cervix, where resident microbiota are highly abundant. This contamination has such an impact on the pregnancy rates after IVF that some treatment

centres administer prophylactic antibiotics to reduce the chance of contamination (70, 71). However, the clinical usefulness of these prophylactic antibiotics, especially concerning non-discriminatory use of prophylaxis, is still under debate (71, 72).

There is still uncertainty about the source and mechanisms behind the influence of microbiota on the reproductive process. As discussed in an earlier section of this discussion, microbiota can be found in the uterus and in the fallopian tubes as well (31, 32, 34-36). Although these microbiota have not been as extensively studied as the vaginal microbiota, associations between the presence and compositions of these microbiota and conception have been described (73, 74). Specifically the inflammation related to the presence of these microbiota has been pointed at as the main mechanisms through which conception and pregnancy outcomes are affected (75). The novelty of these topics makes it so that opportunity for further research focussing on confirmation and discovery is still plentiful.

The opportunities for vaginal modulation before pregnancy

The concept of adjusting or replacing an unwanted vaginal microbiome has been a focus point for a number of recent studies. The most frequently researched way of altering the vaginal microbiome is through the administration of probiotics, which are usually introduced to the vagina through vaginal tablets. In one study *Lactobacillus* based vaginal tablets were able to replace BV with a normal or intermediate vaginal microbiome, while only 12% of non-treated patients showed improvement (76). Treated patients were also less likely to have recurring instances of BV. More effective and long-term adjustment to a *Lactobacillus* dominated microbiome was obtained through the application of a combination of antibiotics and probiotics (56, 77, 78). Additionally, improvement of the vaginal microbiome during the later stages of pregnancy was also seen in women taking a probiotic mixture of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* strains (79). Most notably, there was an overall increase of anti-inflammatory effects observed in the vaginal microbiome. Local inflammation during pregnancy has long been related to pre-term births, so probiotics promoting an anti-inflammatory state likely reduce the amount of morbidity and mortality related to pre-term births.

Another way to adjust the vaginal microbiota is through the use of antimicrobials, often primarily used as a treatment of infection or BV. Giving a patient with BV the antibiotic Rifaximin over a course of five days has been shown to decrease BV related bacteria in the vaginal microbiome (80). Notably it was also shown that there was an increase in *Lactobacillus*

