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The cervical microbiome in female infectious infertility: clinical trial and experimental mouse models

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Abbreviations

°C ..... degree celsius
µg ..... microgram
µl ..... microliter
3D ..... three-dimensional space
ATCC ..... American Type Cell Culture Collection
AUC ..... area under curve
B. bifidum ..... *Bifidobacterium bifidum*
B. breve ..... *Bifidobacterium breve*
bp ..... base pairs
BV ..... bacterial vaginosis
BVAB ..... bacterial vaginosis associated bacterium
C. difficile ..... *Clostridium difficile*
C. muridarum ..... *Chlamydia muridarum*
C. trachomatis ..... *Chlamydia trachomatis*
CDC ..... Centers for Disease Control and Prevention
CI ..... confidence interval
cm^2 ..... square centimetre
CO_2 ..... carbon dioxide
CPAF ..... *Chlamydia* protease-like activity factor
CT ..... community type
CVM ..... cervicovaginal mucus
div ..... diverse
DMEM ..... Dulbecco's Modified Eagle Medium
DNA ..... desoxy ribonucleic acid
dNTP ..... desoxynucleotidetriphosphate
dpi ..... days post infection
E. coli ..... *Escherichia coli*
EB ..... elementory body
EDTA ..... ethylenediaminetetraacetic acid
ELISA ..... enzyme-linked immunosorbent assay
et al. ..... *et alii*, and others
FBS ..... fetal bovine serum

FITC ..... fluorescein isothiocyanate
FMT ..... fecal microbiota transplantation
FSW ..... female sex worker
fUGT ..... female urogenital tract
FUT2 ..... fucosyltransferase 2
FZ ..... *Forschungszentrum, Research Center*
g ..... gram
G. vaginalis ..... *Gardnerella vaginalis*
GBS ..... Group B *Streptococcus*
GmbH ..... *Gesellschaft mit beschränkter Haftung*
Gva ..... *Gardnerella vaginalis*-dominated
GVA cocktail ..... antibiotic cocktail containing Gentamycin, Vancomycin and Amphotericin B
GWAS ..... genome wide association study
h ..... hour(s)
HEp-2 cells ..... human epithelial type 2 cells
HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV ..... human immunodeficiency virus
HPV ..... human papillomavirus
HSP60 ..... heat shock protein 60
HSV2 ..... herpes simplex virus 2
i.p. ..... intraperitoneal
IBD ..... inflammatory bowel disease
IFN-γ ..... gamma-interferon
IFT ..... immunofluorescence test
IFU ..... inclusion forming unit
IgA ..... Immunoglobuline A
IgG ..... Immunoglobuline G
INC ..... incorporated
ININF ..... infectious infertility
IVF ..... *in vitro* ferilization
KCl ..... kalium chloride
KY ..... Kentucky
L. crispatus ..... *L. crispatus*
L. gasseri ..... *Lactobacillus gasseri*

L. iners ..... *Lactobacillus iners*

L. jensenii ..... *Lactobacillus jensenii*

Lcr ..... *Lactobacillus crispatus*-dominated

Lga ..... *Lactobacillus gasseri*-dominated

Lin ..... *Lactobacillus iners* dominated

Lje ..... *Lactobacillus jensenii*-dominated

LPS ..... lipopolysaccharide

Ltd ..... Limited Company

M ..... mol/liter

M. genitalium ..... *Mycoplasma genitalium*

M. hominis ..... *Mycoplasma hominis*

min ..... minute(s)

ml ..... millilitre

mM ..... millimol/liter

MOMP ..... major outer membrane protein

MoPn ..... mouse pneumoniae strain

N. gonorrhoeae ..... *Neisseria gonorrhoeae*

NaCl ..... natrium chloride

NGS ..... next-generation sequencing

nINING ..... non-infectious infertility

NSET ..... non-surgical embryo transfer

NY ..... New York

OMP2 ..... outer membrane protein2

OTU ..... operational taxonomic unit

PBS ..... phosphate buffered saline

PCoA ..... Principal Coordinates Analysis

PCOS ..... polycystic ovary syndrome

PCR ..... polymerase chain reaction

pH ..... negative decimal logarithm of the H^+ ion concentration

PID ..... pelvic inflammatory disease

PmpD ..... polymorphic membrane protein D

qPCR ..... quantitative PCR

QTL ..... quantitative trait loci

RB ..... reticulate body

rcf.....relative centrifugal force(s)
RDP ..... ribosomal database project
ROC..... receiver operating characteristics
rpm ..... rounds per minute
RPMI medium..... Roswell Park Memorial Institute medium
rRNA .....ribosomal ribonucleic acid
RT-PCR ..... real-time PCR
S ..... Svedberg
spp. .....*species pluralis,* species (plural)
STI ..... sexually transmitted infection
T. vaginalis..... *Trichomonas vaginalis*
TARP ..... translocated actin-recruiting phosphoprotein
™ ..... trademark
U. myomatosus.....Uterus myomatosus
U. urealyticum .....*Ureaplasma urealyticum*
USA .....United States of America
UTM ..... universal transport medium
V1/V2 region.....hypervariable region 1 and 2
V3/V4 region.....hypervariable region 3 and 4
vs.....versus
WHO .....World Health Organization

Abstract

Infertility affects more than ten percent of couples which desire to have children. This infertility is often caused by infections within the female urogenital tract, causing tubal factor infectious infertility (ININF). While the infectious causes of ININF (such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*) are well known, and while a role of the microbiome of the female urogenital tract (fUGT) in the progeny of infectious agents has been demonstrated recently, nothing is known about the role of the urogenital microbiota in ININF. For this reason, the present study assesses the cervical microbiome of females with ININF using next-generation sequencing of partial 16S rRNA genes and describes it in comparison to females with non-infectious infertility (nININF), female sex workers (FSW) and fertile females.

The microbiome of fertile females and nININF mostly comprised members of the genus *Lactobacillus*. In contrast, the cervical microbiome of females with ININF and FSW featured increased abundances of anaerobic bacteria and an enhanced diversity typical for an adverse microbiome, comprising species which are not well studied so far. Correspondingly, the proportion of *Lactobacillus*-dominated community types (CTs) was reduced in ININF and FSW, while a *Gardnerella vaginalis*-dominated CT was enhanced in ININF. Diverse CTs were prominent in both these study groups. Interestingly, sexual behavior had only a minor effect on the cervical microbiome. Integrating microbiome sequencing data and diagnostic analysis, a prediction model could be established with high precision for infectious infertility. Comparison of two common strategies analyzing microbiome sequencing data showed that careful consideration of primers used for amplification of partial 16S rRNA genes is important for comprehensive assessment of the microbiota and correct interpretation concerning microbial composition.

It has been shown previously that lactobacilli are responsible for a low pH microenvironment and are able to prevent sexually transmitted infections (STIs). The findings from the present study of changes of the microbiome in ININF indicate changes in the microenvironment. Such changes might favor the growth of sexually transmitted infections such as *C. trachomatis* since the protective mechanisms within the urogenital tract might be impaired due to enhanced pH, lower availability of oxygen and loss of competitive exclusion by *Lactobacillus* species. Remarkably, the results of the present study support the hypothesis that different *Lactobacillus* species differ in their capability to protect against STIs.

Assessment of the implications of changes of the microbiome is still hampered by the lack of functional models for hypothesis based experimental testing. Therefore, STI models using chlamydial infection in mice were developed within the framework of this thesis. Using *C. trachomatis* serovar D and the mouse-specific *C. muridarum* it could be shown that two models are now available for studying different aspects of microbiome-pathogen interaction. These models will be used in future studies to modulate the microbiome and study the impact of changes of the microbial composition in STIs.

Zusammenfassung

Infertilität betrifft mehr als zehn Prozent aller Paare mit Kinderwunsch. Dabei ist Infertilität oft bedingt durch Infektionen im Urogenitaltrakt von Frauen. Obwohl die infektiösen Ursachen wie z. B. *Chlamydia trachomatis* und *Neisseria gonorrhoeae* durchaus bekannt sind und erst kürzlich gezeigt werden konnte, dass das Mikrobiom des weiblichen Urogenitaltraktes Einfluss nimmt auf sexuell übertragbare Infektionen, ist die Rolle des Mikrobioms in infektiöser Infertilität unbekannt. Daher wurde in dieser Studie mit Hilfe von modernen Sequenziermethoden (engl. *next-generation-sequencing*, NGS) das zervikale Mikrobiom von Frauen mit infektiöser Infertilität (ININF) im Vergleich zu Frauen mit nicht-infektiöser Infertilität (nININF), weiblichen Sexarbeitern (FSW) und fertilen Frauen untersucht.

Fertile Frauen und Frauen mit nicht-infektiöser Infertilität wiesen vor allem Arten der Gattung *Lactobacillus* auf. Im Gegensatz dazu beinhaltete das Mikrobiom von ININF und FSW verstärkt anaerobe Bakterien und eine erhöhte Diversität, die typisch sind für ein abnormes Mikrobiom, mit in Teilen nur geringfügig erforschten Organismen. Im selben Rahmen wiesen ININF erhöhte Zahlen eines von *Gardnerella vaginalis* dominierten bakteriellen Konsortiums auf und Konsortien mit hoher Diversität waren bei ININF und FSW prominent vertreten, während von *Lactobacillus*-Arten dominierte Konsortien in diesen beiden Studiengruppen reduziert waren. Mit Hilfe von diagnostischen Daten und den Daten der Mikrobiomanalyse konnte im Rahmen dieser Arbeit ein Modell entwickelt werden, dass infektiöse Infertilität mit großer Präzision vorhersagen kann. Der Vergleich zweier gängiger Strategien der Mikrobiomanalyse zeigte, dass die Wahl der Primer zur Amplifikation der partiellen 16S rRNA Gene kritisch zu betrachten ist, da diese Wahl erheblichen Einfluss nimmt auf die Ergebnisse und die Wahl eines unpassenden Primerpaares zu falschen Interpretationen führen kann.

Es wurde bereits gezeigt, dass lactobacilli verantwortlich zeichnen für ein Umgebungsmilieu mit geringem pH-Wert, welches sexuell übertragbare Infektionen verhindern kann. Die Ergebnisse dieser Studie über Veränderungen im Mikrobiom in ININF lassen auf Veränderungen im Umgebungsmilieu schließen. Dies könnte das Wachstum von Pathogenen wie z. B. *C. trachomatis* fördern, da schützende Mechanismen gegen sexuell übertragbare Krankheiten durch Erhöhung des pH-Werts, Erniedrigung des Sauerstoffgehalts und fehlendem Konkurrenzausschluss durch *Lactobacillen* reduziert sein dürften. Bemerkenswerterweise unterstützen die vorliegenden Ergebnisse frühere Hinweise auf

Unterschiede in der Verhinderung sexuell übertragbarer Krankheiten durch verschiedene *Lactobacillus*-Arten.

Untersuchungen zur Bedeutung des Mikrobioms sind nach wie vor erschwert durch das Fehlen von funktionalen Modellen zur hypothesengebundenen Forschung. Diesbezüglich wurden im Rahmen dieser Arbeit Maus-Modelle für Chlamydieninfektionen entwickelt. Es konnte gezeigt werden, dass mittels *C. trachomatis* serovar D und dem mausspezifischen *C. muridarum* nun zwei Modelle zur Untersuchung verschiedener Aspekte von Mikrobiom-Pathogen-Interaktionen verfügbar sind. Diese sollen in zukünftigen Arbeiten genutzt werden, um das urogenitale Mikrobiom zu modulieren und den Einfluss dieser Modulierungen auf sexuell übertragbare Infektionen zu testen.

1 Introduction

1.1 Female infectious infertility

1.1.1 Female infertility is an important worldwide health issue

Tubal factor female infertility is a health issue of growing importance in developing as well as in developed countries. Although assessing infertility is difficult, the World Health Organization (WHO) reports a total of 48.5 million couples worldwide being affected by infertility [1]. In more than ten percent of couples that wish to have children the woman is unable to become pregnant as according to the WHO and the Centers for Disease Control and Prevention (CDC) [1,2]. Furthermore, the likelihood of becoming infertile increases with higher age and couples are postponing their childbirth more and more due to socioeconomic reasons [3]. It is worth mentioning, that current state-of-the-art techniques such as reproductive tubal surgery and *in vitro* fertilization (IVF) are driving factors accelerating health system costs worldwide [4]. There are many factors that can cause infertility in females: on the one hand non-infectious causes such as polycystic ovary syndrome (PCOS) [5], endometriosis, abdomino-pelvic surgery, use of intrauterine devices, and induced surgical abortion [4] play a role. On the other hand, sexually transmitted infections are the main causes of tubal obstruction, endosalpingeal destruction, periadnexal adhesions, and pelvic inflammatory disease (PID) [4]. Infectious causes of infertility have been studied over decades. The current knowledge can be used on a translational basis answering unsolved questions with the aim to reduce infectious infertility in human populations. This will be the main focus of the present study.

1.1.2 Sexually transmitted diseases cause tubal factor infertility

Infertility in females is often caused by infections that are sexually transmitted. Eukaryotic parasites, viruses, and bacteria are able to induce pathologies in the upper urogenital tract ultimately leading to infertility [6]. The two most commonly considered bacterial species playing a role in the development of tubal factor infertility are *Chlamydia trachomatis* and *Neisseria gonorrhoeae* [7], with *C. trachomatis* being the most common sexually transmitted bacterial pathogen worldwide [8]. In most cases asymptomatic [9], infections with *C. trachomatis* can ascend to the upper genital tract causing pathologies such as PID (Figure 1-1), and an estimated three percent of chlamydial infections ultimately lead to tubal factor infertility [10]. Although the mechanisms remain elusive, reinfections and persistent

infections are thought to drive *Chlamydia*-induced pathologies leading to infertility [11,12]. *N. gonorrhoeae* infections ascending to the upper genital tract are considered as a second important agent causing female infertility [13], but in contrast to urogenital chlamydial infections, already the primary acute infection is believed to as cause similar pathological sequels [14] as ascending *C. trachomatis* infections. However, the prevalence of *N. gonorrhoeae* is lower than that of *C. trachomatis* in developed countries [15,16].

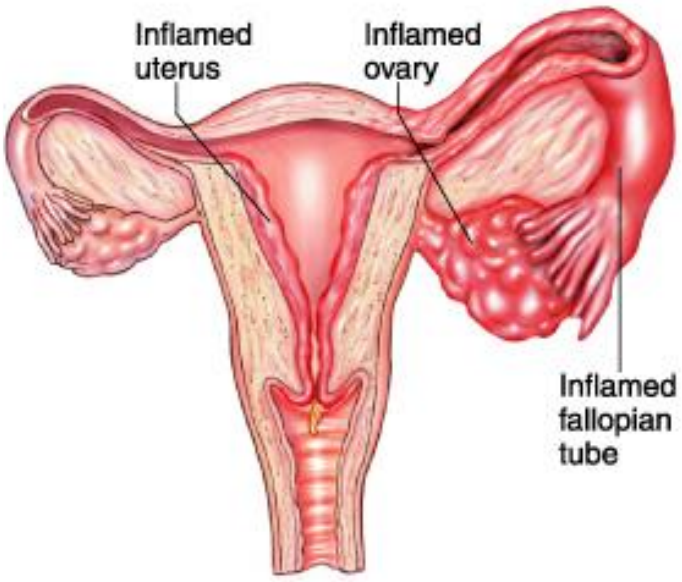


Figure 1-1: Schematic representation of the female urogenital tract showing salpingitis, typically seen in patients with pelvic inflammatory disease (PID). Infection caused PID increases the risk for tubal factor infertility in females. Picture © Reed Group.

Several members of the family Mollicutes are discussed as causing upper genital tract sequels in females that lead to tubal factor infertility. Here, *Mycoplasma genitalium* is an emerging pathogen that was found in infertile females in several studies [17-20], showing strong association with infertility and is therefore considered a causal agent [21,22]. However, although the closely related *Mycoplasma hominis* and *Ureaplasma urealyticum* are often found in females with infertility, adverse pregnancy outcomes, and upper genital tract pathologies, their causal role is not yet clarified. Studies which assessed the relation between bacterial vaginosis (BV) and infertility revealed higher rates of BV in infertile females compared to the healthy population, speaking for a more general impact of vaginal bacterial communities (microbiome) on reproductive health. However, no clear characterization of the causalities between BV and infertility has yet been forthcoming (reviewed in [23,24]). Notably, species such as *M. hominis* and *U. urealyticum* are typical members of BV and the

same is true for *Gardnerella vaginalis*, which is a microbial factor also associated with tubal factor infertility in females [25].

Besides the several bacteria that can cause tubal factor infertility, among the wide range of eukaryotic parasites infecting humans *Trichomonas vaginalis* is the only sexually transmitted one with implications on reproductive outcome. *T. vaginalis* has the potential to cause a range of adverse health outcomes including tubal factor infertility in females [26,27]. Furthermore, sexually transmitted viruses have been linked to adverse reproductive health, with indications for herpes simplex virus 2 (HSV2) to play a role in infertility [28,29] as well as HIV [29]. Patients with HSV2 positive serology were linked to endometritis [28] and HSV2 and HIV were predictive for infertility in a case-control study from Rwanda [29]. In contrast, the most common sexually transmitted virus, human papillomavirus (HPV), is known to cause abortion but has not been shown to impact on female infertility [6].

While STIs have been proven to be a major reason for tubal factor infertility, the derivation of a direct correlation between STI and infertility is hampered by several limitations: (i) acute STIs are often asymptomatic and are therefore not recognized, (ii) chronic infections are missed by current diagnostic strategies, (iii) there is often a long time span between an acute STI-related inflammatory disease (eg. PID) and the recognition of female infertility. Identification of the infectious cause of tubal factor infertility is, therefore, not completely ensured by using anamnestic and serological analysis on previous STIs in combination with visualization of the occluded fallopian tubes (which is currently done in clinical routines). This study therefore takes into account a so far overlooked factor in developing tubal factor infertility: the urogenital microbiome.

1.2 The microbiome of the female urogenital tract

1.2.1 Characterization of the vaginal microbiota in health and disease

The vaginal microbiota has been studied for a long period, and it is broadly accepted that an intact vaginal microbial community is an important factor in vaginal health. The most common members of a healthy vaginal microbiome have thereby been shown to be *Lactobacillus* species [30]. In clinical settings, vaginal health is assessed by microscopic measures such as the Nugent score, which is a health score on the basis of presence/absence and abundance of (i) *Lactobacillus* morphotypes (ii) *Gardnerella vaginalis* or Bacteroides morphotypes (small gram-variable or gram-negative rods) and (iii) curved gram variable rods.

High numbers of *Lactobacillus* morphotypes along with low numbers of the other above-named morphotypes characterize a healthy vaginal microbiome, while high numbers of the latter types in conjunction with low *Lactobacillus* numbers are indicative of bacterial vaginosis (BV), an adverse composition of the vaginal microbiota [31]. However, the etiology of adverse microbial communities in the female urogenital tract is not yet well understood, although next-generation sequencing (NGS) based techniques are offering new perspectives in understanding the complexity of the urogenital microbial communities.

1.2.2 Next-generation-sequencing drives the knowledge on the urogenital microbiome

Modern sequencing technologies, next-generation sequencing (NGS), have enabled huge steps towards the exploration of non-culturable bacteria. In the field of sexually transmitted diseases in connection to commensal bacteria of the urogenital epithelium this has lead to a vast amount of reports on the vaginal microbiota in the past years. In 2011, Ravel et al. published the first study on the vaginal microbiome of reproductive-age, healthy women using 454-pyrosequencing [32]. The authors used vaginal swabs, extracted DNA and sequenced partial 16S rRNA gene amplicons from bacteria. They could show that the vaginal tract of humans can be colonized by different community types. In brief, they described 5 general types (Figure 1-2): Community types 1, 2, 3 and 5 were characterized by one of *Lactobacillus crispatus*, *L. gasseri*, *L. iners* or *L. jensenii* being the dominant bacterial species, meaning that 50 % of all sequences of a sample belonged to one of these species. Community type 4 comprised all samples which could not be assigned to one of the other groups. Typically these were samples with high diversity and low *Lactobacillus* content. This community type consisted predominantly of the taxa *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia* and *Mobiluncus*. In addition, Ravel et al. reported some singletons, ie. rare samples dominated by other bacteria. While most of the females had a *Lactobacillus*-dominated community type, the portion of females with a diverse community was higher in Hispanics and Black compared to Asian and White females [32]. Further have studies elucidated that other community types exist, including a community type dominated by *Gardnerella vaginalis* [33].

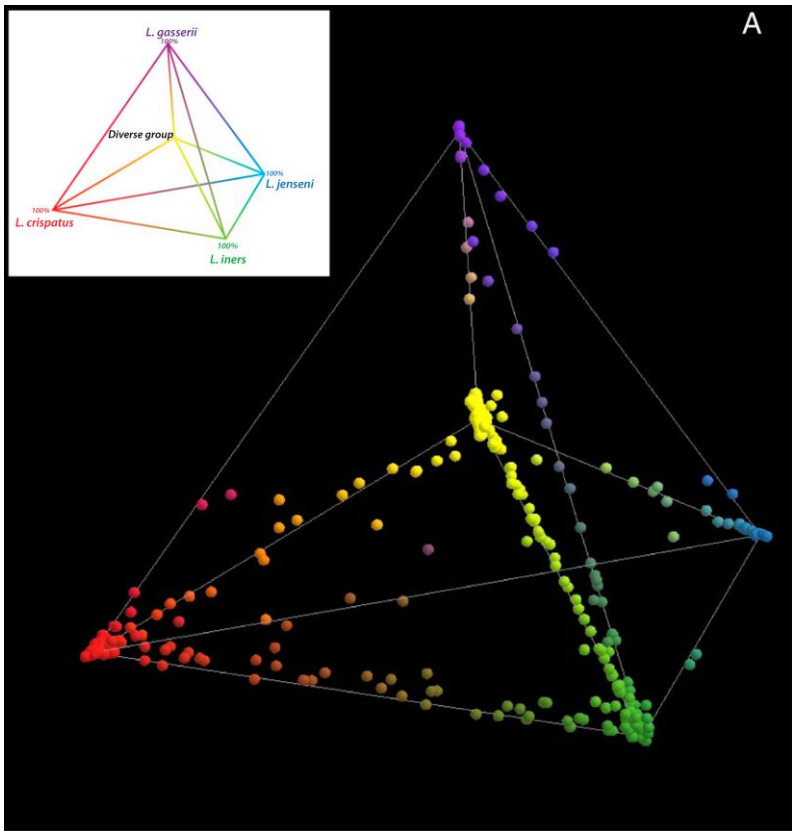


Figure 1-2: 3D representation of the bacterial communities observed by the study of Ravel et al. [32] based on a principal component analysis. Each dot resembles the vaginal microbial community of a single female. Color and position indicate the proportion of four *Lactobacillus* species and the proportion of diversity as depicted in the legend at the upper left part of the figure.

Interestingly, all community types may undergo rapid changes over time or may stay as they are. Although changes occur more often during mensis and in diverse communities, all community types can undergo changes and it is rarely predictable, from which community type to which other type a change might occur and whether or not the new state will change back to the initial state, will remain stable or change to a third state [34,35]. Although increased instability of the community types was associated with the predicted hormonal status during the estrous cycle [35,36], oral contraceptives and medroxyprogesteron-acetat (both substances that impact on the hormonal status of women) showed no evidence of an influence on the microbiome in African sex workers [37]. To understand the role of stability of the microbiome better, several researchers have addressed the impact of sexual activity on the microbial communities. In the past, sexual activity has been shown to be associated with recurrent and enhanced rates of BV [38-41]. However, although sexual activity reduces the stability of the vaginal community types [35], a direct impact of sexual activity on microbial diversity could not yet be demonstrated using modern sequencing technologies [37].

The presence of *Lactobacillus* species in the vaginal tract correlates with low pH, although pH also differs between the *Lactobacillus*-dominated community types [32]. The low pH is mainly due to lactic acid production by *Lactobacillus* species [42], thereby influencing the microenvironment within the female urogenital tract (fUGT). This feature is thought partially to control the urogenital microbiome [43]. Furthermore, lactobacilli are discussed as producers of hydrogen peroxide and bacteriocin-like substances; however the role of these substances in the fUGT is debated controversially [43]. It should be noted here, that other micro-environmental factors such as oxygen availability, might be influenced by the vaginal microbiota and that changing oxygen levels might play a role in maintenance of the fUGT microbiome. The female urogenital tract displays a low oxygen environment (O_2 concentration below six percent) [44] and oxygen concentrations eg. below two percent may have an impact on growth and immune control of *C. trachomatis*, the most common bacterial STI [45].

The high portion of diverse communities in some groups of females without symptoms of BV or other signs of vaginal disease have lead some researchers to question the dogma of a disturbed microbiome associated with increasing Nugent scores. Some authors argue, that these diverse communities or at least some of them might also reflect a healthy microbiome, questioning the validity of classical Nugent score testing for the diagnosis of a healthy vaginal microbiota [32,35]. On the other hand, when looking at women with a diagnosed BV, communities dominated by *Lactobacillus* spp. occur only very rarely and especially the taxa *Gardnerella, Atopobium, Prevotella, Peptostreptococcus, Mobiluncus, Sneathia, Leptotrichia, Mycoplasma,* BV-associated bacterium 1 (BVAB1) to BVAB3, and Lachnospiraceae dominate the vaginal bacterial community [25,46]. Furthermore, BV patients display a high variability in the composition of the microbiome [47]. Accordingly, DiGiulio et al. were able to show that diverse communities are associated with preterm birth [48] and the same study showed a dramatic change of the vaginal microbiome following child delivery that persists for a period up to one year.

1.2.3 The urogenital microbiome and sexually transmitted pathogens: recent findings

In general, many studies have demonstrated a role of the composition of the vaginal microbiome in infectious diseases; for example: *Escherichia coli* colonization in the vaginal tract has been shown to be associated with recurrent urogenital tract infections and to be

inversely correlated with the presence of *Lactobacillus* spp. [49]. Wiesenfeld et al. described BV to be predictive of infections with *N. gonorrhoeae* and *C. trachomatis* upon exposure to infected partners [50] and a role of the BV was also shown in viral infections such as HSV2 and HIV (e.g. [51,52]). However, due to the limitations of the classical methods used in such studies, an analysis of complete microbial communities was not possible neither in healthy nor in pathogenic states until NGS techniques have become available for microbiome and metagenomic sequencing.

Following the analytical possibilities opened up by NGS-based techniques, several studies have shown the variations of the vaginal microbiome as described in chapter 1.2.2. Modern sequencing technologies have also enabled targeted studies of the association of sexually transmitted pathogens with the vaginal microbiome: in the case of *T. vaginalis* infection such studies have shown that the microbiome differs from that of uninfected women when they are diagnosed with a low or intermediate Nugent score. Only uninfected women with a high Nugent score cannot be distinguished from infected women based on the microbiome. It is worthwhile to note that infection with *T. vaginalis* coincides with the presence of either *Mycoplasma hominis* or another, not yet described *Mycoplasma* species [53]. Nunn et al. could show that cervicovaginal mucus (CVM) from women that contained mainly *Lactobacillus crispatus* was able to trap HIV particles while other CMVs (including CMVs that were *L. iners*-dominated, *G. vaginalis*-dominated or featured diverse communities) failed to do so [33]. Similar results have been shown for *L. crispatus*-dominated genital tract secretions when their inhibitory capacity on *E. coli* was tested. *L. crispatus*-dominated secretions displayed high inhibitory activity against *E. coli* while this was not true for secretions dominated by *L. iners* or *G. vaginalis* [54]. While these studies suggest, that only *L. crispatus*-dominated communites have a preventive character with regard to STIs, a recent study proved *Lactobacillus crispatus*, *L. gasseri* and *L. jensenii* to kill *Chlamydia trachomatis* in culture experiments with the acidification by lactobacilli in particular having been been shown to be responsible for this effect [55,56]. However, Nardinini et al. described *L. crispatus* to be the most effective *Lactobacillus* species inhibiting *C. trachomatis* elementory bodies infectivity *in vitro* [57] and females with a *L. crispatus*-dominated community could be shown to have a reduced association with *C. trachomatis* following sexual contact with a *C. trachomatis* infected male partner compared to diverse and *L. iners*-dominated communities [58].

1.2.4 The role of the urogenital microbiome in infectious infertility remains elusive

As described above, many studies point to the influence of the microbial composition in the female urogenital tract on sexually transmitted diseases, which have been linked to upper genital tract sequels ultimately leading to infertility. While numerous studies have been conducted on the influence of an adverse microbiome on pregnancy outcomes such as preterm birth (eg. [48,59,60]), no information is available about the role of the urogenital microbiome in infectious infertility. One part of this study therefore aims to give the first insights into the microbiome of the female urogenital tract in infectious infertility compared to females with non-infectious infertility, fertile controls and female sex workers using NGS-based microbiome sequencing of partial 16S rRNA gene sequences from cervical swabs.

The second part of the present study will address the use of animal models in STI research, since there are currently no models available to test the role of the urogenital microbiome functionally in STIs *in vivo*. Regarding sexually transmitted diseases mouse models have been established for the most important, infertility-causing bacteria. This study now aims to establish and compare different mouse models for the major bacterial cause of infertility: *C. trachomatis.* The intention is to establish a model which can serve as a basis for studying the impact of modulation of the microbiome on sexually transmitted diseases.

1.3 Mice as model organisms for studying sexually transmitted diseases

Studies in humans indicate a role of the microbiome in the defense against sexually transmitted diseases, a global health problem ultimately leading to infectious infertility (see chapter 1.2.3). However, no functional *in vivo* data describing the influence of the microbiome on STIs are yet available. Regarding *in vivo* STI research, the most progress has been made in chlamydial mouse models. Urogenital chlamydial infection models in mice comprise the human pathogenic strain *C. trachomatis* as well as the mouse specific strain *C. muridarum* (formerly known as *C. trachomatis* MoPn) both of which differ in the outcome of the infection [61] and are described in detail in the following chapter.

1.3.1 *Chlamydia trachomatis* as a model for sexually transmitted pathogens in mice

Chlamydia trachomatis is an obligate intracellular bacterium. Its different serovars infect the urogenital tract, the eyes, and the lymphatic system of humans, and it is the most common bacterial STI worldwide. An infection with *C. trachomatis* can lead to severe sequels such as pelvic inflammatory disease (PID) that has the potential of tubal factor infertility in females [10] (see chapter 1.1.2). In most cases the infection can be eliminated using antibiotics such as azithromycin or doxycycline. However, in some cases, treatment failures for unknown reasons are reported [62]. *Chlamydia* possess a characteristic and unique biphasic life cycle consisting of a non-replicating, infectious form (elementory body, EB) and a replicative, non-infectious and strict intracellular form (reticulate body, RB). An aberrant, non-infectious and non-replicating form (persistent *Chlamydia*) is linked to reinfections and severe sequels of the infection including infertility [11,12]. The microenvironment has been shown to influence infection progression. In particular, oxygen supply [45], nutrient availability [63], and pH [55] are important factors influencing the course of the disease. As the microbiome of the female urogenital tract is known to play a key role in maintaining a low-pH microenvironment it is therefore discussed as playing a role in disease prevention in humans [55,56,64].

In the field of chlamydial infections mouse models have been established with varying success. The most widely used model uses a *Chlamydia muridarum* infection in the mouse [65], which leads to a self-limiting infection approximately four weeks lasting causing pathologies similar to human *C. trachomatis* infections (eg. hydrosalpinx) [66]. As in humans, infertility can be induced by this model [61]. Typically, the course of the infection is measured by recovery of viable *Chlamydia* from vaginal swabs and sometimes also via qPCR using gene copy number assays. The pathological outcome is mostly scored by the visibility and severity of hydrosalpinx. Models using vaginal *C. trachomatis* infection are also in use, however, they are hampered by the fact, that *C. trachomatis* does not ascend to the upper genital tract in mice, thereby leading only to a mild infection without pathological symptoms [61]. With the intention of bypassing this limitation, a new technique was developed more recently using *C. trachomatis* serovar L2 as infectious agent causing macroscopically visually pathologies in the mouse urogenital tract. In this context, a non-surgical embryo transfer (NSET) device [67] is used to inoculate the bacteria directly into the uterus horns via the cervical opening [68,69] (see Figure 1-3 for comparison of the pathological outcome of the different models).

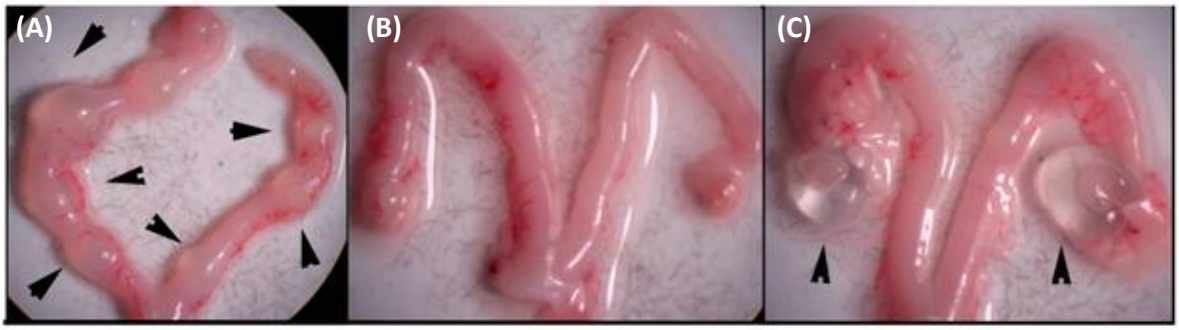


Figure 1-3: Pathological outcome of different chlamydial infection models. Non-surgical transcervical infection using *C. trachomatis* strain L2 leads to swollen nodules along the uterus horns (A), while the same strain inoculated vaginally does not lead to upper genital tract pathologies (B). Vaginal infection with *C. muridarum* leads to hydrosalpinx (C). Black arrows indicate pathologies. Pictures taken from [69].

Animal models in chlamydial research have been mostly used to assess the interplay between host and pathogen, deciphering the host´s innate and adaptive immunity, impact of the ostreus cycle on the infection and the pathological outcome of the infection [61]. More recently coinfection models have been added to this field, taking into account the importance of bacterial interaction in infectious diseases [70,71]. In one such model, co-infection with *N. gonorrhoeae* lead to enhanced gonococcal shedding in the presence of *C. muridarum* [70]. A second, recently introduced co-infection model using *C. muridarum* and a HSV2 infection has shown an impact of a chlamydial pre-infection on a HSV2 infection [71].

A large number of studies have shown the utility of mouse models in STI research on the one hand. On the other hand, an important role of the microbiome for infections has been demonstrated. However, no efforts have been made to study the relevance of the microbial communities as a whole in sexually transmitted infections *in vivo* until today. Setting the basis for functional studies on the influence of the microbiome on STIs, a part of the present study will focus on chlamydial infections, establishing urogenital infection models with (i) the mouse specific *C. muridarum* and (ii) the human *C. trachomatis* serovar D and comparing their suitability for subsequent functional studies on the influence of the microbiome on sexually transmitted diseases.

1.4 Aims of the study

The microbiome of the female urogenital tract contributes significantly to a healthy microenvironment. So far, however, the role of the microbiome in infectious infertility remains unknown so far. Furthermore, diagnosing infectious infertility is complicated and a precise method which is able to diagnose all cases of infertility correctly is still missing. Additionally, no *in vivo* model is yet available for studying the interactions between the

microbiome and sexually transmitted pathogens and their pathogenetic mechanisms. All these open questions need to be addressed to expand our knowledge about the relevance of the microbiome in infectious infertility. This study therefore aims to address the following questions:

(i) Does the cervical microbiome in females with infectious infertility differ from females with non-infectious infertility, female sex workers and fertile females?

(ii) Does the choice of two different, commonly used regions of the bacterial 16S gene impact on the results concerning the urogenital microbiome of the females?

(iii) Are changes in the microbiome predictive for infectious infertility?

(iv) Are mouse models suitable for studying the interactions between the urogenital microbiome and sexually transmitted pathogens?

2 Material and Methods

2.1 Material

2.1.1 Devices

The following table shows all devices used for this thesis.

Table 2-1: Devices

Device	Version	Supplier
Centrifuge	5417R	Eppendorf, Hamburg, Germany
	Megafuge 2.0 R	Heraeus Instruments GmbH, Hanau, Germany
	Multifuge 3 S-R	Heraeus Instruments GmbH, Hanau, Germany
	Rotina 38R	Hettich Lab Technology, Tuttlingen, Germany
Cleanbench	EN 12469	Clean Air Techniek B.V. Woerden, Netherlands
	SterilGard Hood Class II A/B3	Baker Company, Sanford, USA
	Lamin Air HB2472	Heraeus Instruments, Hanau, Germany
Cryocontainer	Mr. Frosty	Nalgene Labware, Rochester, NY, USA
Dispenser pipette	Ripette®	Ritter GmbH, Schwabmünchen, Germany
	Multipette	Eppendorf AG, Hamburg, Germany
Fluorescence microscope	BZ-9000	Keyence, Osaka, Japan
Fluorometer	Qubit 2.0	Invitrogen, Carlsbad, California, USA
Freezer	Ultra low -80 °C	Sanyo, Leicestershire, Great Britain
	Comfort -20 °C	Liebherr, Biberach an der Riß, Germany
	Sportline -20 °C	Bosch, Munich, Germany
Fridge	Profiline	Liebherr, Biberach an der Riß, Germany
Heating block	PCH-2	Grants-Instruments Ltd, Shepreth, Great Britain
	Thermomixer comfort	Eppendorf AG, Hamburg, Germany
Horizontal gel chamber	EasyPhor Maxi	Biozym, Hessisch Oldendorf, Germany

	Horizon 11.14	Biometra, Göttingen, Germany
Imagingsystem Fusion	FX7	Vilber Lourmat GmbH, Eberhardzell, Germany
Incubator (37°C)	H2200-H	Benchmark Scientific, Edison, USA
Incubator (37°C, 5%	CB Series	Binder GmbH, Tuftlingen, Germany
CO_2)	Forma Series II 3131	Thermo Fisher Scientific, Waltham, USA
Magnetic stirrer	RET basic	IKA®-Labortechnik, Staufen, Germany
Microscope	Axiovert 25	Carl Zeiss Microscopy GmbH, Göttingen, Germany
Mouse cage	IVC Green Line II	Tecnicplast, Buguggiate, Italy
Multichannel pipette	Research plus (10µl, 1000 µl)	Eppendorf AG, Hamburg, Germany
Multipette	M4	Eppendorf AG, Hamburg, Germany
pH meter	MP220	Mettler Toledo, Gießen, Germany
Pipette	Reference 10, 100, 1000 µl	Eppendorf AG, Hamburg, Germany
Pipette controller	accu-jet	Brand GmbH, Weimheim, Germany
Power supply	EC-105	Biometra, Göttingen, Germany
Scale	KB 600-2	Kern & Sohn GmbH, Ballingen-Frommen, Germany
Sequencer	GS FLX+	Roche, Basel, Switzerland
	MiSeq	Illumina®, San Diego, California, USA
Shaker	Polymax 1040	Heidolph Instruments GmbH, Schwalbach, Germany
Thermocycler	C1000	Bio-Rad, Munich, Germany
	Light Cycler 480 II	Roche, Basel, Switzerland
Transilluminator UV	TFX 20M	Vilber Lourmat GmbH, Eberhardzell, Germany
Vibrating shaker	VXR basic	IKA®-Labortechnik, Staufen, Germany
Vortex device	REAX 2000	Heidolph Instruments GmbH, Schwalbach, Germany

2.1.2 Consumable supplies

Table 2-2 lists all consumable supplies, which were necessary for completion of this thesis.

Table 2-2: Consumable supplies

Consumable supplies	Supplier
175cm^2-tissue culture flask	Greiner Bio-One GmbH, Frickenhausen, Germany
24-well-tissue culture plate (1.8 cm^2 per well)	Greiner Bio-One GmbH, Frickenhausen, Germany
AlumaSeal® II film for PCR and cold storage, sterile	Sigma-Aldrich Corporation, St. Louis, USA
Biosphere® SafeSeal tubes (1.5 ml, 2 ml)	Sarstedt AG &Co, Nürnbrecht, Germany
Costar® Thermowell® 96-well-PCR Plate	Corning Incorporated, Corning, New York,USA
Cryotubes	Nalge Nunc, NY, USA
Falcon tubes (12, 15, 50 ml)	Sarstedt AG &Co, Nürnbrecht
GeneCatcher	Gelcompany, San Francisco, California, USA
Glass beads	Karl Hecht GmbH & Co KG, Sondheim, Germany
Neubauer counting chamber	Hassa, Laborbedarf, Lübeck, Germany
Non-Surgical Embryo Transfer Device (NSET)	ParaTechs, Lexington, KY, USA
Parafilm	Carl Roth GmbH, Karlsruhe, Germany
Pipette tips	Sarstedt AG &Co, Nürnbrecht, Germany
Reaction tubes (0.5, 1.5 ml)	Sarstedt AG &Co, Nürnbrecht, Germany
Serologic pipette (5, 10, 25 ml)	Sarstedt AG &Co, Nürnbrecht, Germany
Tissue culture dish (60x15 mm)	BD Bioscience, San Jose, USA
Transfer pipette (1 ml)	Sarstedt AG &Co, Nürnbrecht, Germany
Transsystem Amies w/o CH Alluminium Apllicator Rayon Tipped	Copan Diagnostics INC., Murrieta, USA

2.1.3 Chemicals

All chemicals and media used in this thesis are displayed in the following table.

Table 2-3: Chemicals and media

Chemicals	Supplier
Bambanker cryopreservation	Wako Chemicals GmbH, Neuss, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich Corporation, St. Louis, USA
Disodium hydrogen phosphate	Merck KGaA, Darmstadt, Germany
DMSO	Sigma-Aldrich Corporation, St. Louis, USA
dNTP-Set	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Ethanol, absolute	Merck KgaA, Darmstadt, Germany
Fetal bovine serum	PAN-Biotech, Aidenbach, Germany
GeneRuler 100 bp DNA Ladder	Thermo Fisher Scientific, Waltham, USA
Glycerol	Merck KgaA, Darmstadt, Germany
Glycine	Sigma-Aldrich Corporation, St. Louis, USA
HEPES	Invitrogen GmbH, Darmstadt, Germany
Isopropanol	Fisher Scientific, Waltham, USA
L-glutamine	Lonza, Veriers, Belgium
Loading dye (6x)	Thermo Fisher Scientific, Waltham, USA
Methanol	Merck KgaA, Darmstadt, Germany
Non-essential amino acids	HyClone, Thermo Fischer Scientific, Schwerte, Germany
Potassium chloride (KCl)	Merck KgaA, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck KgaA, Darmstadt, Germany
RedSafe	iNTRON Biotechnology, Korea
Saccharose	Merck KgaA, Darmstadt, Germany
Sodium chloride (NaCl)	Merck KgaA, Darmstadt, Germany
Sodium hydrogen phosphate monohydrate ($NaH_2PO_4xH_2O$)	Merck KgaA, Darmstadt, Germany
Tris HCl	Sigma-Aldrich Corporation, St. Louis, USA
Tris(hydroxylmethyl)aminomethane	Bio-Rad Laboratories GmbH, Munich, Germany
Trypan blue (0.4%)	Sigma-Aldrich Corporation, St. Louis, USA
Trypsin/EDTA	Invitrogen GmbH, Darmstadt, Germany

Tween20	Serva Electrophoresis GmbH, Heidelberg, Germany

2.1.4 Buffers and solutions

Used buffers and solutions used for this thesis as well as their ingredients are listed below in Table 2-4.

Table 2-4: Buffers and solutions

Buffers/ solutions	Ingredients
Antibiotic GVA cocktail	16.6 mg Gentamycin, 41.6 mg Vancomycin, 1.6 g Amphotericin B, ad 10 ml destilled water
Cycloheximide	1 g cycloheximide, ad 1 ml destilled water
Loading dye colorless	50 mM Tris (pH8), 40 mM EDTA, 40 % Sucrose, ad 100% destilled water
PBS buffer	80 g NaCl, 2 g KCl, 11.5 g $NaH_2PO_4x12H_2O$, 2 g KH_2PO_4, ad 1 l destilled water, pH 7.2
SPG buffer	75 g saccharose, 2.47 g Na_2HPO4, 0.36 g NaH_2PO_4, 0.72 g L-glutamic acid, ad 1 l destilled water, pH 7.3
TBS buffer (10x)	24.2 g Tris, 80 g NaCl, ad 1 l destilled water, pH 7.6

2.1.5 Cultivation media

Table 2-5 comprises the cultivation media for bacteria and cell culture lines used in this thesis.

Table 2-5: Cultivation media

Cultivation media	Ingredients	Supplier
Hep-2 standard medium	RPMI medium 1640 with 5 % FBS, 10 mg/ml L-glutamine (Lonza), NEAA (100x)	Invitrogen GmbH, Darmstadt, Germany
Long-time cultivation medium	DMEM (4.5 g/l) with 10% FBS, 1 M HEPES	Invitrogen GmbH, Darmstadt, Germany

2.1.6 Antibodies

Antibodies used in this thesis for immunostaining are listed in the following Table 2-6.

Table 2-6: Immunostaining antibodies

Antibody	Dilution	Supplier
primary antibody		
Anti-Chlamydia-LPS (mouse)	1:50	Prof. H. Brade, FZ Borstel, Germany
secondary antibody		
Anti-mouse, FITC conjugated	1:250	Dako Germany

2.1.7 Enzymes

The following table shows the Enzymes used in this thesis.

Table 2-7: Enzymes

Enzyme	Supplier
Phusion High Fidelity Polymerase	Thermo Fisher Scientific, Waltham, USA
OB Protease	Peqlab, Darmstadt, Germany

2.1.8 Kits

The table below lists all commercially available kits, which have been used in this thesis.

Table 2-8: Kits

Kit	Supplier
GS FLX Titanium Sequencing Kit	Roche, Basel, Switzerland
MinElute Gel Extraction Kit	Qiagen, Düsseldorf, Germany
MiSeq Reagent Kit v3 (600 cycles)	Illumina®, San Diego, California, USA
PowerSoil® DNA Isolation Kit	MO BIO Laboratories,INC., Carlsbad, California, USA
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific, Waltham, Massachusetts, USA
UTM Kit	Copan Diagnostics INC., Murrieta, USA
LightMix® Kit *C. trachomatis*	TIB MOLBIOL, Berlin, Germany
LightMix® Kit *N. gonorrhoea*	TIB MOLBIOL, Berlin, Germany
LightMix® Kit M. *hominis/genitalium*	TIB MOLBIOL, Berlin, Germany
LightMix® Kit *U. urealyticum*	TIB MOLBIOL, Berlin, Germany

2.1.9 Primer for PCR

All primers were obtained as lyophilisate from Metabion (Planegg). Primers were dissolved according to the supplier's protocol to 100 µM in RT-PCR Grade Water (Thermo Fisher Scientific). Aliquots were diluted to 10 µM and 2 µM final concentrations. The Sequences of the primers are listed at the appendix (Table 6-1 and Table 6-2).

2.1.10 Organisms, cell lines and human samples

2.1.10.1 Organisms

In the following table all organisms used in this thesis are depicted.

Table 2-9: Organisms

Organism	Supplier
Chlamydia trachomatis serovar D	ATCC, Manassas, USA
Chlamydia muridarum NIGGII	ATCC, Manassas, USA
Mus musculus C57BL/6	Charles River

2.1.10.2 Cell lines

Table 2-10 shows the used cell lines and their corresponding tissues/ organisms.

Table 2-10: Cell lines

Cell line	Organism/ tissue	Reference
HEp-2	Human / epidermoid laryngeal carcinoma	ATCC, Manassas, USA

2.1.10.3 Human samples

Cervical swabs were obtained from different medical surgeries using UTM™ kits from Copan Diagnostics INC. (Murrieta, USA) Swabs were taken by expert physicians avoiding contamination of the swabs with other parts of the human body. Swabs were stored in UTM media at -80 °C.

Serum of all study participants was collected from 10 ml venous blood per individual. The blood was therefore centrifuged at 3500 rpm for 3 min and the supernatant collected.

2.2 Ethics declaration

The work on human samples within this study was approved by the ethics commitee of the University of Lübeck at the 05.02.2012 with the reference number 11-185.

2.3 Declaration of consent and information of study participants

All study participants which provided samples for this study, were informed about the purpose of the study and usage of the data (see appendix, chapter 6.2) and signed a declaration of consent (see appendix, chapter 6.4) for participation in this study.

2.4 Questionnaire

Each study participant answered a questionnaire regarding personal demographic and anamnestic data (see appendix, chapter 6.5). The raw data which were used for graphical visualization and statistical analysis (see chapter 2.11.3) were kindly provided by Kathrin Gillmann from her medical doctoral thesis [72].

2.5 Clinical diagnostic analysis

The raw data concerning diagnostics for acute sexually transmitted infections and serological testing for antibodies against *C. trachomatis* which were used for the clinical characterization of the study groups in this thesis were kindly provided by Kathrin Gillmann from her medical doctoral thesis [72], except for the data for *M. genitalium* and *M. hominis* PCR-detection (see chapter 2.5.1). Graphical visualizations and statistical analysis of this clinical characterization were carried out as part of this thesis (see chapter 2.11.3).

2.5.1 Pathogen detection by PCR

For the direct detection of pathogenic bacteria of the human female urogenital tract, cervical swabs were subjected to PCR-testing of *Chlamydia trachomatis*, *Mycoplasma genitalium*, *M. hominis, Ureaplasma urealyticum* and *Neisseria gonorrhoeae* using the corresponding LightMix® Kits (TIB MOLBIOL) and following the distributor´s instructions. qPCRs were carried out using Light Cycler 480 II (Roche).