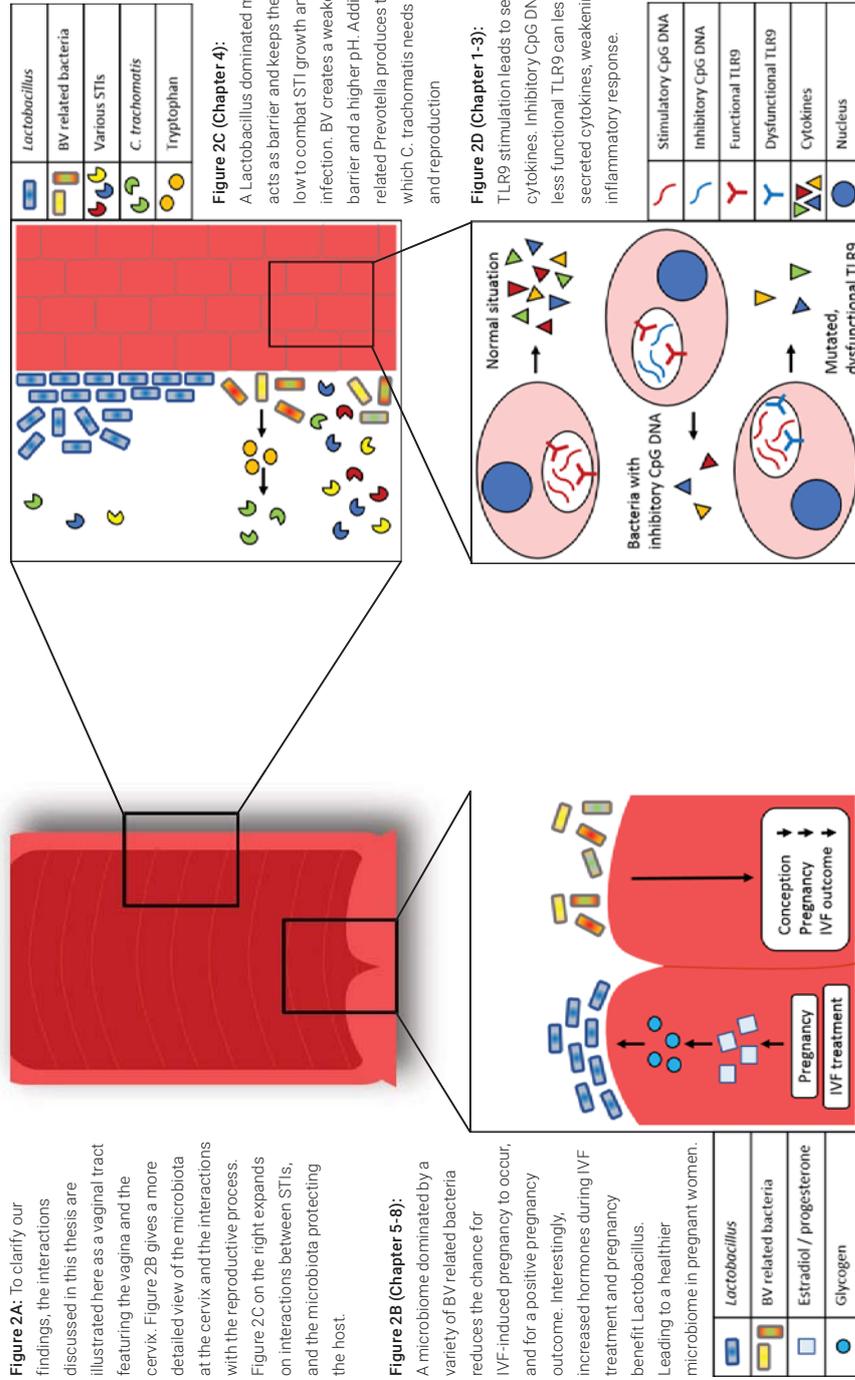
during and after the Rifaximin treatment. Similarly, treatment of BV using Metronidazole secreted by an intra-vaginal ring showed decrease of BV related characteristics in the vaginal microbiome (81). More recently it was shown that a combination of tinidazole treatment and probiotic *L. rhamnosus* treatment increased the amount of other *Lactobacillus* species in the vagina (51). Probiotic treatment is important, as BV related bacteria quickly recolonize the vaginal space after antibiotic treatment has stopped (82).

Although in a previous section of this discussion it was shown that hormonal contraceptives have been related to healthy microbiota compositions, the use of these hormonal contraceptives with the intent of modulating the vaginal microbiota did not result in the shift from a dysbiotic to a healthy microbiota. These results suggest that a strategy of hormonal change may not be the best avenue to alter the vaginal microbiome (83). Vaginal application of a sucrose based gel does show a capacity to change the vaginal microbiota from BV to a *Lactobacillus* based microbiota composition (84). Besides these methods a number of other female hygiene products, such as vaginal douches, have the unintended side-effect of altering the vaginal microbiome. Unfortunately, this alteration was shown to make the user more susceptible to BV (85).

It is important to note that, even though modulation of the microbiota in theory should have a positive effect on pregnancy rates and outcomes, there is still some controversy on this topic. One study has shown that, even though there was successful modulation of the vaginal microbiota, there was no noticeable effect on clinical pregnancy rates (73). It is likely that the topics covered in this discussion, such as the relation between microbiota in the upper female genital tract and pregnancy, make intervention strategies more complex than they may first appear.

The second part of this thesis has shown that there is a significant association between the composition of the vaginal microbiota and the way the immune system of women interacts with STDs. Additionally, it shows an important association between the composition of the vaginal microbiota and the outcome of IVF treatments. The contributions of this thesis have led to better definitions regarding the vaginal microbiota, but through them we have also taken valuable steps towards implementing the current knowledge of the vaginal microbiota into clinical practice.

Overview: Bacterial interactions in the female genital tract seen at a glance



CONCLUSIONS, RECOMMENDATIONS, AND FUTURE PERSPECTIVES

Part 1: Pathogens in the vaginal tract and their interaction with the immune system

Conclusions

In conclusion, the research described in part 1 of this thesis has led to an increased understanding of the interactions between bacterial STIs and the host. Our knowledge of these interactions has contributed on the following three levels. First, we revealed that differences in cytokine expression of hosts impacts the severity of *C. trachomatis*, *N. gonorrhoeae*, and *T. pallidum* infections. Second, we have shown the inflammatory potential of bacterial STIs based on the presence and abundance of CpG DNA motifs in bacterial STI DNA. Third, the current understanding of host genetic factors was expanded by our study showing that *TLR9* and *IL10* are innate immune response related genes that influenced the production of pustules during *H. ducreyi* infections. Depending on the SNPs present in *TLR9* and *IL10*, the influence could both be an increased or decreased production of pustules through polymorphisms in either gene.