2.5.2 Pathogen detection by cultivation

Standard bacterial cultures for *Candida* spp., Group B *Streptococcus*, *Gardnerella vaginalis*, *Escherichia coli,* and *Klebsiella* spp. were used for diagnostic analysis from cervical swabs. The agar plates used are displayed in Table 2-11.

Table 2-11: Agar plates used for standart bacterial culture from cervical swabs

Agar	Supplier
Columbia agar + 5 % sheep blood	bioMérieux, Marcy-l´Étoile, France
Gardnerella agar	bioMérieux, Marcy-l´Étoile, France
Mac Conkey agar	bioMérieux, Marcy-l´Étoile, France
chromID™ CPS® Elite	bioMérieux, Marcy-l´Étoile, France
Chocolate agar + PolyViteX™	bioMérieux, Marcy-l´Étoile, France

2.5.3 Serology of *C. trachomatis*

The serum of each study participant was tested for the presence of IgG- and IgA-antibodies targeting *C. trachomatis* using *recom*Well *C. trachomatis* IgG/IgA ELISA (Mikrogen) following the distributor´s instructions. *recom*Line immunoblots for IgG- and IgA-antibodies targeting

the *C. trachomatis* antigens MOMP, OMP2, TARP, CPAF, and HSP60 (Mikrogen) were carried out following the distributor´s instructions.

2.6 Molecular biological methods

2.6.1 DNA isolation

Bacterial DNA was isolated from cervical swabs using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). In a first step, the swabs were thawed at RT vortexed for 1 min on highest speed. One ml buffer was used to perform DNA isolation using the supplied protocol of the kit with some exceptions: A 2 h incubation with 0.4 units OB Potease (Peqlab) was included after adding solution C1 and the sample to the PowerBead Tubes and instead of vortexing this mixture, the samples underwent homogenization using the PowerLyzer supplied by MO BIO Laboratories. As the sample volume was very high, solutions C2, C3 and C4 were used in double amounts.

The obtained DNA was stored at -20 °C until further usage.

2.6.2 Polymerase chain reaction

Partial sequences of bacterial 16S rRNA genes were amplified using polymerase chain reaction (PCR) following a barcoding approach. Therefore, the primer each contained an overhang with a unique sequence (index) and every sample received a unique combination of indices using forward and reverse primers for sequencing on a MiSeq sequencer. For sequencing on a GS FLX+ sequencing system only the reverse primer contained an index. For amplification of bacterial DNA following mix was pipetted:

RT-PCR grade water	8.25 µl
5 x HF Buffer	5 µl
10 µM dNTPs	0.5 µl
2 mM Primer	each 5 µl
DNA	1 µl
Phusion Polymerase	0.25 µl
Total	25 µl

The amplification was performed using following cycling conditions:

98 °C	5 min	
98 °C	9 sec	30 x
55 °C	60 sec	
72 °C	90 sec	
4 °C	∞	

2.6.3 Agarose gel electrophoresis

Gel electrophoresis was performed using 1.5 % Agarose (Peqlab) in 1 x TAE. Agarose was dissolved directly prior to usage in a microwave and 1:20,000 RedSafe was added to the gel before cooling.

The size of amplicons was determined using a GeneRuler 100 bp DNA Ladder from Thermo Fisher Scientific.

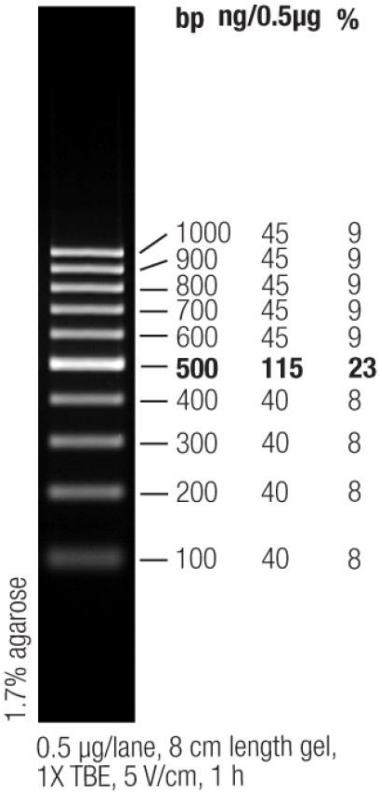


Figure 2-1: Thermo Scientific GeneRuler 100 bp DNA Ladder. This ladder was used for size determination and estimation of DNA amount of amplicons. Source: Thermo Scientific.

Based on the amount of marker used, the DNA concentration of amplicons was estimated using the same marker. The marker was therefore added to the gel with an DNA amount of 0.5 µg and the intensities of the amplicons were compared to the intensities of the 500 bp band (115 ng/0.5 µg marker) for intense amplicons or the 600 bp band (45 ng/0.5 µg marker)

for low-intensity amplicons of the marker using a Fusion FX7 imager and the analytic software Bio1D.

2.6.4 Elution of DNA from agarose gels

DNA was eluted from agarose gels using the MinElute Gel Extraction Kit from Qiagen following the instruction from the supplier´s protocol. Elution was done twice with 10 µl each time and the total volume of 20 µl eluted DNA solution was pooled.

2.6.5 Next-generation-sequencing

2.6.5.1 454-Pyrosequencing (Roche)

The barcoded samples from the PCRs were pooled in equimolar amounts and sequenced on a GS FLX+ sequencing system by colleagues at the Max Planck Institute of Evolutionary Biology (Plön) following the distributors instructions.

2.6.5.2 Sequencing-by-synthesis (Illumina)

Barcoded amplicons of each sample were pooled in equal concentrations prior to sequencing. Sequencing was performed by collegues of the Max Planck Institute of Evolutionary Biology (Plön) using a MiSeq desktop sequencer (Illumina) following the approach from Kozich et al [73].

2.7 Animal experiments

2.7.1 Chlamydial infection mouse models

Female C57BL/6J mice with an age of 8 weeks were purchased from Charles River (Sulzfeld, Germany). The mice were kept in individually ventilated cages (IVC Green Gine II, Tecnicplast) cages with 4 animals per cage. Not earlier than 1 week after the arrival of the mice, their oestreous cycle was synchronized using Depo-Clinovir®. Infection of the mice was performed 7 days after synchronisation. The mice were inoculated with 1 x 10^6 or 4 x 10^6 IFUs *C. trachomatis* serovar D transcervically or 1 x 106 IFUs *C. muridarum* NIGGII intravaginally. Transcervical infection was performed using the Non-Surgical Embryo Transfer (NSET) Device (ParaTechs); each mouse was fixed in a tube and a speculum was inserted into its vagina. The NSET device was used to apply the infectious agent through the cervical opening directly into one of the uterus horns.

Following infection, swabs were taken from infected and control mice every 3 days. The swabs were used to assess the course of infection by measuring revoverable IFUs using a modified recovery assay. The swabs were placed in 750 µl (*C. trachomatis*) or 1 ml (*C. muridarum*) sterile PBS in sterile tubes containing glass beads (10 ml tubes) and put on a vibrating shaker for 5 min to resuspend bacteria from the swab. Between 50 µl and 250 µl of the resuspended bacteria were used for recovery assay as described previously (chapter 2.9.2). The recovery assay was accomplished by using 10 µl/ml antibiotic GVA cocktail to prevent growth of other bacteria than *Chlamydia* and growth of fungi.

200 µl of the resuspended bacteria in PBS were stored at -80 °C for DNA isolation and further analysis.

2.7.2 *C. muridarum* model scoring system

Pathologies following *C. muridarum* infection were scored based on visibility and size of the hydrosalpinx:

Table 2-12: Scoring system for pathologies following *C. muridarum* infection

Score	Visibility/Size of the hydrosalpinx
0	No hydrosalpinx
1	Hydrosalpinx visible only using magnification
2	Hydrosalpinx visible by the eye, smaller than the ovar
3	Hydrosalpinx size equal to ovar
4	Hydrosalpinx size bigger than ovar

2.8 Cell culture

2.8.1 Long term cultivation of HEp-2 cells

HEp-2 cells were cultivated using the long-term cultivation medium in an atmosphere of 5% CO_2 at 37 °C. Sterile 175-cm^2 flasks with 25 ml medium were used for cultivation.

2.8.2 Passaging

For cell subcultivation, the culture medium was removed and discarded. The cells were washed with phosphate buffered saline (PBS). Afterwards, trypsin-EDTA solution was added to detach the cells from the bottom of the flask within 5 min at 37 °C. Fresh medium was

added to the cell-trypsin solution for cell resuspension. The cells were split 1:4 – 1:8 and transferred into a new flask.

2.8.3 Freezing and thawing

Cells were detached with trypsin-EDTA solution after reaching confluence. The cells were then resuspended in 4 ml Bambanker medium. Following centrifugation for 5 min at 200 x g, the supernatant was discarded and the cell pellet was resuspended in 1 ml freezing medium. 1 ml of the cell suspension was transferred into cryotubes, which were temporarily stored in a Mr. Frosty™ Freezing Container (Thermo Scientific) at -80 °C to ensure a cooling rate of 1 °C/minute. Finally, the cryotubes were stored in liquid nitrogen at -210 °C.

Cells were thawed in a preheated water bath at 37 °C and the thawed cells were put in a sterile 175-cm^2 flask supplemented with 25 ml long term cultivation medium. After 24 h the mixture of freezing medium and long term cultivation medium was replaced by 25 ml long term cultivation medium. Cells were then treated further as described above.

2.8.4 Counting

10 µl cell suspension were diluted with 80 µl PBS and 10 µl trypan blue. 10 µl of this dilution were pipetted on a Neubauer counting chamber (depth 0.1 mm). Four squares were counted with a 10x magnification. Intact cells showed no staining, while dead cells appeared blue, since the blue dye diffuses through the cell membrane of dead cells. The cell number of the suspension was determined as follows:

$$cells/ml = \frac{number\ of\ cells\ counted \times dilution\ factor \times 10^4}{number\ of\ counted\ squares}$$

2.9 Infection biology

2.9.1 Preparation of chlamydial stocks

$6x10^6$ HEp-2 cells were seeded per well in twelve 6-well plates in 2 ml long term cultivation medium. 1 µg/ml cycloheximide was added after 24 h incubation at 37 °C with 5% CO_2. The cells were infected with the respective chlamydial species (4 IFUs/cell) by 1 h centrifugation at 700 rcf at 30 °C and incubated for 48 h at 37 °C with 5% CO_2. The infected adherent cells were removed with a cell scraper and disrupted with glass beads for 10 min on a vibrating

shaker. Afterwards, the cell debris was centrifuged 5 min at 200 rcf and 4 °C. An additional centrifugation step with the *Chlamydia* containing supernatant for 90 min at 15500 rcf at 4 °C followed. The *Chlamydia*-pellet was resuspended in 2 ml SPG buffer and stored in 20 µl aliquots at -80 °C.

2.9.2 Recovery assay

Vaginal swabs from mice were transferred into a 10 ml Falcon containing 1 ml glass beads and 0.75 ml or 1 ml sterile and chilled PBS. Bacterial cells attached to the swab were suspended and disrupted on a vibrating shaker for 5 min. A cell layer with long-term cultivation medium in a 24-well plate was supplemented with 1 µg/ml cycloheximide and 10 µl antibiotic GVA cocktail. A 24-well was infected with 5 to 250 µl of the suspension from the swab. Following mixing of the first well, another 250 µl were removed from the first well for infection of the next well. This step was repeated for 6 wells to ensure a dilution series of 1:5 for each sample. Afterwards, the 24-well plate was centrifuged 1h at 700 rcf, 30 °C. The medium was removed after 30 h incubation at 37°C with 5% CO_2 and the cells were fixed with methanol at -20 °C. Chlamydial inclusions were visualized with an indirect immunofluorescence test (IFT, see chapter 2.10.1) and counted to determine the recovery rate.

2.9.3 Quantifying *Chlamydia*

For quantification of *Chlamydia*, chlamydial inclusions within the dilution series from recovery assays were stained with an indirect IFT and counted as described below. Pictures of 24 visual fields of a well were taken and merged into one field using a Keyence microscope and software. The inclusions of the merged field were counted. Based on the volume of the *Chlamydia*-suspension and its dilution factor a formula for the determination of the *Chlamydia* content was obtained by using the size of the merged visual field and the size of a 24-well (200 mm^2). Using a 20-fold magnification the merged field size was 5.3 mm^2.

$$IFU/\mu l = \frac{counted\ inclusions \times size\ of\ a\ 24\ well}{applied\ volume \times dilution\ factor \times size\ merged\ field}$$

2.10 Microscopy

2.10.1 Indirect immunofluorescence of *Chlamydiae*

Indirect immunofluorescence was performed in a 24-well plate. The cell medium was discarded and replaced with -20 °C methanol for fixation. Afterwards the methanol was discarded and the cells were washed with PBS. Then 250 µl of the mouse anti-chlamydial lipopolysaccharide (LPS) antibody were applied to the cells and incubated for 45 min at 37°C. After applying the antibody, cells were washed twice with PBS. The cells were incubated with 250 µl of the FITC conjugated second antibody for 30 min at 37 °C. The cells were washed twice with PBS. The chlamydia content was quantified with a fluorescence microscope.

2.11 Bioinformatics and statistics

2.11.1 Software

The software needed for this thesis is depicted in Table 2-13.

Table 2-13: Software

Software	Distributor	Freeware/License
Acrobat Reader	Adobe INC., Delaware, USA	License
Bio1D	Vilber Lourmat, Eberhardzell, Germany	License
Excel	Microsoft Corporation, Redmond, Washington, USA	License
BZII Analyzer	Keyence, Osaka, Japan	License
BZII Viewer	Keyence, Osaka, Japan	License
mothur	Mothur project	Freeware
PICRUSt	Huttenhower lab	Freeware
R	R-Project	Freeware
RStudio	RStudio	Freeware
Stirrups	SourceForge	Freeware
Usearch	drive5	Freeware
Word	Microsoft Corporation, Redmond, Washington, USA	License

2.11.2 Processing of raw sequencing data

Fastq files were processed using mothur version 1.35.0 [74]. Contigs were produced of forward and reverse sequences and any sequence was removed if it had ambiguous bases, a homopolymer length > 9 or a size longer than the amplified fragment. The remaining sequences were aligned using a customized SILVA reference data base [75] and unaligned sequences were removed. Chimeras were detected using the UCHIME algorithm [76] as implemented in mothur and removed from the data set. Classification of the sequences was performed using the mothur-formatted RDP [77] training set version 9 with a cutoff of 80 and non-bacterial sequences were removed. Further analysis was done on a random subset of 2500 reads/sample either using operational taxonomic units (OTUs) clustered with a similarity threshold of 97 % or based on taxonomic assignment. To keep a consistent classification on genus and species level, Stirrups was used for taxonomic assignment with a reference taxonomy specifically produced for vaginal microbiota [78].

2.11.3 Graphical and statistical evaluation

All statistics and graphical visualizations were produced using R version 3.2.2 [79]. The prevalence of diagnostic parameters was tested globally over all four groups using Pearson´s Chi-square test with Holm correction for the number of calculations within each set of diagnostic tests (PCR-testing, cultivation of pathogens, IgG-serology and IgA-serology). Fisher´s exact test was used for subsequent pairwise comparison of significant parameters. Differences in relative abundances of bacterial genera from the microbiome sequencing were tested for their significance using Kruskal-Wallis rank sum tests with Benjamini-Hochberg correction for the number of performed tests. Subsequent pairwise comparisons were performed for significant results using the Wilcoxon rank sum test with continuity correction. Alpha diversity for the microbiome data was assessed by calculating Simpson´s and Shannon´s diversity indices for each sample on the basis of OTUs using R package vegan [80]. Differences in alpha diversity were calculated using the Kruskal-Wallis rank sum test followed by pairwise Wilcoxon rank sum tests with Benjamini-Hochberg correction. The influence of sexual activity on the bacterial diversity was assessed by performing linear regression analysis and computing Pearson´s product-moment correlation between Shannon´s diversity indices and the frequency of sexual intercourse/month. A heatmap of the 25 main taxa was produced using R package BoutrosLab.plotting.general [81]. Bacterial communities were clustered in community types: A community type was determined as being dominated by *Lactobacillus crispatus*, *L. iners*, *L. gasseri, L. jensenii* or *Gardnerella vaginalis* if a sample consisted of >

50 % sequences of the respective species. Otherwise the community was stated to be diverse. Differences in proportion of community types were assessed using Chi-square testing.

2.11.4 Probability modelling

A prediction model for suffering from infectious infertility was developed based on a subset of the clinical and sequencing data. FSW were excluded from the model. The prediction was performed using binary logistic regression with the following parameters as predictors: (i) detection of bacterial pathogens by culture/PCR, (ii) IgA and IgG- immunoblots against epitopes of *C. trachomatis* and (iii) the most abundant ten taxa from the sequencing analysis. Samples were categorized as suffering from infectious infertility when they reached a threshold of 0.2 in the probability prediction. Other samples were categorized as not suffering from infectious infertility. The prediction outcome and goodness-of-fit of the model was assessed by refrence to (i) the numbers of correct predictions, (ii) the overall accuracy and (iii) using McFadden´s pseudo R^2. ROC curves displaying true vs. false positive rate were generated using r-package ROCR [82], the corresponding areas under curve were computed and a 95 % confidence interval (CI) was given using r-package pROC [83].

3 Results

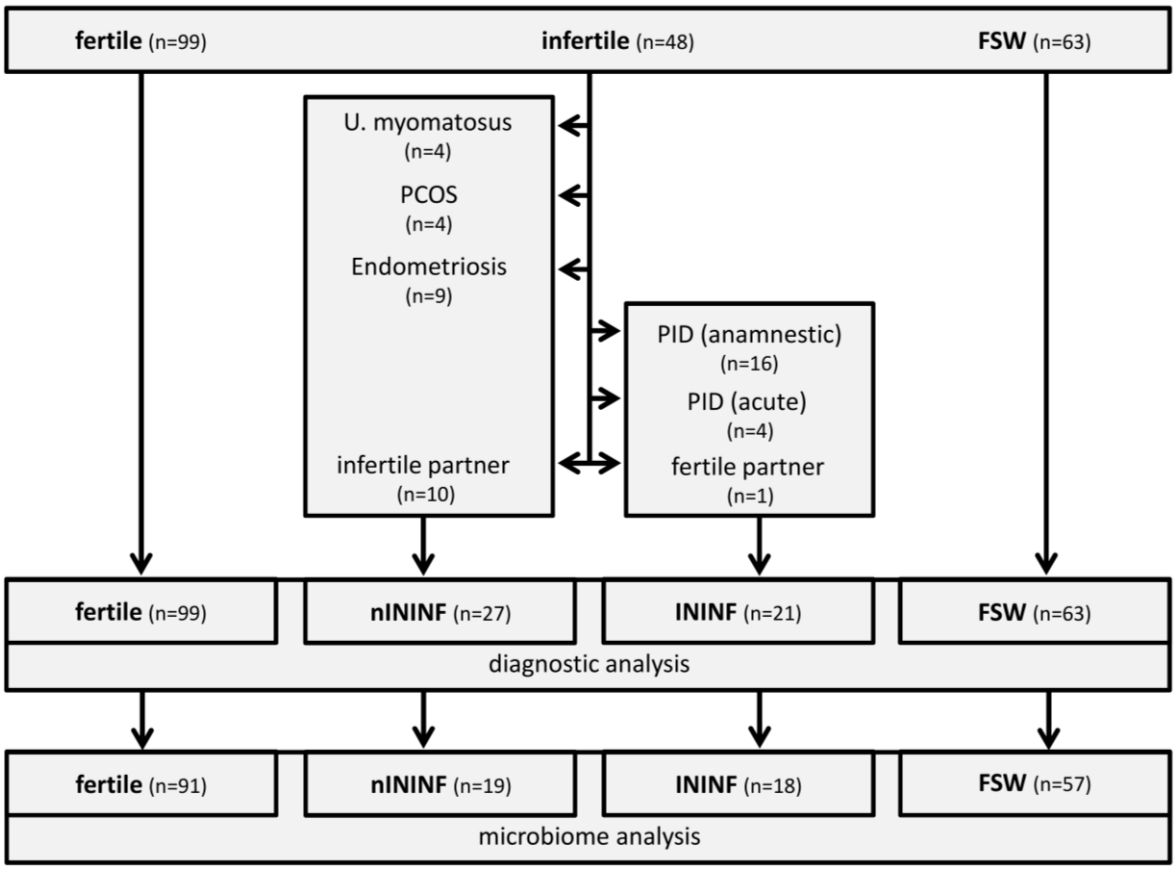
3.1 The microbiome of females suffering from infectious infertility

Infectious infertility in females is a major health issue worldwide, caused mostly by infections ascending from the lower to the upper female genital tract. A lot of research has hitherto focused on the sexually transmitted pathogens causing infectious infertility. Although recent findings indicate a role of the microbiome in the control of sexually transmitted diseases, no information is available about the role of the microbiome in infectious infertility. This study has therefore undertaken a comprehensive assessment of the anamnestic and clinical parameters used in diagnosing infectious infertility with a particular focus on the analysis of the cervical microbiome of females with infectious infertility (ININF), females with non-infectious infertility (nININF), female sex workers (FSW) and fertile females. Microbiome sequencing was assessed using two different regions (V1/V2 and V3/V4) of the bacterial 16S gene, which were compared during all steps of analysis. Finally, a prediction model for infectious infertility was developed.

3.1.1 Study inclusion criteria and study groups

A total of 210 females were recruited for this study of the cervical microbiome in infectious infertility from 2012 to 2014 (Table 3-1). Three main groups were prospectively sampled by expert physicians: a group of fertile females from an Outpatient Clinic in Lübeck (fertile females, n=99), females from couples which consulted the Fertility Clinic of the University Clinic Schleswig-Holstein, Campus Lübeck (infertile females) and female sex workers from the Center of Sexual Health in Berlin (FSW, n=63). Within the infertile group, 27 females were classified as having nININF on the basis of the following clinical parameters: Uterus myomatosus, polycystic ovarian syndrome (PCOS), endometriosis or proven infertility of the male partner. History of pelvic inflammatory disease (PID) and females with tubal occlusion due to acute or chronic PID and infertile females with a fertile male partner without evidence for a nININF were combined in the infectious infertility group (ININF, n=21). A subset of 99 fertile females, 19 females with nININF, 18 females with ININF and 57 FSW yielded sufficient sequencinig reads and underwent cervical microbiome analysis of the V1/V2- and V3/V4 region of the bacterial 16S rRNA gene.

Table 3-1: Classification of the study groups. The group of fertile women and female sex workers (FSW) were randomly sampled in the Center of Sexual Health and the Outpatient Clinic. Women from the Fertility Clinic were separated into women with non-infectious infertility (nININF) and infectious infertility (ININF) on the basis of clinical criteria.



U. myomatosus: Uterus myomatosus; PCOS: Polycystic ovarian syndrome; PID: Pelvic inflammatory disease.

3.1.2 Clinical characterization of the study groups

Evaluations of the self-reported questionnaire concerning age, frequencies of sexual intercourse and the use of contraception are depicted in Table 3-2. Fertile females were older and reported less sexual intercourse compared to the other groups. nININF and ININF reported nearly no use of contraception, while contraception was standard for fertile females and FSW reported that they are mostly using condoms.

Table 3-2: Demographic characteristics and sexual behaviour of the study groups

		Fertile	nININF	ININF	FSW
Age [years]	Min	26	24	23	18
	Median	38	32	35	28
	Mean	37.5	31.8	33.8	31.5
	Max	45	43	43	57
Sexual intercourse/month [%]	<6	66.6	33.3	31.6	16.7
	7-10	23.1	41.7	31.6	11.7
	>10	10.3	25	36.8	71.7
Contraception [%]	Yes	23.5	96	90.5	8.2
	No	76.5	4	9.5	91.2
Type of Contraception [%]	Condom	17.3	4	9.5	85.2
	Oral contraception	37.8	0	0	24.6
	Intrauterine device	22.4	0	0	3.3

Within the questionnaire the group of FSW reported significantly more infections with *C. trachomatis* ($p<0.01$) and *N. gonorrhoeae* ($p<0.05$) in comparison to fertile controls (Figure 3-1). Furthermore, the group of ININF significantly differed in the frequency of previous *C trachomatis* infections in comparison to fertile controls ($p<0.01$) and females with nININF ($p<0.05$). No differences between the groups were observed for reported infections with HPV, HSV, *Treponema pallidum*, HIV or Hepatitis B and C.

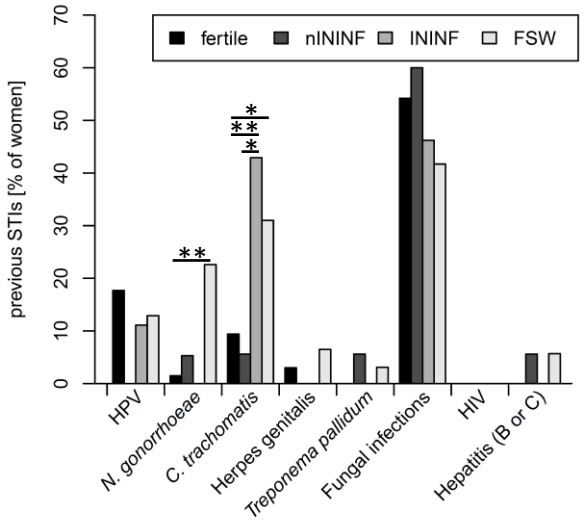


Figure 3-1: Self-reported data on previous sexually transmitted infection in the female cohort according to the study groups. Significantly enhanced rates of females reporting STIs are present in FSW (*N. gonorrhoeae* and *C. trachomatis)* and ININF (*C. trachomatis)* only (Fisher´s exact test for groups passing Chi-square testing with Holm correction for number of tests: **p*<0.05, ***p*<0.01).

Increased rates of testing positive for *U. urealyticum* (41.30 %), *N. gonorrhoeae* (7.90 %), *M. genitalium* (9.50 %) and *M. hominis* (34.90 %) in FSW were observed, while no significant differences were observed for other pathogens between the the groups (Figure 3-2A/B). Subsequently, immunoblotting against the chlamydial proteins MOMP, OMP2, TARP, CPAF and HSP60 was performed. In contrast to fertile controls females with ININF had IgG antibodies targeting chlamydial antigens MOMP, OMP2, CPAF and HSP60 significantly more often. IgG antibodies against CPAF and HSP60 were also significantly enhanced in ININF when compared to nININF. FSW exhibited significantly higher frequencies in the detection of IgG antibodies against MOMP, OMP2, TARP and CPAF compared to both fertile controls and nININF. Furthermore, HSP60 IgG antibodies showed a significant reduction in FSW compared to ININF (Figure 3-2C). Significantly higher frequencies of IgA antibodies against OMP2 and TARP were observed for FSW, and OMP2 and HSP60 for ININF, when compared to the fertile controls (Figure 3-2D). *C. trachomatis* antibodies against IgG and IgA were additionally tested using ELISA, showing significantly enhanced prevalence for Antibodies against *C. trachomatis* in ININF and FSW compared to fertile females and nININF (Figure 3-3). When all Immunoblot results were taken together, the ELISA results were confirmed for IgG, however, IgA antibodies were significantly enhanced only in FSW compared to fertile females (Figure 3-3).

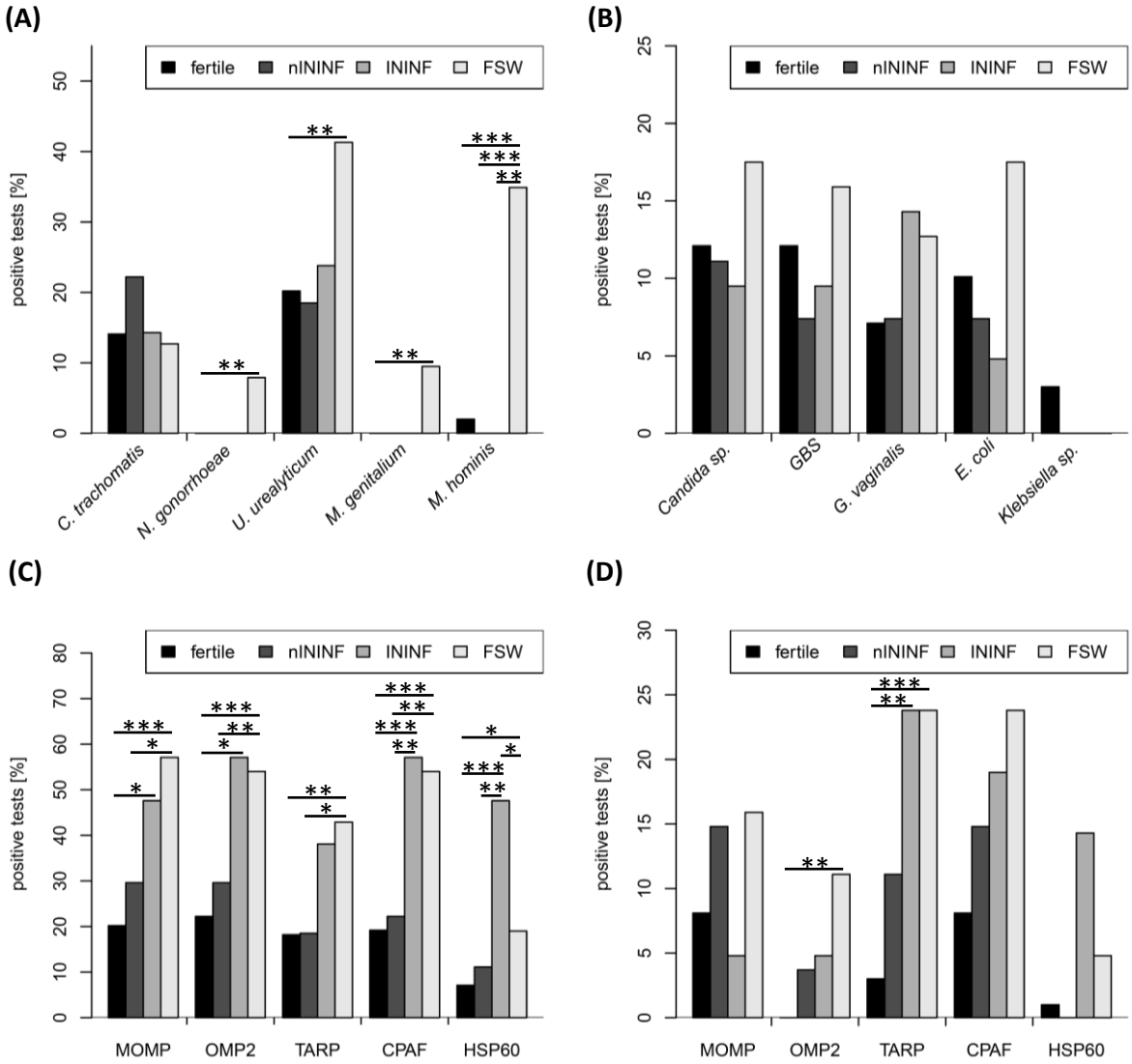


Figure 3-2: Results of the diagnostic testing of the study groups. Tests for sexually transmitted infections by PCR *(A)* showed enhanced rates for *N. gonorrhoeae, U. urealyticum, M. genitalium*, and *M. hominis* in FSW. Conventional bacterial cultures *(B)* from cervical swabs showed no significant differences between the study groups. Serological testing of IgG *(C)* antibodies targeting the epitopes MOMP, OMP2, TARP, CPAF and HSP60 revealed significantly enhanced rates in ININF and FSW for all targets except for TARP in INFINF, while ININF and FSW differed only in their prevalence for HSP60. IgA antibodies *(D)* targeting the same epitopes were significantly enhanced for TARP in ININF and OMP2 and TARP in FSW (Fisher´s exact test for groups passing Chi-square testing with Holm correction for number of tests: *p<0.05, **p<0.01, ***p<0.001). GBS: Group B *Streptococcus*.

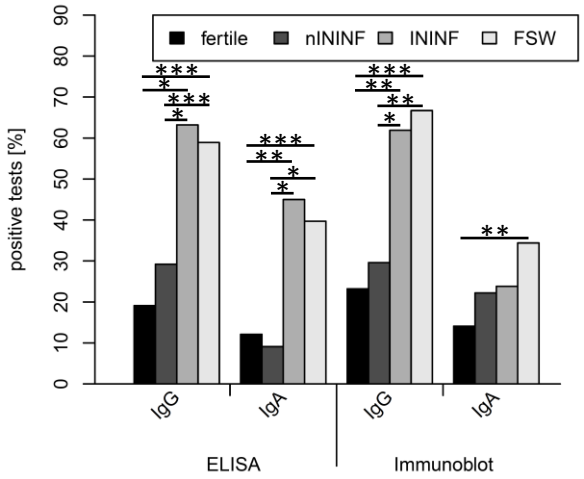


Figure 3-3: IgG and IgA antibodies targeting *C. trachomatis* display enhanced prevalence in ININF and FSW compared to fertile females and nININF. The results from ELISA in comparison to the total of the immunoblots are displayed. ININF and FSW were significantly enhanced compared to fertile females and nININF except for IgA immunoblots, which showed enhanced rate for FSW compared to fertile females only (Fisher`s exact test with Holm correction: *p<0.05, **p<0.01, ***p<0.001). ININF: infectious infertility; FSW: female sex workers; nININF: non-infectious infertility.

3.1.3 General characteristics of the cervical microbiome

In a first step in describing the cervical microbiome, a phylotype-based analysis was carried out, assessing the taxonomic classification of the processed sequencing reads. The analysis found that the microbiome of females was mainly dominated by *Lactobacillus* species, while other taxa such as *Prevotella, Atopobium, Streptococcus*, Lachnospiraceae, *Sneathia* etc. were represented to a lesser extent (Figure 3-4A). Comparing both sequenced regions, the V3/V4 region showed additional taxa, namely *Gardnerella* and *Bifidobacterium*, which were not represented using the V1/V2 region (Figure 3-4A). Among the pathogens tested with diagnostic techniques, the presence of *C. trachomatis* sequences could not be proven using the V1/V2 region (Table 6-4) while all pathogens, which were present in diagnostic testing also occurred in the microbiome sequencing using the V3/V4 region (Table 6-5).

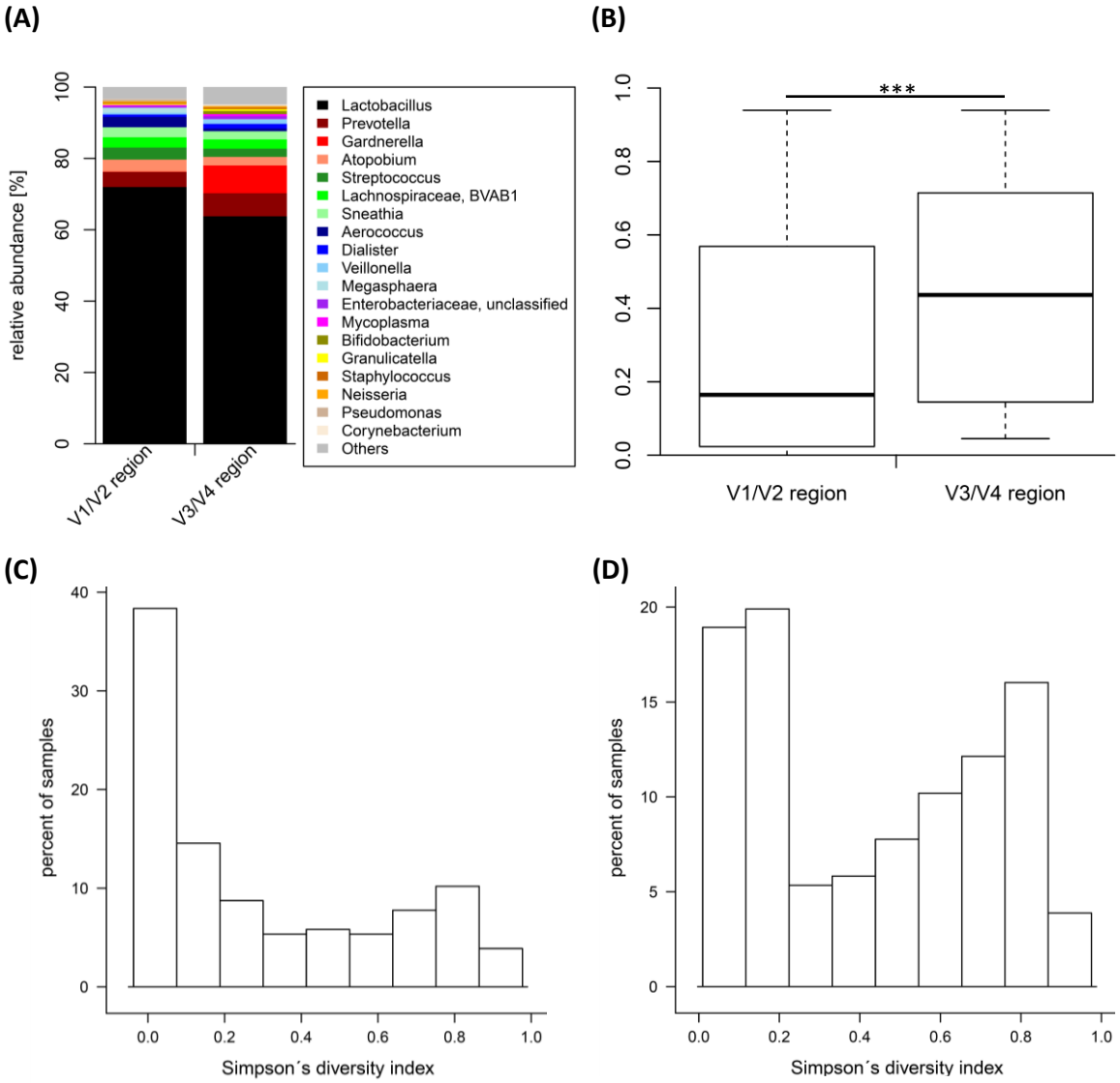


Figure 3-4: Comparison of sequencing based assessment of relative abundance of bacterial taxa and bacterial diversity from cervical swabs using the V1/V2 and the V3/V4 region of bacterial 16S rRNA genes. Lactobacilli make up the majority of the sequences, however, a substancial fraction of sequencing reads belongs to other bacterial taxa, which are shown in *(A)*. Additional taxa, which are not represented using the V1/V2 region are present in the V3/V4 data set (*Gardnerella* and *Bifidobacterium*) *(A)*. The diversity of the bacterial communities displays significantly higher values using the V3/V4 region *(B)* (Wilcoxon rank-sum test with continuity correction: ***p<0.001). While a majority of diversity indices is below 0.2 in the V1/V2 data set *(C)*, a shift is observed towards higher diversity indices using the V3/V4 region *(D)*.

Following the above observations, the microbial diversity (alpha diversity) was assessed using Simpson´s diversity index. Diversity was mostly low when it was assessed using the V1/V2 region but high diversity also occured and diversity varied from 0 to 0.939 using V1/V2 (variance: 0.096). The diversity measured was significantly higher when diversity for the V3/V4 region was calculated; however, the variance was slightly lower (0.083) in the V3/V4 region with indices ranging from 0.045 to 0.939 (Figure 3-4B). Histograms of distributions of

diversity indices showed a majority of indices below 0.2 in the V1/V2 data set, while fewer indices below 0.1 and a shift towards high indices up to 0.8 were observed in the V3/V4 region. However, nearly 40 % of the indices still remained below 0.2 (Figure 3-4C/D) in the latter case.

To assess beta diversity, sequences were clustered into operational taxonomic units (OTUs) with a global identity threshold of 97 %; furthermore Principal Coordinates Analysis (PCoA) with the Bray-Curtis index was carried out. Different clusters could be observed using both sequencing region, however, samples clustered closer to each other in the V1/V2 data set while they were more spread using the V3/V4 region. The first axis explained the variability of the data set to a higher degree when using the V1/V2 region. By the same token, the total explained variability was lower using the V3/V4 region when analyzing the first two principal coordinates of the PCoA (Figure 3-5).

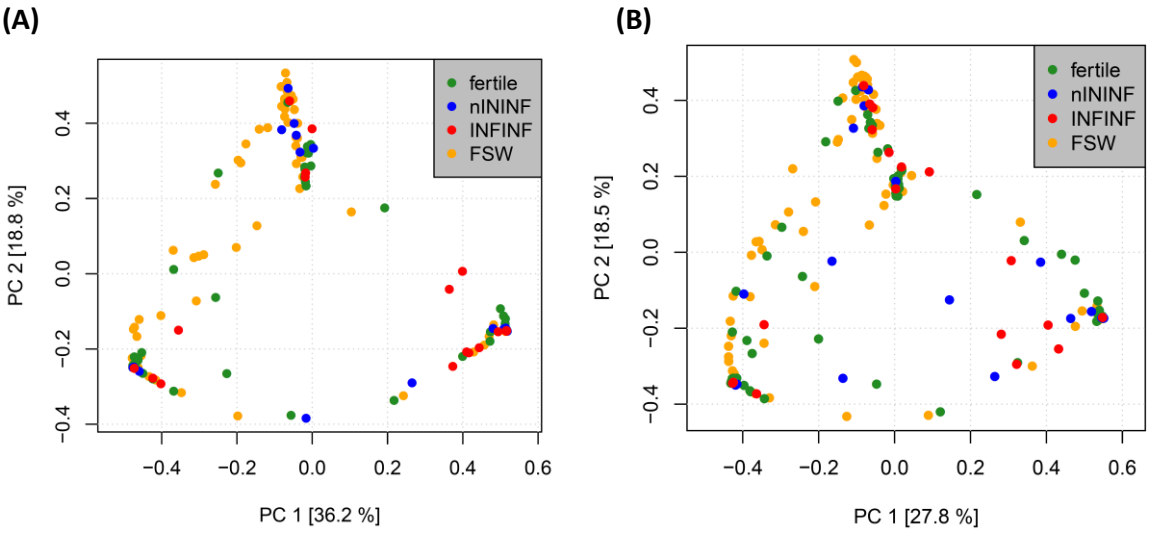


Figure 3-5: Principle coordinate analysis using Bray-Curtis index of bacterial OTUs. *(A)* displays the results achieved using primers for the V1/V2 region of the 16S rRNA gene. *(B)* displays the results gained using primers for the V3/V4 region. The percentage of explained variability of the corresponding data set is given with the axis labels. Different clusters of samples could be observed. The V3/V4 region showed a more widespread pattern while the variability explained was lower compared to the V1/V2 region. nIININF: non-infectious infertility; ININF: Infectious infertility; FSW: Female sex worker; PC: principal coordinate.

Procrustes rotation was used to assess the similarity of both ordinations. Repeated symmetric Procrustes rotation (PROTEST, number of permutations=999) displayed a significant overlap of both ordinations with a sum of squares of 0.44, a correlation in symmetric Procrustes rotation of 0.75 and a p-value below 0.001.

Bacterial communities were further classified to species level. The total number of taxa on species level was 307 in the V1/V2 data set compared to 363 using the V3/V4 region (a complete list of all species-level classifications is given in the appendix, Table 6-4 and Table 6-5). A heatmap was constructed using the 25 most abundant taxa on species level. Heatmaps for the V1/V2 region and the V3/V4 region showed *Lactobacillus crispatus*, *L. iners* and *L. gasseri* highly abundant in the majority of samples. The V1/V2 region additionally showed *L. jensenii* as a prominent species in some samples, while in the V3/V4 region *G. vaginalis* turned out to be a prominent part of some microbial communities. Rare cases of other species dominating a community were observed in both data sets. The Bifidobacteria present only in the V3/V4 region turned out to be *B. breve* and *B. bifidum* (Figure 3-6).

Based on the observations from the heatmap analysis and following a previously published analysis strategy [32], the bacterial communities were assigned to community types (CTs) based on the criteria of domination by one bacterial taxon. Domination was defined as a proportion of >50 % of all reads of a sample belonging to one taxon. In the V1/V2 data set four community types were assigned which were dominated by *L. crispatus, L. iners, L. gasseri* or *L. jensenii* respectivly. In the V3/V4 data set the CTs dominated by *L. crispatus*, *L. iners*, *L. gasseri* or *G. vaginalis* were defined. Other samples were combined into the community type “diverse” in both data sets (Table 3-3).

Based on phylotype definition, alpha diversity calculation, heatmap analysis, and CT assignment the microbiomes of the study groups were compared against each other.

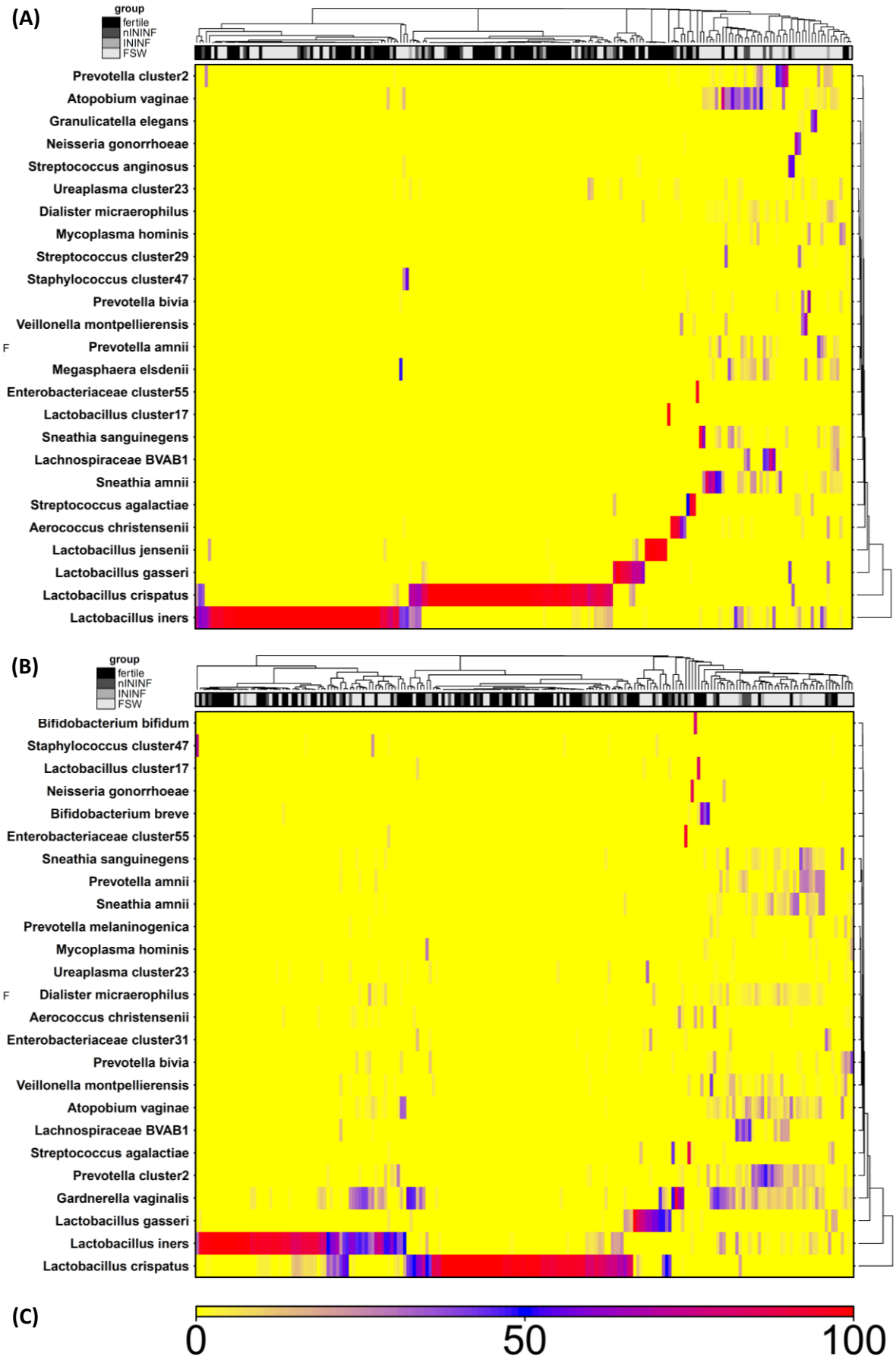


Figure 3-6: Figure legend on the next page.

Figure 3-6: Heatmaps describing relative abundances of the 25 most abundant bacterial taxa on species level for the sequencing regions V1/V2 *(A)* and V3/V4 *(B)*. Complete linkage clustering of samples based on the abundance of bacteria, sample assignment to study groups, and color key for study group assignment is shown at the top of the heatmaps. Complete linkage clustering of the 25 most abundant bacterial taxa based on Spearman´s correlation coefficient is shown at the right of the heatmaps. *(C)* shows the color coding for the relative abundance of the heatmaps. Most samples are dominated by one of the *Lactobacillus* species *L. crispatus*, *L. iners*, *L. gasseri* and *L. jensenii*. Apart6 from samples dominated by *G. vaginalis* (V3/V4 data set), the other samples mostly comprised several other taxa. nININF: non-infectious infertility; ININF: infectious infertility; FSW: female sex workers.