Recommendations

Based on our findings, we recommend more attention is paid to the interactions between the host and lesser known STIs. **Chapter 1** and **chapter 3** showed that there is a lot of potential for discovery regarding the interactions between the host and less common bacterial STIs. These interactions are likely to affect the severity of the disease, as is shown for *C. trachomatis*, *N. gonorrhoeae* and *T. pallidum* in **chapter 1**. As an example of a lesser known STI, the influence of host genetics on the severity of *H. ducreyi* is shown in **chapter 3**. Studying these interactions may lead to improvement of the treatment of the lesser known infections in the future by taking the potential severity of the diseases into account.

Future perspectives

There are many future opportunities regarding the interactions between bacterial STIs and the host immune system. An effort is currently being made to implement a host genetic test which determines the potential for tubal factor infertility due to a previous *C. trachomatis* infection (25). If this test proves successful, similar tests may be developed for late complications of other bacterial STIs as well. As a possible example, a similar test may be

used to determine the likelihood of a *T. pallidum* infection to develop into the most severe stage of neurosyphilis.

Besides potential clinical benefits, opportunities for basic science exist as well. The *in silico* CpG index data from **chapter 2** can be followed up in an *in vitro* experiment to corroborate the inflammatory potential of the bacterial DNA. Using available strictly TLR9 expressing cell lines, the expression of inflammatory cytokines after adding bacterial DNA and oligonucleotides of the inhibitory and stimulatory CpG motifs can be effectively measured. This would provide a nice direct comparison of *in silico* and *in vitro* data.

Part 2: The interaction between the vaginal microbiota and host reproductive health

Conclusions

From our research into the host and pathogen interactions with the vaginal microbiota we can conclude that these interactions are important factors for the general and reproductive health of the host. We reviewed current literature showing that *C. trachomatis* and *M. genitalium* are differentially affected by the microbiota, primarily based on molecular differences such as the pH or H₂O₂ levels, which can be related to dysbiosis of the vaginal microbiome. To experimentally point out another risk related to dysbiosis in the vaginal microbiota, we showed in a cohort of women attempting an IVF treatment that BV can be linked to the failure of IVF. The effect is so well-defined, that we were able to produce an algorithm that accurately predicts the failure of IVF based on the vaginal microbiota.

Recommendations

A need for additional study of upper female genital tract residing microbiota, such as microbiota in the uterus and fallopian tubes, was shown in the discussion of this thesis. Currently, there is a small number of studies showing potential associations between these microbiota and the reproductive process. More research is needed to clarify if these associations are different from the currently known associations between the vaginal microbiota and the host's reproductive health. Additionally, we recommend that additional studies be performed to confirm current understandings regarding upper female genital tract residing microbiota. The associations between the vaginal microbiota and host reproductive health were, over time, found to be highly complex. **Chapter 7** exemplifies this by showing that specific bacterial species impact the host reproductive health, rather than the entire composition

of (dysbiotic) microbiota. It is likely that similar specific interactions also apply in the case of microbiota in the upper female genital tract.

Future perspectives

Finally, we must now look into translating the results found in **chapter 7** into a standard health care practices. Before this can be done, we need to know how long a (dysbiotic) microbiota composition remains stable over time. This will help us determine when a new IVF treatment and ReceptIVFity test can be offered to a patient after a dysbiotic microbiota has been found initially. A study to examine this is currently being set up, and is expected to start inclusion of patients in 2019. Additionally, we would like to be able to offer women with a microbiota composition that predicts a negative IVF outcome a solution to this problem. Therefore, a strategy for vaginal modulation is being devised, to help women obtain a vaginal microbiota that is more likely to lead to a successful IVF treatment. This study is expected to finish in 2020. Lastly, efforts are being made to include the prediction test into insurance covered health care in 2019.

Clinically there are exciting new prospects on the horizon. Implementation of microbiota related diagnostics and interventions are just now starting to become available. Vaginal microbiome modulation is likely to be a topic that garners increasing attention in the coming years. The treatment of recurring BV, which is estimated to occur in roughly 30% of the treated BV cases, using *Lactobacillus* based probiotic vaginal modulation has already shown a number of successes.

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