Table 3-3: Community types assigned to bacterial communities depending on the sequencing region

Sequencing region	Community types	Abbreviation
V1/V2	*Lactobacillus crispatus*-dominated	Lcr
	Lactobacillus iners-dominated	Lin
	Lactobacillus gasseri-dominated	Lga
	Lactobacillus jensenii-dominated	Lje
	diverse	div
V3/V4	*Lactobacillus crispatus*-dominated	Lcr
	Lactobacillus iners-dominated	Lin
	Lactobacillus gasseri-dominated	Lga
	Gardnerella vaginalis-dominated	Gva
	diverse	div

3.1.4 Reduced *Lactobacillus* abundance with increased *Gardnerella* abundance in females with infectious infertility and FSW

Relative abundances of bacterial taxa on genus level within the cervical microbiome were compared between the study groups: ININF, fertile females, nININF and FSW. Although *Lactobacillus* was the most prominent taxon, its relative abundance differed between groups: the highest relative abundance was registered in fertile females while it was lower in nININF and ININF. The lowest relative abundance was observed in FSW. The differences for *Lactobacillus* were significant for ININF and FSW compared to fertile females using the V3/V4 region.

In general, the lower relative abundance of *Lactobacillus* coincided with an increase of other taxa such as *Prevotella, Atopobium, Sneathia, Dialister, Veillonella*, *Megasphaera,* and *Gardnerella*. BVAB1 showed an increase in their relative abundance in all groups compared to fertile females which was most prominent in nININF. The genus *Mycoplasma* showed the highest relative abundance in ININF, although the increase compared to fertile females and nININF was significant only for FSW. The relative abundance of other taxa than the ones mentioned in Figure 3-7, Table 3-4, and Table 3-5 was especially high in ININF and FSW compared to fertile females.

Gardnerella was significantly enhanced in ININF and FSW – as shown by its scores in the V3/V4 region - while the genus *Bifidobacterium* was present in higher amounts in fertile females compared to the other groups. Detailed comparisons between study groups and regions for all taxa representing at least one percent of bacterial scores in a minimum of one group are provided in Figure 3-7, Table 3-4, and Table 3-5.

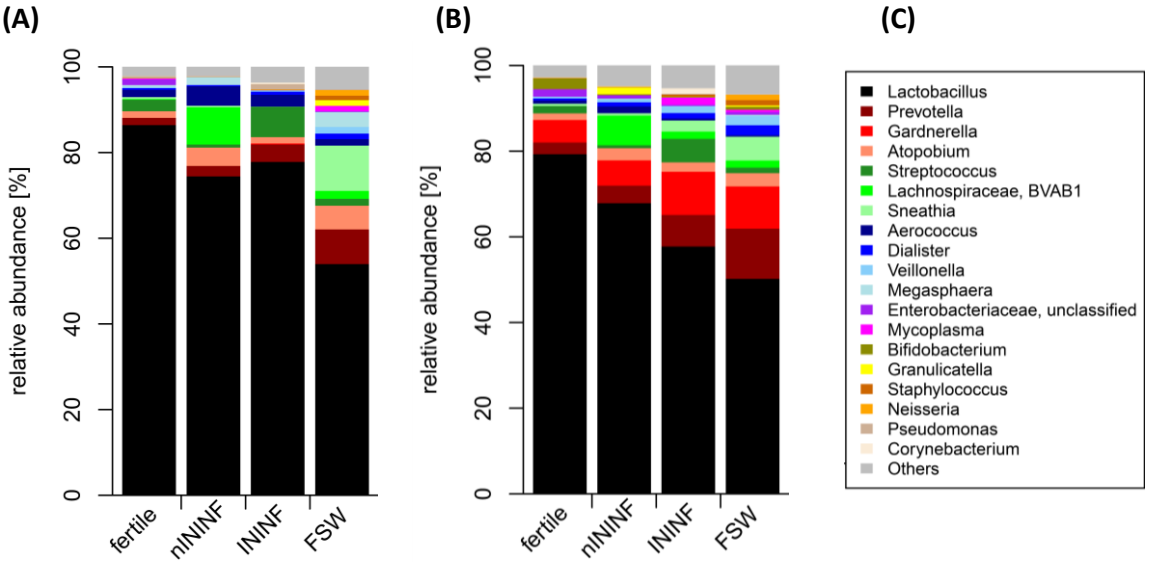


Figure 3-7: Relative abundance of bacterial taxa on genus level. The relative abundance of genera is displayed according to the study groups. Genera are displayed if they account for a minimum of one percent in at least one of the groups. *(A)* displays the results achieved using primers for the V1/V2 region of the 16S rRNA gene. *(B)* displays the results gained using primers for the V3/V4 region. *(C)* Legend for (A) and (B). While *Lactobacillus* was the most prominent taxon in fertile females, it was found at reduced levels in the other study groups, in which several other taxa were correspondingly more prominent. nININF: non-infectious infertility; ININF: Infectious infertility; FSW: Female sex worker; BVAB1: bacterial vaginosis associated bacterium 1.

Table 3-4: Relative abundance of taxa using V1/V2 region. The table displays the relative abundance of taxa, which are classified to genus level to the extent possible. Only those taxa are shown that contribute at least one percent of bacterial scores in any one group in the V1/V2 or the V3/V4 data set.

Taxon	Fertile [%]	nININF [%]	ININF [%]	FSW [%]
Lactobacillus	87.98	71.81	74.13	53.99
Prevotella	1.56	2.67	4.76	8.06
Atopobium	1.44 d***	4.74	1.74 d*	5.53 a***, c*
Sneathia	0.25 d***, b**	0.37 d**	0.01	10.59 a***, b**
Streptococcus	2.87	0.79	8.27	1.61
Aerococcus	1.80	5.04	3.26	1.51
Enterobacteriaceae, unclassified	1.30	0.00	0.06	0.18
Megasphaera	0.05 d***, b*	2.05 a*	0.02 d*	3.51 a***, c*
Lachnospiraceae, BVAB1	0.00 b***, d**	9.63 a***	0.01	1.83 a**
Mycoplasma	0.22 d***	0.00 d*	0.00	1.26 a***, b*
Pseudomonas	0.25	0.21	1.46	0.20
Dialister	0.19 d***	0.31	0.82	1.29 a***
Veillonella	0.30	0.00	0.30	1.53
Staphylococcus	0.06	0.06	0.12	1.03
Neisseria	0.00	0.00	0.10	1.32
Granulicatella	0.00	0.00	0.00	1.32
Corynebacterium	0.03 d**	0.04	0.40 d**	0.01 a**, c**
Gardnerella	0.01	0.01	0.28	0.02
Bifidobacterium	0.02	0.00	0.00	0.00
Others	1.60	2.28	4.25	5.20

The significance of differences between groups was assessed using the Kruskal-Wallis test with Hochberg correction for the number of taxa tested. Subsequently, Wilcoxon rank-sum testing with continuity correction was carried out for those taxa that were significant in the first step: *p<0.05, **p<0.01, ***p<0.001. a=significant against fertile, b=significant against nININF, c=significant against ININF, d=significant against FSW.

Table 3-5: Relative abundance of taxa using V3/V4 region. The table displays the relative abundance of taxa, which are classified to genus level to the extent possible. Only those taxa are shown that contribute at least one percent of the bacterial counts in any one group in the V1/V2 or the V3/V4 data set.

Taxon	Fertile [%]	nININF [%]	ININF [%]	FSW [%]
Lactobacillus	79.30 c*, d***	67.86	57.75 a*	50.22 a***
Gardnerella	5.25 c*, d***	5.90 d*	10.08 a*	9.86 a***, b*
Prevotella	2.73 d***	4.09	7.37	11.70 a***
Atopobium	1.54 d***	2.86	2.18	3.06 a***
Sneathia	0.54 d***	0.53 d*	2.58	5.58 a***, b*
Streptococcus	1.73	0.68	5.50	1.30
Bifidobacterium	2.49	0.22	0.18	0.69
Enterobacteriaceae, unclassified	1.69	0.78	0.27	0.62
Dialister	0.51 c*, d**	0.99	1.25 a*	2.34 a**
Lachnospiraceae, BVAB1	0.00 b***, c**, d*	6.94 a***	1.69 a**	1.66 a*
Veillonella	0.39 c*, d***	0.80	1.63 a*	2.20 a***
Staphylococcus	0.10	0.11	0.47	1.22
Aerococcus	0.71 b**, d***	1.57 a**	0.48	0.34 a***
Neisseria	0.01	0.01	0.02	1.26
Mycoplasma	0.07 d***	0.02 d*	1.71	0.57 a***, b*
Granulicatella	0.00	1.64	0.04	0.32
Corynebacterium	0.07	0.07	1.28	0.04
Pseudomonas	0.10	0.09	0.17	0.07
Megasphaera	0.01 b*, c*, d***	0.04 a*	0.03 a*	0.24 a***
Others	2.74	4.80	5.33	6.72

The significance of differences between groups was assessed using the Kruskal-Wallis test with Hochberg correction for the number of taxa tested. Subsequently, Wilcoxon rank-sum testing with continuity correction was carried out for those taxa that were significant in the first step: *p<0.05, **p<0.01, ***p<0.001. a=significant against fertile, b=significant against nININF, c=significant against ININF, d=significant against FSW.

3.1.5 Low alpha diversity is a characteristic displayed by fertile females only

Operational taxonomic units (OTUs) were established with a 97 % global identity threshold using mothur. Based on these OTUs the diversity of each sample (alpha diversity) was computed using Simpson´s diversity index. Diversity was low in fertile females (median: 0.050 using V1/V2 and 0.207 using V3/V4), increased in nIININF (median: 0.172 using V1/V2 and 0.568 using V3/V4) and ININF (median: 0.198 using V1/V2 and 0.567 using V3/V4) and significantly increased in FSW (median: 0.478 using V1/V2 and 0.592 using V3/V4, p<0.001) (Figure 3-8A). The variances between samples in each group were smaller in fertile females compared to the other groups (variances V1/V2: fertile: 0.047, nIININF: 0.072, ININF: 0.085, FSW: 0.112, variances V3/V4: fertile: 0.060, nIININF: 0.086, ININF: 0.080, FSW: 0.084) (Table 3-6).

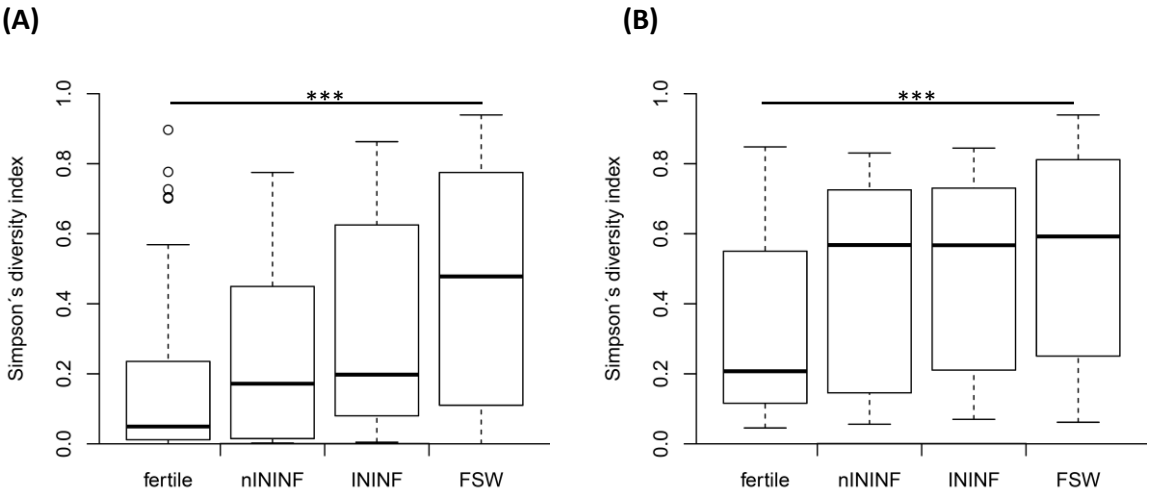


Figure 3-8: Compared to fertile females, the diversity of microbial communities from cervical swabs is higher in all other study groups compared to fertile females. The diversity of each sample was assessed by Simpson´s diversity index. *(A)* displays the results achieved using primers for the V1/V2 region of the 16S rRNA gene. *(B)* displays the results gained using primers for the V3/V4 region. FSW displayed significantly enhanced diversity compared to fertile females, while a trend towards an increase was observed for nIININF and ININF (Wilcoxon rank-sum testing with Hochberg correction for multiple comparisons was carried out following significant Kruskal-Wallis testing: ***p-value<0.001). In general, the diversity was increased in in the V3/V4 data set compared to the V1/V2 region. nIININF: non-infectious infertility; ININF: Infectious infertility; FSW: Female sex worker.

Table 3-6: Variances of Simpson´s diversity indices according to distribution within groups

Region	Fertile	nININIF	ININF	FSW
V1/V2	0.047	0.072	0.085	0.112
V3/V4	0.060	0.086	0.080	0.084

3.1.6 Community type assignment reveals differences between the study groups

Using PCoA based on OTUs, clusters of samples with high similarity were observed; however, no strict overlap between clustering of samples and groups could be shown (Figure 3-5). Only the centroid of FSW could be separated from the other groups using permutational multivariate analysis of variance (adonis, $p<0.001$, r-package "vegan"[80]) using V1/V2 as well as V3/V4. Therefore, a heatmap based on the relative abundance of the 25 most abundant taxa, where possible classified to species level, was carried out (Figure 3-6). A high proportion of samples were dominated by one of the species *L. crispatus*, *L. iners*, *L. gasseri, L. jensenii*, and *G. vaginalis,* while the remaining samples showed the presence of several taxa which were mostly not present in those cases in which *Lactobacillus* species dominated.

Bacterial communities were assigned to community types as described earlier (Table 3-3). *Lactobacillus*-dominated community types were most common in fertile females while their proportion decreased in nININF as well as ININF and was lowest in FSW. Instead, diverse communities were observed in nININF and ININF with the highest proportion of diverse CTs being present in FSW. *L. jensenii* CTs were missing in ININF using the V1/V2 region, while their presence was not confirmed using the V3/V4 region. Using the V3/V4 region, *G vaginalis* CTs were most prominent in ININF. The proportion of the *L. iners* CT was relatively stable throughout all groups (Figure 3-9). The differences between the study groups were found to be significant (p-value<0.001) using Chi-square testing.

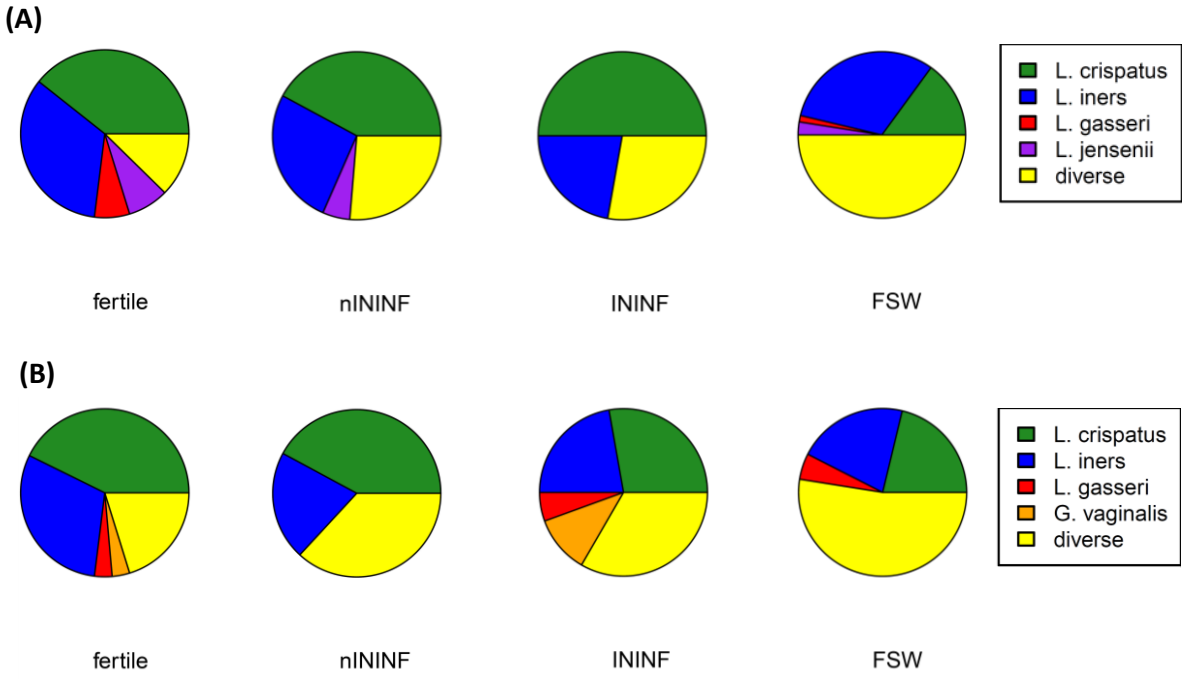


Figure 3-9: Proportion of different community types in the study groups. Women harbor one of five main community types that are differentially distributed between the study groups. The samples cluster into five main community types based on the relative abundance of several species which are defined as follows: Lcr: *L crispatus*-dominated community; Lin: *L. iners*-dominated community; Lga: *L. gasseri*-dominated community; Gva: *G. vaginalis*-dominated community; div: diverse communities. *(A)* V1/V2 and *(B)* V3/V4. The CT proportions differed between the study group with the *G. vaginalis* CT being enhanced in ININF using the V3/V4 region. Compared to fertile females, higher proportions of the diverse CT were observed in the other three groups, with FSW having more than 50 %. Total *Lactobacillus* CTs were reduced in these three groups compared to fertile females; however, the *L. iners* CT was constant throughout all groups. Chi-square testing proved the differences between the study groups to be significant (p-value<0.001).

3.1.7 Sexual behaviour impacts on the urogenital microbiome of women

The impact of sexual activity on alpha diversity was assessed by correlating the frequency of sexual intercourse per month with Shannon´s diversity index of bacterial communities based on OTUs. Fractional polynomials were calculated to choose a fitting correlation model. These showed linearity to be the best correlation model. For this reason, linear regressions were computed and correlations were calculated using Pearson´s product-moment correlation coefficient. The contribution of sexual intercourse to diversity was significant (p-value<0.001) with R^2=0.241 (V1/V2) and 0.080 (V3/V4) (Figure 3-10 and Table 3-7).

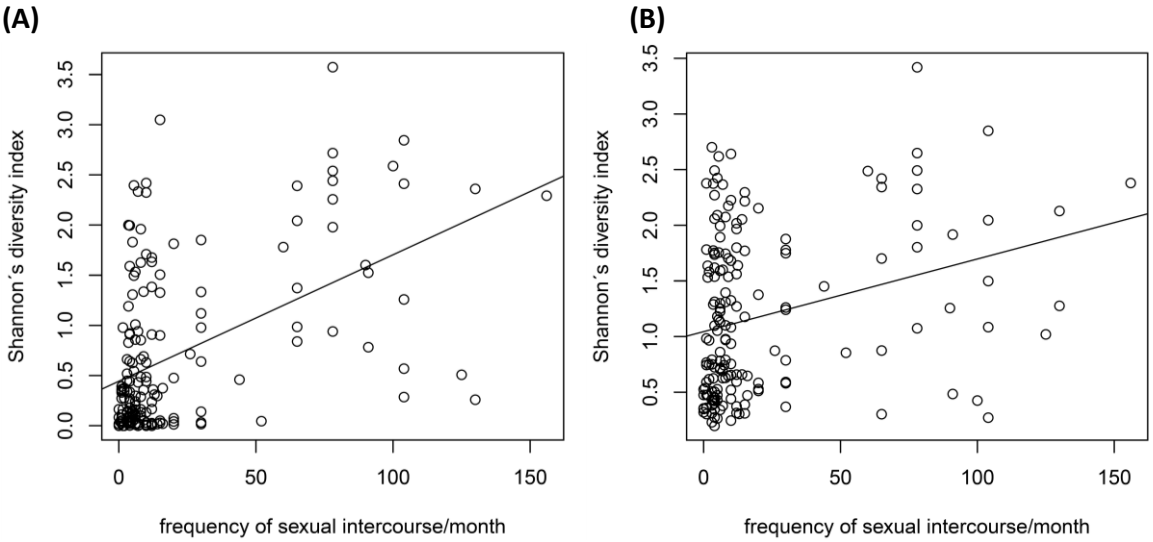


Figure 3-10: Correlation between frequency of sexual intercourse/month and diversity of the bacterial communities. Linear regression was computed using Shannon´s diversity index of microbial communities and the self-declared frequency of sexual intercourse/month using the V1/V2 region (A) and the V3/V4 region (B). Correlation was strongest using the V1/V2 data set. Corresponding coefficients and estimates using Pearson´s product-moment correlation are given in Table 3-7.

Table 3-7: Coefficients of the linear regression analysis in Figure 3-10 and estimates of the Pearson´s product-moment correlation including p-values

Region	Intercept	Coefficient	R	R^2	p-value
V1/V2	0.443	0.013	0.491	0.241	<0.001
V3/V4	1.044	0.007	0.284	0.080	<0.001

In a second step, the females were grouped into those with low (0-6), intermediate (7-10) and high (>10) frequency of sexual intercourse per month and the proportions of community types were compared. The higher the frequency of sexual intercourse per month was, the higher was the frequency of diverse communities and the lower were the CTs dominated by *Lactobacillus crispatus* and *L. jensenii*. *L. gasseri* was increased in females with an intermediate frequency of sexual intercourse using the V1/V2 region, and the same was true for *G. vaginalis* using the V3/V4 region. *L. iners* CTs displayed its highest proportion in females with intermediate frequency of sexual intercourse (Figure 3-11); however, Chi-square testing was not significant for any of these changes.

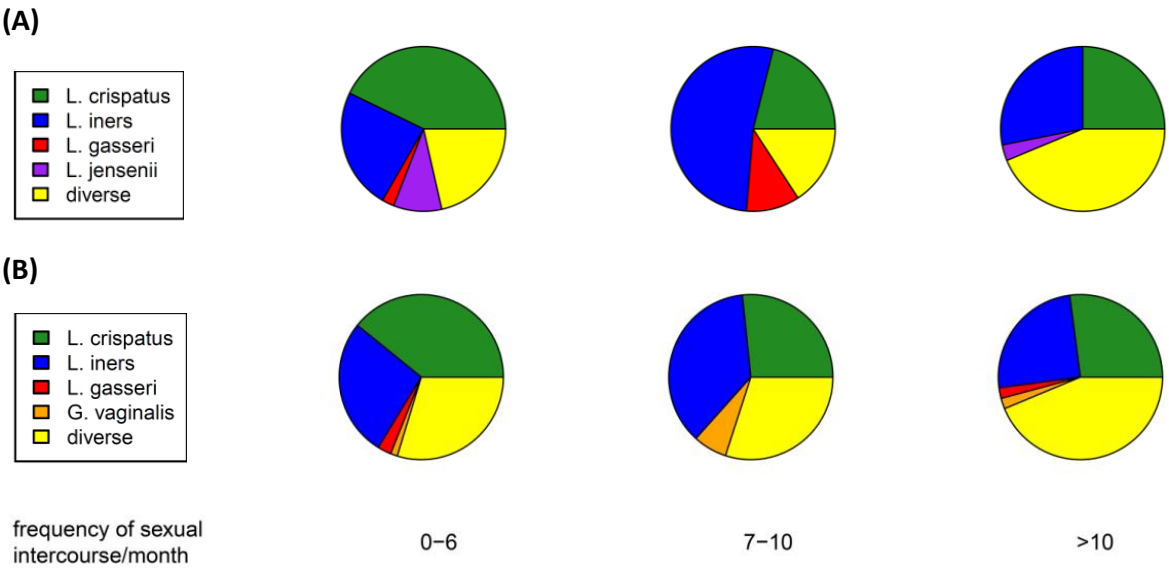


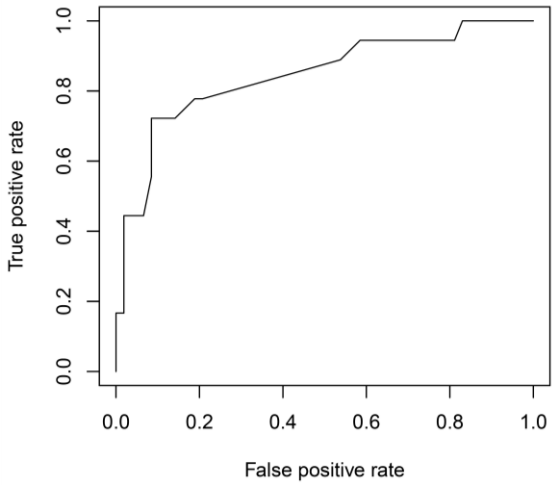
Figure 3-11: Community type distribution according to sexual activity. In women with sexual intercourse more than ten times per month the proportion of diverse communities was increased in both the V1/V2 *(A)* and the V3/V4 data set *(B)* while the proportion of *Lactobacillus*-dominated CTs decreased. While the proportion of CTs varied in general between study groups and sequencing regions, these differences were not significant when tested using the Chi-Square test.

3.1.8 Development of a microbiome-based prediction model for infectious infertility

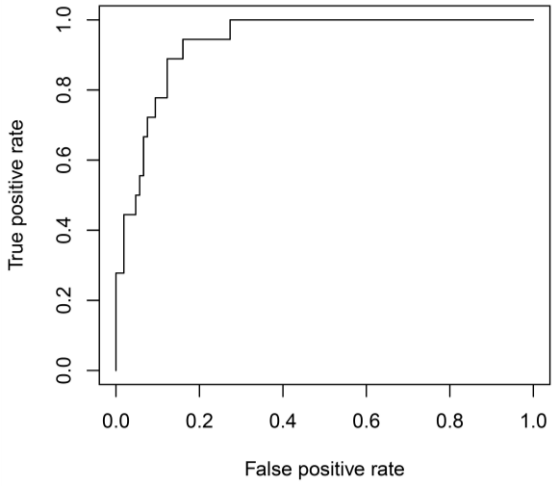
The diagnosis of infectious infertility is currently a lengthy progress with limited precision. Therefore, a binary logistic regression model was developed to predict infectious infertility where females with infectious infertility were taken as cases and females with non-infectious infertility as well as fertile females were taken as controls to train the model. FSW were excluded from this analysis as their fertility status was unknown. The following predictors were used: (i) PCR-based and cultivation-based diagnostics for STIs (see Figure 3-2A/B), (ii) serological testing for antibodies targeting *C. trachomatis* epitopes (see Figure 3-2C/D) and (iii) the first ten taxa, on species level, from the microbiome sequencing (see Table 6-4 and Table 6-5). The model was built for both sequenced regions and compared against the same model without the sequencing data. The model without the microbiome data was able to predict 13 of 18 cases of infectious infertility. The model including microbiome data enhanced the correct positive prediction to 14 of 18 cases using the V1/V2 region and to 17 of 18 cases using the V3/V4 region instead. It is worth noting that the model had also the highest total accuracy using the V3/V4 region and that all nININF could be correctly separated from ININF in that case (Table 3-8). Receiver operating characteristic (ROC) curves were established describing true vs. false positivity rates to measure the performance of the model. The areas under curve were assessed; these were 0.845 in the case without microbiome data, 0.937 in

the case integrating the V1/V2 region and 0.977 in the case using the V3/V4 region (Figure 3-12). McFadden R^2 testing was used to assess the goodness-of-fit of the model and showed the highest value when microbiome sequencing of the V3/V4 region was integrated into the model.

(A)



(B)



(C)

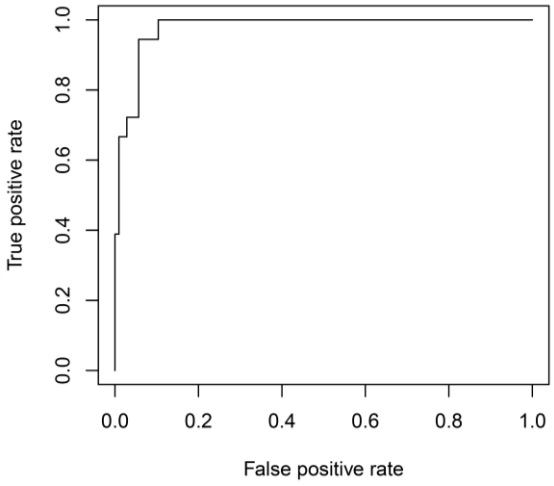


Figure 3-12: ROC curves for the binary logistic regression model predicting infectious infertility depicting true *vs*. false positive rates. *(A)* Using Pathogen detection by PCR and cultivation as well as serologic antibody testing (AUC: 0.845, 95% CI: 0.734-0.955), *(B)* including the first ten taxa from the microbiome data using the V1/V2 region (AUC: 0.937, 95% CI: 0.892-0.981) and **(C)** including the first ten taxa from the microbiome data using the V3/V4 region (AUC: 0.977, 95% CI: 0.956-0.999). AUC: Area under curve.

Table 3-8: Validation of the prediction models for infectious infertility

Predictors	Prediction [ININF/total]			Accuracy	McFadden R^2
	Fertile	nININF	ININF	Total	
PCR/Cultivation/Serology	10/87	1/19	13/18	0.871	0.320
PCR/Cultivation/Serology/ Microbiome V1/V2	9/87	1/19	14/18	0.879	0.456
PCR/Cultivation/Serology/ Microbiome V3/V4	7/87	0/19	17/18	0.936	0.685

3.2 Establishing a chlamydial mouse model for microbiome-pathogen interaction studies in sexually transmitted diseases

This study aims to set the basis for further functional studies of microbiome-pathogen interaction in sexually transmitted diseases. To this effect, different models using the most common sexually transmitted bacterium *C. trachomatis* as well as the closely related mouse specific *C. muridarum* were established, compared and further developed. A vaginal infection with *C. trachomatis* serovar D was compared with a newly established transcervical *C. trachomatis* infection using the Non-Surgical Embryo Transfer (NSET) Device. Subsequently the latter model was compared to the currently most widely employed chlamydial model using *C. muridarum*.

3.2.1 Validation of a new application strategy using the NSET device

In order to compare a vaginal with a transcervical *C. trachomatis* infection in the mouse, the workflow for non-surgical transcervical infection was tested. To ensure a successful transcervical application to the uterus horns using the NSET device, PBS with 1 % trypan blue was applied to dead C75BL/6 mice. Subsequently, the abdominal cavity of the mice was opened to check successful application through observation of a blue color in the uterus horns. Blue color was observed in one of the uterus horns of each of the three tested mice (Figure 3-13).

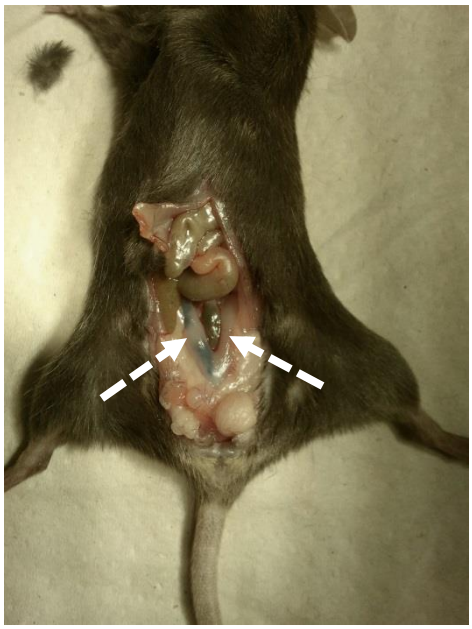


Figure 3-13: Representative picture of the validation of the application method using the NSET device for application of solutions to the uterus horns via the cervical opening. White arrows indicate the uterus horns. The blue colored buffer shows successful application to one of the uterus horns.

3.2.2 Comparison of the outcome of vaginal vs. transcervical *C. trachomatis* infection

To establish a *C. trachomatis* infection model, two different infection strategies were used at outset. The bacteria were either inoculated vaginally in a volume of 4 µl using a 10 µl pipette or transcervically using the NSET device and the same volume. 1 x 10^6 inclusion-forming units (IFUs) were subjected to each mouse. Mock-infected mice were used as controls and a total of eight mice per group were used. During the course of the infection, swabs were taken from the vagina every third day and the number of recoverable IFUs was determined using recovery assays. At day 25 post infection the animals were killed and the urogenital tract was removed and examined for pathologies. In both groups chlamydial inclusions could be recovered from the vagina over a period of 21 days (Figure 3-14A). It was noted that vaginal infection lead to high variances between animals concerning the bacterial shedding, especially at later time points (12 days post infection (dpi) and later) with some animals showing no recoverable IFUs while other animals continued to shed bacteria in a range of more than 10,000 bacteria/swab. In contrast, variances declined over time following transcervical infection (Figure 3-14B). 25 days after the infection, no pathologies could be observed macroscopically, whether in mice infected vaginally or in mice infected transcervically (Figure 3-15).

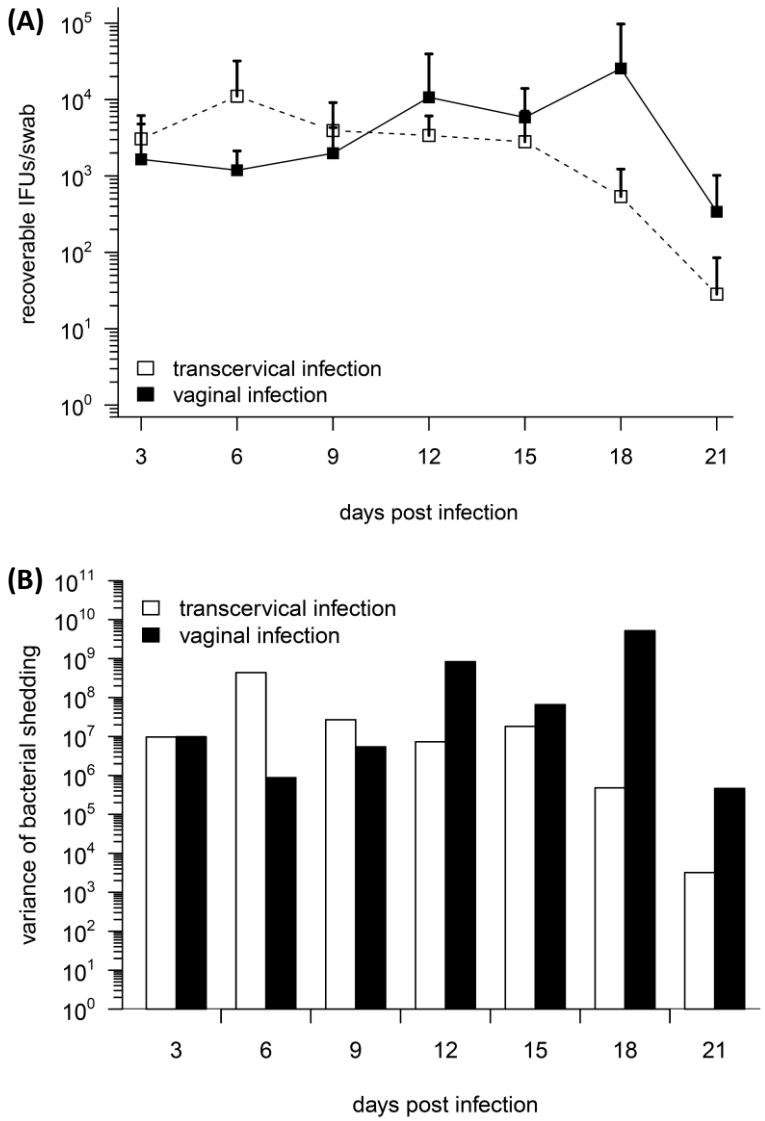


Figure 3-14: Bacterial shedding of *C. trachomatis* infected mice. Transcervically infected and vaginally infected mice displayed bacterial shedding on a relative constant level over a period of approximately 3 weeks *(A)* as shown by recoverable IFUs from vaginal swabs. However, at later time points the variances between vaginally infected mice increased compared to transcervically infected mice *(B)*. n=8, error bars indicate standard deviation.

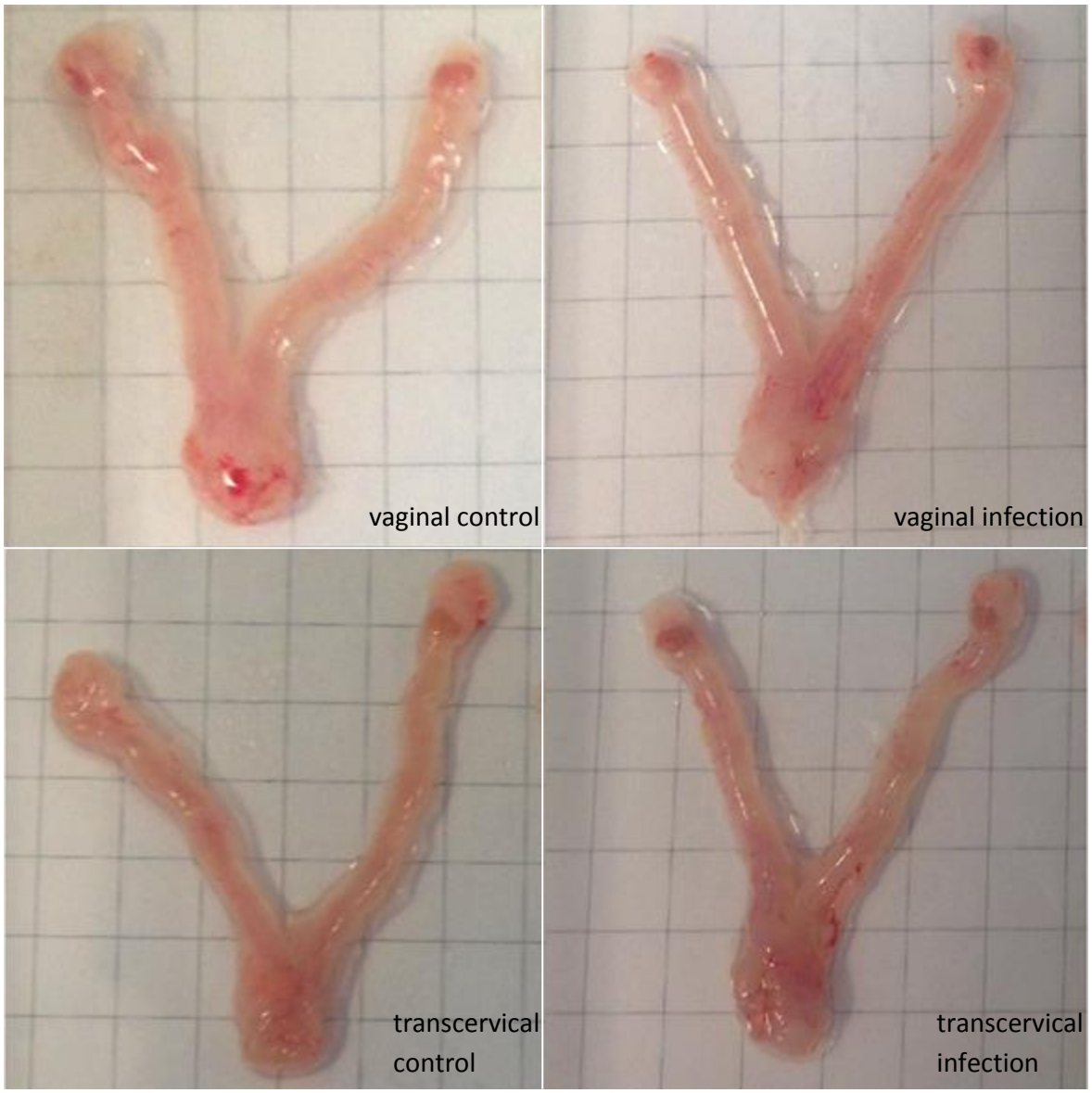


Figure 3-15: Representative pictures of the urogenital tract of uninfected mice (controls) and mice infected with *C. trachomatis* vaginally or transcervically. No pathologies could be observed using vaginal and transcvervical infection with 1 x 10^6 IFUs *C. trachomatis* strain D. A total of eight mice per group were dissected.

3.2.3 Comparison of murine infections with *C. trachomatis* and *C. muridarum*

In a second step, the *C. trachomatis* model was compared to a murine infection model using *C. muridarum*. To this effect, transcervical infection with *C. trachomatis* was compared with the most frequently used chlamydial mouse model, vaginal *C. muridarum* infection. In comparison to the previous *C. trachomatis* infections described above, the inoculated dose was increased to 4x10^6 IFUs while *C. muridarum* was inoculated with 1x10^6 IFUs. Again, mock infected mice served as controls and eight animals per group were used. The mice were killed at 43 dpi. Bacterial shedding of *C. muridarum* was characterized by a constant decline over a

period of 21 days with no detectable bacteria at day 24. Initial shedding of *C. muridarum* was approximatly 10^5-fold higher than *C. trachomatis* shedding. The latter lasted for 24 days with, on average, constant numbers of recoverable IFUs (Figure 3-16). No macroscopically visible pathological signs could be observed following *C. trachomatis* infection. In contrast, all animals infected with *C. muridarum* showed hydrosalpinx on at least one fallopian tube. The fallopian tubes were scored according to the size of the hydrosalpinx as described in 2.7.2 (Table 3-9 and Figure 3-17).

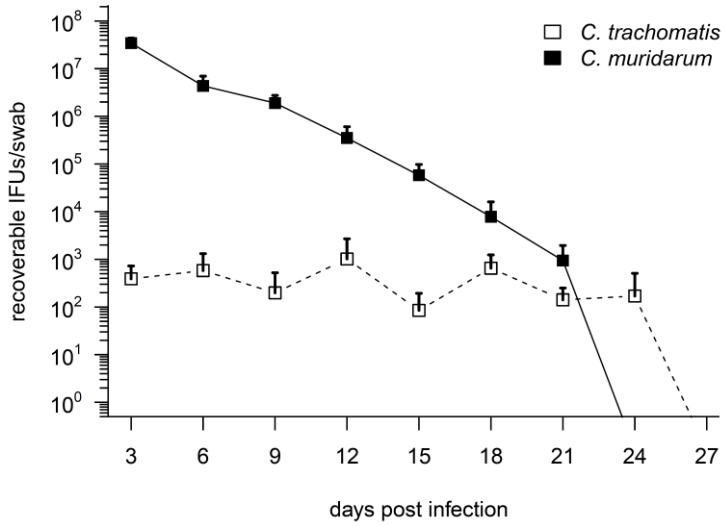


Figure 3-16: **Bacterial shedding of mice infected with *C. trachomatis* or *C. muridarum*.** Mice infected vaginally with *C. muridarum* showed increased bacterial shedding that declines during the course of infection when compared to transcervical infection with *C. trachomatis*, which displayed low but constant shedding. n=8, error bars indicate standard deviation.

Table 3-9: Pathology scoring of mice infected vaginally with *C. muridarum*

Mouse #	Left uterus horn (from ventral)	Right uterus horn (from ventral)
Mouse 1	0	3
Mouse 2	4	4
Mouse 3	0	4
Mouse 4	0	1
Mouse 5	0	3
Mouse 6	4	4
Mouse 7	0	4
Mouse 8	0	1

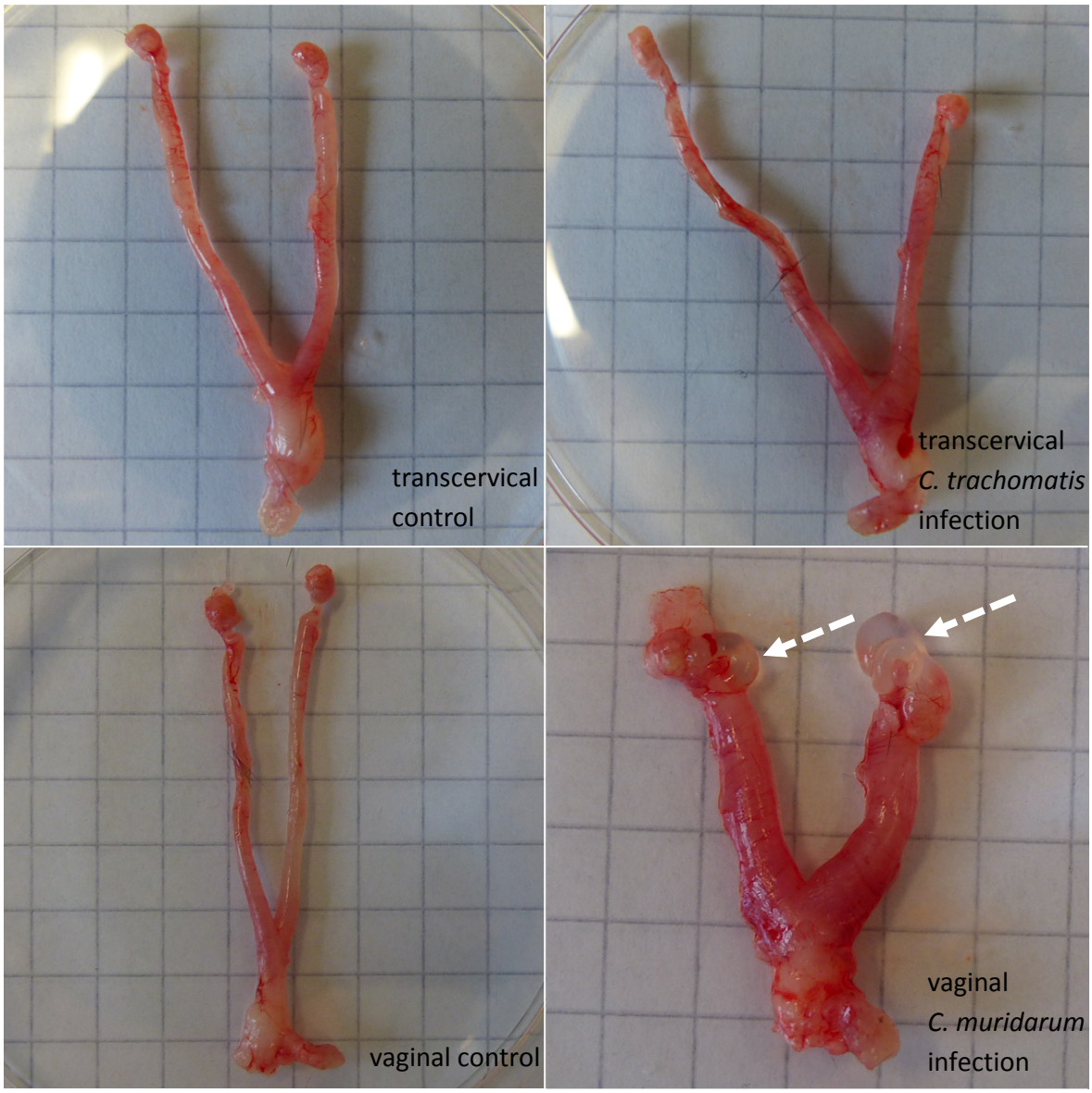


Figure 3-17: Representative pictures of the urogenital tract of uninfected mice (controls) and mice infected with *C. trachomatis* transcervically or *C. muridarum* vaginally. While no pathologies could be observed from transcervical infection with 4 x 10^6 IFUs *C. trachomatis*, vaginal infection with 1 x 10^6 *C. muridarum* led to hydrosalpinx (white arrows) and a swollen urogenital tract. A total of eight mice per group were dissected.

3.2.4 Testing the utility of the established mouse models using doxycycline

As a last step, it was tested whether antibiotic treatment can be used for modification of the vaginal microbiome. As a measure for this purpose, the depletion of *Chlamydiae* from the urogenital tract was assessed. Mice infected with *C. trachomatis* and *C. muridarum* were treated with doxycycline given i.p. one day post infection. The bacterial shedding and pathological outcome was assessed as previously described. While the infected control mice showed the same respective pattern of shedding as shown before for animals infected with

C. trachomatis and *C. muridarum*, the initial shedding (3 dpi) in mice treated with doxycycline was reduced and after 6 days no viable bacteria could be recovered from the vagina in both cases (Figure 3-18). The pathologies observed in mice infected with *C. muridarum* without doxycycline treatment were not present following the corresponding infection with antibiotic treatment (Table 3-10). As previously, no pathologies could be observed macroscopically following *C. trachomatis* infection 43 dpi (Figure 3-19). A total of 4 mice were used per group.

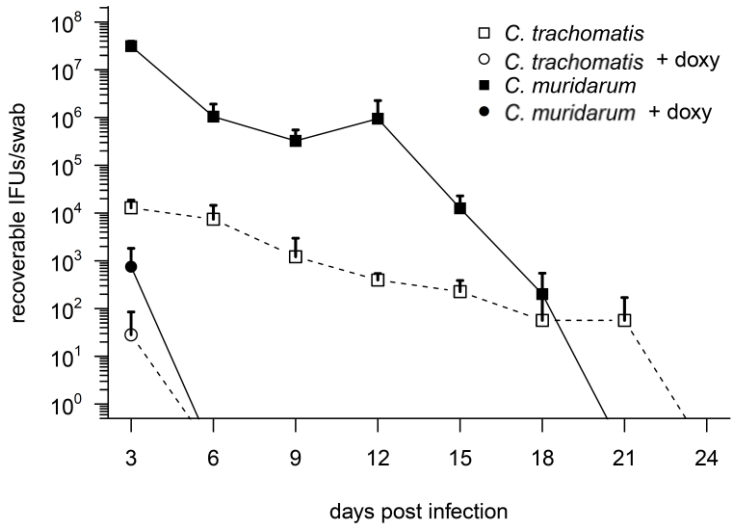


Figure 3-18: I.p. injection of doxycycline abrogates bacterial shedding of *C. trachomatis* and *C. muridarum*. Treatment with doxycycline one day post infection led to a reduced infectious burden at day three post infection while no infectious bacteria could be recovered from vaginal swabs six days post infection. IFUs: inclusion forming units; doxy: doxycycline. n=4 except for *C. muridarum* where n=3 as one mouse showed no bacterial shedding and was therefore removed from the analysis, error bars indicate standard deviation.

Table 3-10: Pathology scoring of mice infected vaginally with *C. muridarum*

Mouse #	*C. muridarum* [left/right] (uterus horn from ventral)	*C. muridarum* + doxycycline [left/right] (uterus horn from ventral)
Mouse 1	2/1	0/0
Mouse 2	4/4	0/0
Mouse 3	0/0*	0/0
Mouse 4	4/1	0/0

*This mouse showed no signs of bacterial shedding

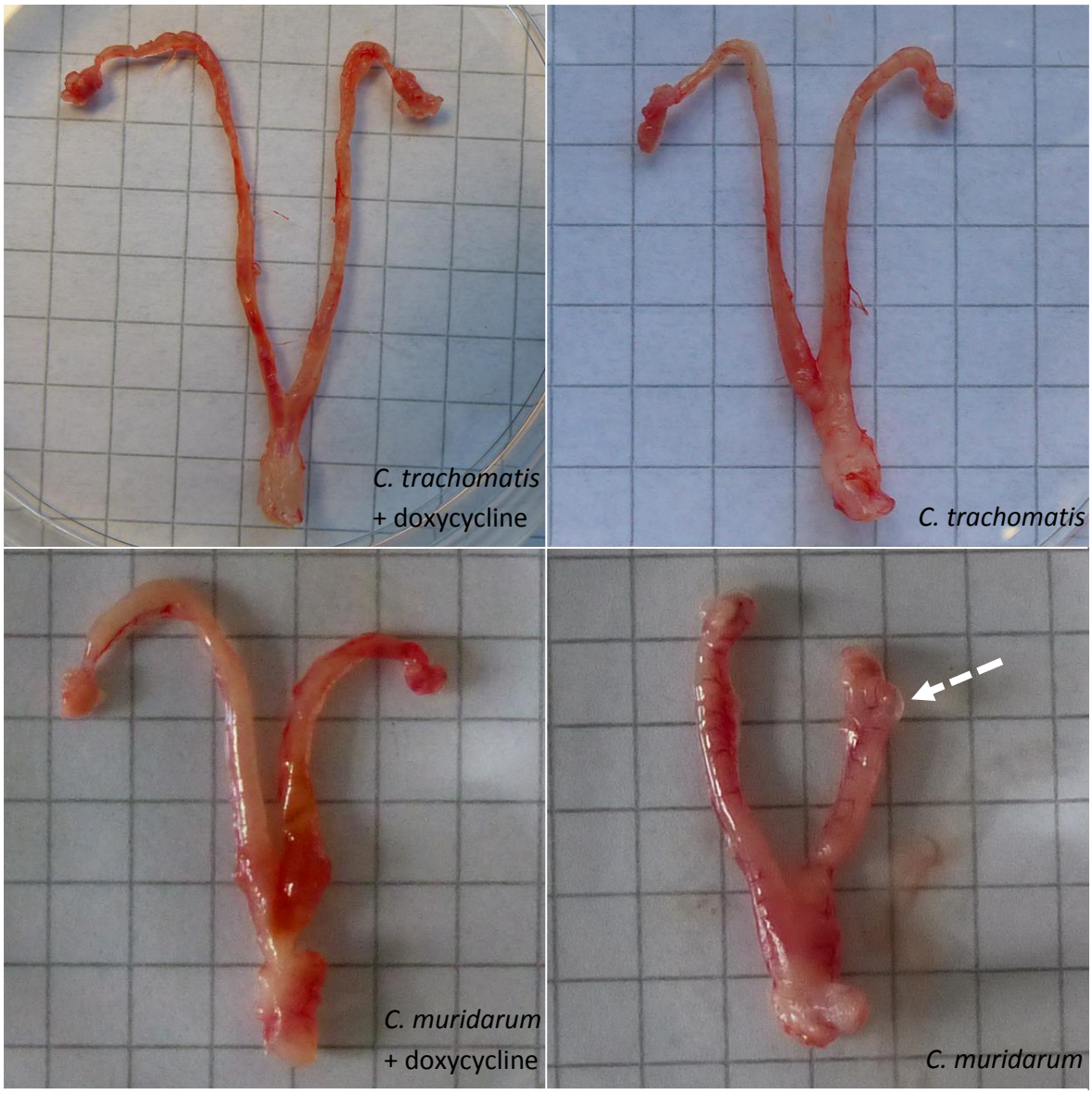


Figure 3-19: Representative pictures of the urogenital tract of mice infected with *C. trachomatis* or *C. muridarum* and corresponding infection with doxycycline treatment following infection. *C. muridarum* infected mice showed macroscopically visible pathologies such as a swollen urogenital tract and hydrosalpinx (white arrow). Doxycycline treatment reduced the swelling and prevented hydrosalpinx formation. A total of four mice per group were dissected.

4 Discussion

4.1 The microbiome of females with infectious infertility is characterized by a community structure indicating an altered susceptibility to sexually transmitted infections

While the role of the microbiome is currently discussed in the context of sexually transmitted infections [32,33,54,56], its role in the development of tubal factor infectious infertility in females has so far remained unexplored. In order to to decipher this role, the present study described the clinical characteristics of females with infectious infertility (ININF) and specifically assessed their microbiome, leading to a comparison of the females with the microbiome of females with non-infectious infertility (nININF), female sex workers (FSW), and fertile females. The presented data can be discussed as follows in the context of the potential contribution of the microbiota of the female urogenital tract (fUGT) to prevention and development of infectious infertility.

4.1.1 The prevalence of sexually transmitted infections of the study participants reflects reports on the European population

The prevalence of sexually transmitted infections (STIs) is regularly assessed by PCR, cultivation and serology. Serological testing is not recommended in many cases for diagnosing acute diseases such as uncomplicated *C. trachomatis* infections. Instead, amplification of nucleic acid of a pathogen or successful cultivation of viable pathogens is required in many cases for detection of an acute infectious disease in clinical diagnostics [84]. Often, PCR-based methods represent the fastest and most sensitive approaches [84]. The diagnostic testing of the cohort of this study can, therefore, be compared to previously published studies. While the prevalence of *N. gonorrhoeae* is generally very low in European countries (prevalence: 0.5 %) [15], its prevalence is increased among FSW as shown by earlier studies and confirmed by the data of the present study [16]. In respect of *M. genitalium*, a prevalence of 2 % for low-risk groups and a prevalence of 7 % for high-risk groups has been published [21], supporting the findings of increased detection rates of *M. genitalium* in FSW. The same trend is visible in respect of *M. hominis* (Figure 3-2A). Epidemiologic studies of *C. trachomatis* have revealed high interstudy variations concerning its prevalence in European countries, ranging from below 1 % up 12 % with confidence intervals ranging up to 20 % [85,86]. In the present study, the prevalence of *C. trachomatis* was generally above 10 %, but did not differ

between groups (Figure 3-2A). Among the tested pathogens, all groups had the highest positivity rate for *Ureaplasma urealyticum*, which corresponds well to recently published findings by Ikonomidis et al. [87]. The good concordance of the pathogen prevalence observed in the present study with that found in the published literature suggests that the conclusions on the human microbiome drawn in this study are informative for population-wide interpretations.

Interestingly, fertile females and nININF have reported lower previous infection rates with *C. trachomatis* than those yielded by the positive tests for acute infections (compare Figure 3-1 and Figure 3-2A). This might be explained by an underestimation of infections with *C. trachomatis* due to the high number of asymptomatic infections [9] and may thus not reflect true rates of previous infections. It has to be mentioned that other studies have indicated the need for screening programs for *C. trachomatis* infections [88], as they are often undetected but have severe long-term complications. The interpretation from the present study supports this view.

While PCR-based detection is recommended for acute testing for infections with *C. trachomatis*, it provides only limited information about the complications of ascending infections so that serologic analysis of anti-*C. trachomatis* antibodies has been suggested as being more effective for such purposes [89]. The present study added *C. trachomatis* antibody responses to the classic diagnostic parameters, which has not been done before for females with the diagnosis of infectious infertility. In general, antibodies targeting *C. trachomatis* were increased in ININF and FSW compared to fertile females and nININF. In particular, MOMP, OMP2, CPAF and HSP60 IgG antibodies were enhanced in ININF (Figure 3-2C/D). In some clinical settings, antibody responses to *C. trachomatis* have been investigated. Detection of high levels of CPAF antibodies in females with *C. trachomatis* cervicitis was first reported by Sharma et al., suggesting that CPAF is immunogenic during human chlamydial infection [90]. Forsbach-Birk et al. have previously analyzed sera from 13 female patients with upper genital tract infections. In this group MOMP, CPAF, OMP2, TARP and PmpD showed the highest overall diagnostic sensitivity and specificity [89]. In contrast to the findings in acute infections, the results from the present thesis highlight a role of HSP60, CPAF, MOMP and OMP2 in host-pathogen interactions in ININF. In particular, HSP60 IgG antibodies separated the ININF group from all other groups, including FSW (Figure 3-2C). Interestingly, antibodies against HSP60 and CPAF were also detected at significantly higher levels in inflammatory ocular *C. trachomatis* infections, suggesting both as markers for

disease severity, and IgG antibodies against CPAF were found significantly more often among the trachomatous trichiasis cases, which may be seen as a consequence of chronic inflammation in the tarsal conjunctiva [91].

While clinical parameters assessed by diagnostic PCR, cultivation and serology are routinely used in clinical settings, the microbiome of the female urogenital tract has not been in the focus of research on infectious infertility. Although the microbiome is of growing interest in clinical research, its potential role in infectious infertility has so far been neglected. The cervical microbiome described in this study will be discussed below in the light of previous findings as well as the current knowledge. Furthermore, implications for the clinical impact and future research considering the impact of the microbiota on STIs and their consequences will be presented.

4.1.2 Careful consideration of primers used in urogenital microbiome studies ensures reliability of results

Numerous combinations of primers have been employed in microbiome studies using 16S rRNA gene sequencing, but for the most part little attention has so far been paid to the impact of the particular primer pair used on the outcome of any particular study. This is somewhat surprising, as the strengths and weaknesses of the common primer pairs have already been studied several years ago. Although generally termed to be “universal”, no primer pair used for amplification is able to amplify 100 % of bacterial sequences [92]. Some important publications on the vaginal microbiome feature a primer combination amplifying the V1 and V2 region of the bacterial 16S rRNA gene [32,35], and the same combination was also used in the present thesis. However, the results presented in this thesis show discrepancies between the outcome of the microbiome sequencing and the results from diagnostic testing, performed in each case on the same samples. Briefly, the presence of two bacterial species found in classical diagnostics (cultivation and diagnostic PCR) could not be confirmed using microbiome sequencing targeting the V1/V2 region of the 16S rRNA gene (Table 6-4). This is, in fact, not surprising, as the inability of the used forward primer to bind to sqequences from members of the taxa Bifidobacteriales (which include the genera *Gardnerella* and *Bifidobacterium*) and *Chlamydia* has been reported previously [93]. It has to be noted here, that these taxa play an important role in vaginal health and disease in humans and it is obvious that missing out such clinically relevant taxa is a disadvantage of the methodical setting that should be avoided. To assess the impact of different target regions, a

different primer pair was used to resequence the same samples amplifying the V3/V4 region of the bacterial 16S rRNA gene. These primers have previously been termed the "most promising bacterial primer pair" for microbiome studies using partial 16S rRNA gene sequencing as they display a high overall coverage for bacterial sequences (89.0 %) and have the lowest number of undetectable phyla of all primer pairs used in 16S microbiome sequencing [92] (there are different primer terms in different publications for the same primer sequences used, a list with corresponding names is given in the appendix, Table 6-3). It is worth highlighting that, the undetectable phyla (*Chloroflexi*, *Elusimicrobia*, BHI80-139 and Candidate division OP11 [92]) do not play a role in the vaginal microbiome.

In sequencing the V3/V4 region, sequences belonging to *C. trachomatis* and *G. vaginalis* could be identified (Table 6-5) and the presence of these species could thereby be verified, in line with the findings of the clinical testing. However, not only these two species were identified as missing in the V1/V2 data. Also the presence of two species from the genus *Bifidobacterium* could only be observed using the V3/V4 region*: Bifidobacterium breve* and *B. bifidum* (Figure 3-6B). It is worth emphasizing that both these species seem to play an important role in a limited number of vaginal communities, as they were present only in a small number of women but, if present, mostly constituted the major part of the community (Figure 3-6).

When comparing the results obtained for the regions V1/V2 and V3/V4, it thus becomes apparent that the former region appears to lack several taxa which are all important in vaginal health and disease and which play a prominent role in the present data on urogenital bacterial communities. When considering earlier publications on the microbiome of the urogenital tract using the V1/V2 region, the aspect of missing species should always be kept in mind. The possibility can not be excluded that above-named species went completely or partly undetected, as it was the case in the data using the V1/V2 region which were analyzed in the present study. This leads to an overrepresentation of other species, such as *Lactobacillus* spp. in all groups and to an underestimation of the diversity present in the samples, as the present data confirm (Figure 3-7, Table 3-4, Table 3-5, and Figure 3-8). The same issue might be relevant in respect if studies focusing only on the V1/V2 region. However, one must not ingnore here that more recent publications using the V1/V2 region have handled this issue by using a modified mixture of primers with variation in the binding region to the bacterial 16S rRNA gene, as suggested by Frank et al. [93], and seem thereby partially to have overcome previous limitations of this region [94]. But a drawback of this method has

to be mentioned: the specificity and sensitivity is decreased in this setting [93], and Kalle et al. have termed such primer mixtures a “very questionable practice” in such settings as the primer mixtures introduce artefacts to the amplification [95]. In addition, it cannot be excluded that a modified mixture of the V1/V2 region still leads to a skew in the results, as the primers might be used in different proportions depending on the template. It is well known that primers are a limiting factor in all PCR settings [96] and the likelihood of primers to find a complementary target sequence might be influenced by the primer-to-target ratio. In general, comparison between microbiome studies using different regions is critical, as the data of the present thesis suggest in respect of urogenital microbiome studies and as has been shown elsewhere for different human body sites and environmental samples [97-100]. To facilitate a more thorough statement on the comparability of the different regions in the urogenital microbiome further studies are necessary, comparing a set of urogenital samples using the modified method of the V1/V2 region, as used by Brotman et al. [94], and the V3/V4 region, as it was used in the data set of the present study. Furthermore, the region and method used in vaginal/urogenital microbiome studies should be considered carefully when conducting a study or reading a publication to ensure a critical evaluation of the data.

In broader terms, this issue not only applies to the vaginal microbiome but also to microbiome studies dealing with other sites of the human body. An example is provided by the study of Koren et al., where the authors claimed that they failed to detect chlamydial sequences in their microbiome data while being able to prove their presence by qPCR [101]. This aspect also gains importance for studies in infants, where *Bifidobacteria* play an important role [102]. To cite just two additional possible instances, this has to be taken into account in studies of rectal chlamydial infections and in urinary tract studies in men.

As the V3/V4 region seems to show more comprehensive data on the urogenital microbiome, which are closer to the real bacterial communities, the following discussion of the microbiome will mostly rely on this region for data interpretation.

4.1.3 The female urogenital tract displays a conserved bacterial community structure throughout the human population

It has been known for decades that the bacterial communities in the vagina of human females consist mostly of *Lactobacillus* species and that the communities colonizing the vagina have rather a low diversity [31]. Ravel et al. published the first report on the female urogenital microbiome using next-generation-sequencing. The authors describe an analysis strategy that

has become the standard for all research on the urogenital microbiota. They used a threshold of 50 % relative abundance to determine if a species dominates a bacterial community from vaginal swabs and described five community types: Community types dominated by *L. crispatus, L. iners, L. gasseri, L. jensenii,* and a community type described as diverse, in which no dominant species occurred with a relative abundance of more than 50 % [32]. Although the exact algorithm used for assigning the bacterial communities to community types differs between publications (e.g. [32,35]), all publications use the above and further community types for analyzing the urogenital microbiome. It is worth noting that no complete consensus on the method for establishing community types has been reached up to now. However, incorporation of machine learning has been suggested for large data sets [103]. Unfortunately, this performing machine learning is not yet suitable for the most commonly used, relatively small data sets, such as those used in this thesis. As it is still the most intuitive way of describing community types, the initial approach described by Ravel et al. was used for the purpose of the present study.

Reviewing the published literature on the female urogenital microbiome using NGS leads to the conclusion that there is a remarkable similarity across the entire human population regarding observable community types. While many reports were published using swabs from women living in the USA (eg. [32,35,48,94]), another study observed similar types in Rwanda [37] and the present study observed the same general community types in a German population (Table 3-3). The proportions of community types within populations defined by geography and race seem to differ [32]. All these factors taken together suggest that a strong interaction of the microbiome with the host seems to play an important role in the community structure. Besides the facts, that the glycogen-rich microenvironment of the urogenital tract favors *Lactobacillus* spp. growth and that a low-oxygen content impacts on the composition of the bacteria, not much is known about the host factors which influence the bacterial composition of the urogenital tract. However, in other parts of the human body a strong influence of the genotype of the host has been demonstrated. For example, FUT2 secretor status strongly influences the composition of colonic mucosa-associated bacteria [104]. Future studies assessing the genetic background of females could shed more light on how the host influences the vaginal microbiota. Besides genotyping single genetic loci of particular interest, large scale NGS-based studies like quantitative trait loci (QTL) mapping and genome wide association studies (GWAS) would, therefore, be appropriate methods. The latter methods are still cost-intensive. But understanding how the urogenital tract is built up

is critical for understanding its potential in clinical settings and QTL mapping was recently successful in identifying host-gene microbiota interaction in autoimmune skin blistering disease in mice [105]. However, these mouse experiments were carried out under standardized conditions and thus excluded confounding environmental factors. Such conditions are not achievable in humans, meaning that confounding factors must be appropriately addressed and that knowledge about differences between females due to such factors as diseases is critical.

4.1.4 Anaerobic bacteria combined with enhanced diversity mark the microbiome in infectious infertility

The microenvironment of the female urogenital tract is known to display a low-oxygen microenvironment [44], generally favoring bacteria that are at least facultatively anaerobic. It is, therefore, not surprising that the major part of bacteria within the urogenital tract consist of lactobacilli, ie. bacteria which are facultative anaerobes [32]. It is furthermore not surprising that *Lactobacillus* was the most prominent genus in fertile females with a relative abundance of about 80 %. Interestingly, when lactobacilli are replaced by other bacteria, as in the present study especially in respect of ININF and FSW, the replacing bacteria are often strictly anaerobes as the data from the present study indicate (Figure 3-7) and which is in concordance with other publications (reviewed by [106]). In the present study, the genera *Gardnerella*, *Prevotella*, *Atopobium*, *Sneathia*, BVAB1, *Mycoplasma*, *Megasphaera*, and *Veillonella* were especially prominent in this sense. Of these, only *Gardnerella*, *Mycoplasma* and *Atopobium* can tolerate oxygen. While some of these taxa are well known as comprising pathogens (*Gardnerella* and *Mycoplasma*), much less is known of the role which taxa such as *Sneathia* and BVAB1 play in human health. However, the taxa just named are also discussed as potential pathogens, since *Sneathia* is associated with BV, preterm labor and invasive infections [107,108] and bacterial vaginosis associated bacteria are named after their significant association with BV [109]. An interesting result from the present thesis is that the strictly aerobic taxon *Aerococcus* was found in fertile females but not in the other groups (Table 3-5). Taken together, the relative abundance of the described taxa in the study groups suggests that a urogenital tract with a healthy microbiome dominated by *Lactobacillus* displays a slightly oxygenated microenvironment. Conversely deviations from such a *Lactobacillus*-dominated microbiota seem to coincide with anaerobic conditions.

4.1.5 Females with infectious infertility have a unique community type distribution

As described earlier, various bacterial CTs, which have been described in the past years, were observed in this study. Amongst the findingst, it is worth noting that the *Lactobacillus*-dominated CTs were reduced, and other CTs (*G. vaginalis*-dominated and diverse communities) were increased in ININF and that the diverse community type was most prominent in FSW (Figure 3-9). Interestingly, only one CT showed stable proportions in all groups: the *L. iners*-dominated CT. In the interpretation of the meaning of different CTs, it is important to discuss which CTs are describing healthy or unhealthy states of the urogenital microbiome respectivly. In clinical settings (such as use of the Nugent score), a healthy microbiota in the female urogenital tract is defined by the presence of *Lactobacillus* species, and deviations from this microbial composition are generally accepted as indicative of a disturbed microbiome (generally termed BV) [31]. However, the authors of landmark publications deciphering the microbiome using NGS identified highly diverse CTs with low or no *Lactobacillus* abundance in females which are, nevertheless, described as healthy. Furthermore, the authors could show that in Hispanic and Black women, the general proportion of diverse CTs is much higher than in White and Asian women. They therefore conclude that diverse communities might reflect another healthy state of the urogenital microbiome, featuring other bacteria with a similar metabolic function as lactobacilli. However, the authors do not take into account, that they are indirectly describing metabolic differences between the CTs themselves based on the pH in the urogenital tract (which is enhanced in diverse communities compared to *Lactobacillus* CTs) [32]. Although diverse CTs occurred in fertile females in this study, they were increased in diseased females (ININF) and FSW (Figure 3-9). This seems to be a strong indicator that diverse CTs rather show a dysbiosis than a healthy state. However, diverse CTs are, by definition, not homogenous and display a lot of different bacterial species in different combinations. Conclusions concerning the actual health state indicated by any urogenital bacterial community needs to be based on thorough investigations, especially with rergard to the diverse communities, which is complicated due to low number of diverse CTs in females. Nevertheless, in this thesis, a diverse microbiome is accepted as describing a state of dysbiosis.

Comparison of the CT distribution of FSW with fertile females had never been published before the preparation of the present study. Although FSW have been in the focus of a publication [37] of the vaginal microbiome in African females they did not include a random

control group. This is of particular importance for the interpretation of microbiome data, as CT proportions differ between ethnicities, complicating correct biological interpretation of the presented data. The data of Borgdorf et al. show that the majority of FSW display highly diverse CTs, while less than 50 % were dominated by *L. iners* and, in parts, by *L. crispatus*. However, their study used vaginal swabs from African sex workers and, as described earlier, Black women have been shown often to display diverse CTs. Therefore, no interpretation in comparison to the normal population was possible. In contrast, the present study showed approximately the same ratio of CTs in female sex workers as Borgdorf et al., but also showed that this ratio is enhanced compared to the normal population (Figure 3-9). It should be mentioned here, that Borgdorf et al. tried to describe the impact of hormonal treatment on the cervicovaginal microbiome but could not show any effect [37]. The results of the present study suggest that FSW are not convenient as a study group for such a purpose, as the microbiome seems to be confounded by other factors disturbing the typical *Lactobacillus*-dominated CTs in FSW. One of these factors might be sexual intercourse, a factor investigated in the present study as well.

4.1.6 Sexual activity impacts on the microbiome but does not explain its vast variability

Diverse CTs were enhanced in FSW in this study raising the question of the impact of sexual intercourse on the microbiome. Several groups of scientists have hitherto attempted to describe the influence of sexual behavior on the urogenital microbiome in females. In the era prior to NGS, Schwebke et al. showed that day-to-day variability is influenced by the frequency of sexual intercourse [40]. More recently, biomarker-confirmed unprotected sex has been shown to be associated with BV [39] and sexual intercourse is a risk factor for acquiring single pathogenic bacteria [110-112]. Different types of sex have been investigated, and it has been shown that oral and rectal sex also impact on the fluctuation of the bacteria in the urogenital tract [40] and rates of BV [38]. However, modern NGS-based studies have not provided any clear answers when assessing the direct impact of sexual intercourse on the urogenital microbiome as a whole. Several studies have attempted to describe the impact of sexual behavior by using NGS-based microbiome studies, but only one study was able to show that increased sexual intercourse reduces the stability of the microbiome [35], while other studies could not show a significant effect on the microbiome (eg. [37]). The present study has now been able to show that there is a direct correlation between sexual activity and the diversity of the microbiome. A significant linear correlation proved that increasing frequency

of sexual intercourse impacts on the microbiome through a trend towards higher diversity (Figure 3-10). As low diversity is one of the key features of an intact, *Lactobacillus*-dominated community in the female urogenital tract, high frequency of sexual intercourse seems so serve as risk factor for abnormal bacterial communities, as has been suggested by studies prior to NGS, and has now been proven by modern sequencing technology taking into account the complete variety of the urogenital communities. However, although a significant contribution of sexual activity to alpha diversity has been demonstrated in this thesis, the effect is rather small, explaining only a minor proportion of the variability of the data set (Table 3-7). In combination with other microbiome studies, which were unsuccessful in showing any effect, this might serve as a hint that sexual intercourse impacts much less on the urogenital microbiome than the results from former studies seem to indicate. As high diversity often also occurred in combination with low frequencies of sexual intercourse and, vice versa,, low diversity also occurred in females with high frequencies of sexual intercourse, other factors are definitely involved. As Liu et al. [113] have shown that the penile microbiome can serve as a reservoir for bacteria associated with BV, one might speculate that, while sexual intercourse per se has at most a minor effect, high frequency of sexual intercourse enhances the chances of coming into contact with such a reservoir and that only such contacts influences the vaginal microbiome negatively while.

4.1.7 The potential impact of a changing urogenital microbiome on STIs and their consequences

The present study has described the cervical microbiome of different study groups and has shown how the microbiome can differ among females. In the context of infectious infertility, it is important to discuss how the microbiome can influence STIs in disease initiation, progression, and subsequent pathological sequels.

It is largely accepted that the major players in vaginal health from the bacterial side are *Lactobacillus* spp. Although *Lactobacillus*-produced bacteriocins and H_2O_2 have also been discussed in this context, the most important function of lactobacilli in the female urogenital tract is the acidification of the microenvironment by the production of lactic acid when utilizing glycogen from the cervicovaginal mucus. This acidification has been shown to be a potent barrier against STIs such as *C. trachomatis, E. coli*, and HIV [33,54,55]. Bacterial communities that are lacking a substantial fraction of *Lactobacillus* species have been shown to be impaired in their STI-preventive mechanisms, as has been demonstrated for

G. vaginalis-dominated cervicovaginal mucus [33] and for supernatant from vaginal swabs [54]. It is of particular interest that there are obvious differences in the STI prevention among *Lactobacillus* species. In the cases of both HIV and *E. coli*, the inhibitory effect was only present when the microbiota was dominated by *L. crispatus.* Similar to the *G. vaginalis*-dominated microbiome, when *L. iners* was the dominating species the inhibitory effect was not achieved [33,54]. Also, women with *L. iners*-dominated cervico-vaginal communities seem to have a higher risk of aquiring *C. trachomatis* from infected partners than women with *L. crispatus* as dominant member of the microbiome [58]. These findings contrast with those of publications which have suggested *L. iners* to be a mutualistic persistent member of the urogenital microbiome which can survive under a wide range of conditions, thus making it an important bacteria for the restoration and maintenance of a healthy microbiota [114]. Adherence to the mucosal surface is believed to be important for the exclusion of competing bacteria in the urogenital tract [115]. Thus, it might be speculated that the lack of most of the known adhesion factors important in the urogenital tract [114] reduces the capability of *L. iners* to compete with other bacteria as. Supporting this theory, adherence proteins of *L. crispatus* were tested to competitively exclude *G. vaginalis*, one of the major components in BV [116].

In the light of the importance of the microbiome in STIs, the results presented in this thesis give totally new insights into the potential interactions between the microbiome and STIs in infectious infertility. A decrease in the *L. crispatus*-dominated CT was observed in ININF in comparison to fertile females, while the *G. vaginalis*-dominated CT was more prominent and the same was true for diverse communities (Figure 3-9). The excluding mechanisms of a *Lactobacillus*-dominated microbiome were obviously reduced in ININF. The enhanced proportions of anaerobic bacteria in ININF point to a rather anaerobic microenvironment, and the low number of lactobacilli to a less acidic environment compared to fertile females. Low numbers of lactobacilli might thereby facilitate the growth of pathogenic bacteria within the urogenital tract through the loss of competing exclusion and the lack of killing capacity of the microenvironment due to lactic acid. Additionally, it has been shown for *C. trachomatis*, the major trigger of infectious infertility, that low oxygen (below 3 %) abrogates anti-chlamydial properties of the host, as it blocks IFN-γ-mediated tryptophan depletion, a host defence mechanism in chlamydial infection [45]. It has also been suggested that indole-producing bacteria, which accumulate, for example, in BV, serve as a basis for tryptophan production by *C. trachomatis*, another potential mechanism avoiding IFN-γ-mediated tryptophan depletion

[106,117]. Taken together, the major microbiome-mediated mechanisms for STI prevention were reduced or abrogated in ININF. It is worth noting, that this coincided with enhanced reported rates of previous *C. trachomatis* infections (Figure 3-1). This serves as a strong hint that disturbed bacterial communities are the basis for enhanced rates of STIs, especially *C. trachomatis* infections, which can finally lead to infertility.

No information on the fertility status of the FSW included in this study was forthcoming. However, following the interpretation above, and taking into account that the microbiome is even further disturbed in FSW than in ININF, infection-mediated infertility rates in FSW should be rather high in comparison to those of the population as a whole, since a disturbed microbiome and an enhanced risk of coming into contact with pathogens conjoin in this case. It might be speculated that a more thorough assessment of the urogenital microbiota of females with different underlying risk factors can lead to a better understanding of how to use microbiota as a prevention strategy against STIs, thus ultimately reducing the number of cases of infertility throughout the human population. It is worth mentioning that this opinion is in concordance with that of leading scientists in the field of urogenital microbiome research [118].

When discussing the potential of intervention in the restoration of a healthy, disease-preventing microbiome it is worth taking a look at the best studied human microbiome in the human context, which is the gut. In recurrent *Clostridium difficile* infection, fecal microbiota transplantation (FMT) is attracting more and more interest. Although this treatment is controversially discussed, due to the risk of transferring other diseases from patient to patient, FMT is a method which successfully restores the gut microbiota and, thereby, prevents reinfection with *C. difficile* [119]. In the cure of *C. difficile,* artificial defined microbial compositions will most probably be replacing FMT once the beneficial bacterial traits are defined. Although probiotic lactobacilli formulae exist for such interventions in the urogenital tract, their use is controversially discussed, especially due to the high number of females with non-*Lactobacillus* CT without any sign of disease in some ethnicities [118], and no specific recommendations are available following a systematic review of the current literature [120]. However, given the severity of adverse health outcomes associated with adverse microbiome states (as demonstrated in the present study in respect of infectious infertility), such *Lactobacillus*-based intervention strategies should clearly be in the focus of research. In the context of differences between *Lactobacillus* species described above, the results from this

study provide evidence that *L. crispatus* is a more appropriate species than *L. iners* for such a purpose.

4.1.8 A successful statistical approach for predicting infectious infertility

The predictive power of NGS-based microbial composition data sets has been proven for eg. metabolic [121,122], autoimmune [123], and inflammatory bowel disease (IBD) [122]. The present study has attempted to provide a prediction model for infectious infertility. On the basis of a comprehensive set of data from diagnostic settings and microbiome sequencing, the predictive ability of the model using different combinations of predictor variables was assessed. Incorporating microbiome sequencing data into diagnostic measures increased the predictive power of the model. Interestingly, the increase in predictive power was stronger when using microbiome data generated using the V3/V4 region (Table 3-8 and Figure 3-12). The most probable reason is the higher diversity of the data, resulting in a finer resolution. This predictive model, as established, obviously relies on comprehensive data, including both classic diagnostic measures and microbiome sequencing data, while single parameters are insufficient to distinguish between females with infectious infertility and other females. It might be speculated that increasing the number of cases to train this model would eliminate the need for classical diagnostic testing in the prediction of ININF, as a disease prediction based solely on NGS-techniques has been successful in other circumstances [121-123]. It is worth noting that, a reduced data set of only four bacterial taxa had a predictive power in obesity comparable to that found in the present study [121] using the V3/V4 region. Further refinement by using increased sample numbers, especially for the ININF group, might also reduce the necessary microbiome complexity of this model and should lead to a robust identification of predictor variables that drive the quality of the model.

The prediction model established during the work for this thesis was able correctly to classify all cases of non-infectious infertility, to be separated from infectious infertility (Table 3-8). This is of great importance as this differentiation, in particular, is one of the major obstacles in the diagnosis of infectious infertility, and highlights the importance of the microbiome in infectious infertility. Interestingly, *G. vaginalis* is not only present in infectious infertility in females (Figure 3-9) but also in males that suffer from infertility [7]. It has been demonstrated that the penile microbiome can serve as a reservoir that influences the vaginal microbiota [113]. This further shows that single markers are not useful for diagnosing infectious infertility in females, as they might be misleading, making NGS-based microbiome sequencing an even more powerful approach for the detection of the underlying reason of infertility.

The prediction model was trained on a set of females with established infectious infertility. Whether the microbial signatures, which are used for prediction, are caused by the infertility state or are already present before females suffer from infertility is now one of the open questions to answer. For this purpose, large scale prospective longitudinal studies are required in order to integrate data from females prior to, and during, the establishment of infectious infertility. Additionally, studies in mice modulating the urogenital microbiome will no doubt yield answers to the questions of how the microbiome influences STIs on a functional basis. This can contribute to the elucidation of the role of the microbiome in establishing infectious infertility.

4.2 Future directions for functional microbiome studies

4.2.1 The critical step from descriptive microbiome studies to functional analysis of the microbiota

Management, manipulation and restoration of the urogenital microbiome could improve womens´ health in the future as the leading scientists in urogenital microbiome research, Ravel and Brotman, state. However, they correctly claim that a system-level understanding of the urogenital microbiota and its impact on reproductive health is needed to facilitate interventional strategies [118]. To understand the contribution of the urogenital microbiome to health and disease, functional hypothesis testing is indispensable. Experimental designs *in vivo* using humans as hosts are, however, very problematic, and for most purposes, not achievable for ethical reasons.

Mouse models have been established for some of the major infertility-causing bacterial STIs: chlamydial infections [61,65,68], *N. gonorrhoeae* [124-126], and *M. genitalium* [127]. *C. trachomatis* is the major bacterial factor in developing infertility, making it the most interesting organism in studying the impact of the microbiota on the pathogen.

4.2.2 Successful development of a chlamydial mouse model for studying microbiome-pathogen interaction in sexually transmitted diseases

Mouse models for chlamydial infections have been in use for a long time. However, a major obstacle in *C. trachomatis* models is a low shedding from infected mice, and, especially, a failure to produce macroscopic pathologies in the upper genital tract [65]. For this reason, the most commonly used model comprises infection of mice with *C. muridarum*, a chlamydial species closely related to *C. trachomatis*, and the mouse-specific biovar inducing hydrosalpinx

at the mouse´s fallopian tubes, thereby resembling human *C. trachomatis* infection [61]. Only recently, a non-surgical method has been developed for a *C. trachomatis* infection directly into the uterus horns, leading to pathological signs in the upper genital tract [68,69]. However, this method has not been proven so far by researchers independent from the laboratory which established the method. The present study, therefore, tested the different models, compared them and established their suitability for urogenital microbiome modulation in order to study the impact of changes in the microbiome on STIs.

As expected, infection with *C. trachomatis* using vaginal infection lead to a low bacterial shedding with no signs of pathology following infection. However, results were similar for the same infection using direct transcervical infection to the uterus horns (Figure 3-14 and Figure 3-15). This was interesting as the experiment was an exact copy of the method published by Gondek et al. [69], which could achieve pathologies in the upper genital tract. It should be mentioned that the only difference between the experiments conducted during the preparation of this thesis and the published method is the serovar used. While Gondek et al. used *C. trachomatis* serovar L2, in the present study the serovar D was used, as it is the most frequently transmitted serovar in the urogenital context. The lack of pathology development in transcervical infection in the context of the present study can thus most probably be attributed to differences in infectivity and following sequels between *C. trachomatis* serovars in mice [128], although a comparison between *C trachomatis* serovar L2 and D has not been made so far [128]. As vaginal infection with *C. trachomatis* is generally known not to produce pathological signs in mice, no follow up experiments were conducted. Instead, further experiments focused on the development of the transcervical infection method using *C. trachomatis* serovar D and comparison to vaginal infection with *C. muridarum* as the hitherto most used model.

As the development of pathologies in the urogenital tract is known to be a post-infection process, the time point of animal killing and observation of the urogenital tracts was shifted to a later moment in time, 43 dpi for further experiments. To facilitate an immune response stronger than in the previous experiments, the inoculum of *C. trachomatis* was increased 4-fold. While *C. muridarum* infection showed typical signs of pathology following infection, which enabled scoring of pathology severity, *C. trachomatis* infection showed only an unclear, swollen pathological phenotype (Figure 3-17). An increased infection dose using *C. trachomatis* did not result in enhanced bacterial shedding (Figure 3-16). However, bacterial shedding was assessed from vaginal swabs, while infection was performed directly to the

uterus horns, and theoretically it is possible that the bacterial burden within the vagina does not reflect the course of infection within the upper genital tract in *C. trachomatis* infection in mice. Real-time measurement of bacterial shedding from the uterus without killing the mice has not yet been performed, neither as part of this thesis nor by others. The only technique established to date for measuring bacteria from the uteri involves killing of the mice and performing recovery or qPCR [129]. Rinsing the uterus with buffered solutions would be an option, but most likely such a method is not feasible using the NSET device, which is used for infection. Therefore, performing experiments assessing the bacterial burden over the time from the uteri of killed mice would be necessary to prove whether vaginal swabs are really an appropriate measure for upper genital tract infections. This would increase the numbers of used animals initially, but would ensure using valid methods, which is important considering the 3R principle: reduce, refine and replace.

The final aim of the discussed mouse models is to use modulation of the urogenital microbiota and test the influence of such modulation on STIs. A simple option modulating the microbiota is the use of antibiotics [130]. Therefore, the *C. trachomatis* and *C. muridarum* models were used to test whether antibiotics given via intraperitoneal (i.p.) injection can reach sufficient levels in the urogenital tract to kill the targeted bacteria. As doxycycline given i.p. sufficiently kills both chlamydial species (Figure 3-18), one can expect that antibiotics can be used to modify the microbiome of the urogenital tract. Therefore, treatment with antibiotics targeting the commensal microbiome and infecting treated mice with *Chlamydia* in comparison to mice with unchanged microbiome would be the next step. As a pre-step to that, one should assess the commensal microbiome of the mice in the animal facility. A step which is important because it has been shown that the commensal microbiome of mice strongly depends on vendor, facility, diet and the handling by the staff of the facility [130].

While the *C. muridarum* model is a very common model, and this study has shown that it can be used as presented here, the *C. trachomatis* model still needs refinement to achieve a valid model for the development of pathologies in the upper genital tract. In this context, increasing the number of bacteria inoculated would be feasible. However, as the bacteria are inoculated directly into the uterus horns, the *C. trachomatis* model is not a good option for studies aiming to assess the influence of vaginal microbiota on the course of disease, and the *C. muridarum* model should be used for this purpose instead. The *C. trachomatis* model can be used for studying the effect of co-infections in the upper genital tract. Additionally, since the microbiome may be expected to influence the course of disease, and following

experimental proof using the *C. muridarum* model, the influence of the mirobiome should be tested with *C. trachomatis* inoculated vaginally in order to test, whether the microbiota are a factor behind in the lack of pathology development in mice. So far, the microbiota is not taken into account and the course of disease of *C. trachomatis* infection in mice is thought to be due to bacterial killing mediated by the innate immune system [129]. However, one should not forget that the microbiome influences the immune system of the host, as it has been demonstrated for the human gut [131]. The transfer of specified bacteria of interest to modify the microbiome of the urogenital tract will be of particular interest in the future. For similar purposes in the gut, a recent review has attempted to establish guidelines for optimizing experimental designs [130] that should be taken into account also for the urogenital tract.

4.3 Conclusions

The microbiota of the lower urogenital tract plays an important role in sexually transmitted infectious diseases. Females with infectious infertility display an adverse microbiome consisting of diverse bacterial communities with an increased proportion of anaerobic bacteria compared to fertile females. The adverse bacterial communities indicate changes in the microenvironment that, in combination, favor the growth of pathogenic bacteria within the urogenital tract. Sexual activity has only a minor influence on the cervical microbiome, but together with a microenvironment which is driven by an adverse microbiome, seems to promote infections. This increases severity of the infections and can be the reason for repeated infections or treatment failure, ultimately leading to severe pathological sequels and causing tubal factor infertility.

Diagnosis of infectious infertility is still hampered by the lack of well-defined parameters. This study showed that single markers are not useful for diagnosing infectious infertility. However, by integrating diagnostic parameters and microbiome data into a logistic regression model, infectious infertility can be predicted with high precision.

There are many different methods how 16S-based microbiome studies can be conducted. Studying the microbiome of the female urogenital tract requires careful consideration of the primers to be used for a comprehensive assessment of bacterial communities. Choosing an inappropriate primer combination leads to skewed results and facilitates wrong conclusions.

Several hypothesis are currently discussed as to how the microbiome influences STIs on a systemic, functional level. Chlamydial mouse infection models have, therefore, been established within the framework of this thesis. These models have the potential to facilitate the study of the impact of the urogenital microbiome on pathogenicity of STIs and the severity of the disease through modulation of the vaginal microbiota.

4.4 Outlook

The present study provided new insights into the cervical microbiome of diseased females. However, the sample numbers were relatively small for some study groups. A large-scale, multicenter follow-up study is needed to validate the results and conclusions from this thesis. Such follow-up studies can be supplemented by bioinformatics prediction of metabolic gene content to assess the contribution of the bacteria to the microenvironment, and by metabolome analysis to describe the microenvironment in more detail. Finally, valuable information can likely be gained by adding pH and oxygen measurements for each sample. Due to the possible transfer of bacteria, sexual partners should be taken into account and, for heterosexual couples, the penile microbiome of the respective partner should be assessed. Increased sample numbers of females with infectious infertility are also required for optimizing the prediction model developed during this study.

Using NGS-based data for the analysis of bacterial communities is set to lift microbiological diagnosis of diseases to a totally new level, as not only the actual pathogen is taken into account, but also the microbiome as a factor which determines the microenvironment and which interacts with pathogens and the host. Further ongoing studies of disease-related microbial compositions in different body sites can, therefore, facilitate a general procedure how the microbiome can be applied in diagnostic settings for rapid and precise next-generation diagnostic tools.

In the light of microbiome-host interactions, an interesting and so far not studied aspect of the female urogenital tract as well as sexually transmitted infections and their consequences is the genetic background. A strong interaction between host and microbiota is to be expected and might mediate the outcome of the infections. Furthermore the urogenital virome is an understudied topic and might interact with host, microbiome and sexually transmitted pathogens. NGS-based techniques can help unraveling those interactions.

The established mouse experiments need further refinement for the transcervical *C. trachomatis* model. Increasing the IFUs which are inoculated to the uterus horns might lead to development of pathological sequels in the upper genital tract of the mice. In order to study the impact of the microbiome on *C. trachomatis*, the microbiota of the mice needs to be described first. Subsequently, the microbiota can be modulated using antibiotics before or during infection. In a more specific way, single species can be added to the microbiome to modify specific metabolic functions, such as indole production, which can be tested for its influence on *C trachomatis*. The mouse model can be extended to other STIs, such as *N. gonorrhoeae* and *M. genitalium*, with slight modifications. These experiments can lead to a better understanding of urogenital pathogens and pathogenesis of the female urogenital tract. Such knowledge is important for interventional strategies which are needed in order to prevent and cure infections in the urogenital tract and their severe sequels.

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6 Appendix

6.1 Supplementary material

Table 6-1: List of all primers used for amplification of the V1/V2 region of bacterial 16S rRNA genes for 454-sequencing

Primer	Nucleotide sequence (5´-3´)
MS-27F	TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-338R	AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-index	ACTCCTACGGGAGGCAGCA GG CTGACTGACT
MS-27F-ID-5	AATGATACGGCGACCACCGAGATCTACAC AACCGCAT TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-27F-ID-46	AATGATACGGCGACCACCGAGATCTACAC AAGGCCTT TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-27F-ID-222	AATGATACGGCGACCACCGAGATCTACAC AGAGTGTG TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-27F-ID-422	AATGATACGGCGACCACCGAGATCTACAC CACAAGTC TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-27F-ID-638	AATGATACGGCGACCACCGAGATCTACAC CGTTCCTA TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-27F-ID-967	AATGATACGGCGACCACCGAGATCTACAC GCTTGGAT TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-27F-ID-1063	AATGATACGGCGACCACCGAGATCTACAC GTCAACAC TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-27F-ID-1070	AATGATACGGCGACCACCGAGATCTACAC GTCACTGA TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-27F-ID-1333	AATGATACGGCGACCACCGAGATCTACAC TCTCGTCA TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-27F-ID-1541	AATGATACGGCGACCACCGAGATCTACAC TTGGTACG TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
MS-338R-ID-7	CAAGCAGAAGACGGCATACGAGAT AACCGGAA AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-338R-ID-221	CAAGCAGAAGACGGCATACGAGAT AGAGTGAC AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-338R-ID-402	CAAGCAGAAGACGGCATACGAGAT CAACTGGT AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-338R-ID-640	CAAGCAGAAGACGGCATACGAGAT CGTTCGTT AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT

MS-338R-ID-737	CAAGCAGAAGACGGCATACGAGAT CTGTTCAC AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-338R-ID-965	CAAGCAGAAGACGGCATACGAGAT GCTTGCAA AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-338R-ID-1064	CAAGCAGAAGACGGCATACGAGAT GTCAACTG AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-338R-ID-1280	CAAGCAGAAGACGGCATACGAGAT TCCTCATG AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-338R-ID-1297	CAAGCAGAAGACGGCATACGAGAT TCGACTAG AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-338R-ID-1520	CAAGCAGAAGACGGCATACGAGAT TTGCAAGC AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-338R-noID	CAAGCAGAAGACGGCATACGAGAT AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
MS-27F-noID	AATGATACGGCGACCACCGAGATCTACAC TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
27F-MID-11	AATGATACGGCGACCACCGAGATCTACAC CGTTGGAT TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
27F-MID-12	AATGATACGGCGACCACCGAGATCTACAC CGTTAAGC TATGGTAATT G AGAGTTTGATCCTGGCTCAG
27F-MID-13	AATGATACGGCGACCACCGAGATCTACAC ACAGCTCA TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
27F-MID-14	AATGATACGGCGACCACCGAGATCTACAC GACAAGTG TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
27F-MID-15	AATGATACGGCGACCACCGAGATCTACAC GCATTAGC TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
27F-MID-16	AATGATACGGCGACCACCGAGATCTACAC TGTGGACT TATGGTAATT GT AGAGTTTGATCCTGGCTCAG
338R-MID-K	CAAGCAGAAGACGGCATACGAGAT ACACCTCT AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-L	CAAGCAGAAGACGGCATACGAGAT ATCGTAGC AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-M	CAAGCAGAAGACGGCATACGAGAT CTCTTGAC AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-N	CAAGCAGAAGACGGCATACGAGAT CCTACCAT AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-O	CAAGCAGAAGACGGCATACGAGAT CTGAAGTC AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-P	CAAGCAGAAGACGGCATACGAGAT ACGATCGT AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT

338R-MID-Q	CAAGCAGAAGACGGCATACGAGAT ATATGGCC AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-R	CAAGCAGAAGACGGCATACGAGAT TTCGATGG AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-S	CAAGCAGAAGACGGCATACGAGAT TACGTACG AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-T	CAAGCAGAAGACGGCATACGAGAT GATCACGT AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-U	CAAGCAGAAGACGGCATACGAGAT GTGACAGA AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-V	CAAGCAGAAGACGGCATACGAGAT TGAGTGTC AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-W	CAAGCAGAAGACGGCATACGAGAT GAGAAGAG AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT
338R-MID-X	CAAGCAGAAGACGGCATACGAGAT TCTGGACA AGTCAGTCAG CC TGCTGCCTCCCGTAGGAGT

Table 6-2: List of all primers used for amplification of the V3/V4 region of bacterial 16S rRNA genes for paired-end-sequencing on a MiSeq sequencer

Primer	Nucleotide sequence (5´-3´)
V3F_Seq	TATGGTAATT GG CCTACGGGAGGCAGCAG
V4R_Seq	AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
Index_V3V4	ATTAGAWACCCBDGTAGTCC GG CTGACTGACT
V3F_MID1	AATGATACGGCGACCACCGAGATCTACAC ATCGTACG TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID2	AATGATACGGCGACCACCGAGATCTACAC ACTATCTG TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID3	AATGATACGGCGACCACCGAGATCTACAC TAGCGAGT TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID4	AATGATACGGCGACCACCGAGATCTACAC CTGCGTGT TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID5	AATGATACGGCGACCACCGAGATCTACAC TCATCGAG TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID6	AATGATACGGCGACCACCGAGATCTACAC CGTGAGTG TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID7	AATGATACGGCGACCACCGAGATCTACAC GGATATCT TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID8	AATGATACGGCGACCACCGAGATCTACAC GACACCGT TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID9	AATGATACGGCGACCACCGAGATCTACAC CTACTATA TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID10	AATGATACGGCGACCACCGAGATCTACAC CGTTACTA TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID11	AATGATACGGCGACCACCGAGATCTACAC AGAGTCAC TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID12	AATGATACGGCGACCACCGAGATCTACAC TACGAGAC TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID13	AATGATACGGCGACCACCGAGATCTACAC ACGTCTCG TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID14	AATGATACGGCGACCACCGAGATCTACAC TCGACGAG TATGGTAATT GG CCTACGGGAGGCAGCAG
V3F_MID15	AATGATACGGCGACCACCGAGATCTACAC GATCGTGT TATGGTAATT GG CCTACGGGAGGCAGCAG

V3F_MID16	AATGATACGGCGACCACCGAGATCTACAC GTCAGATA TATGGTAATT GG CCTACGGGAGGCAGCAG
V4R_MID_A	CAAGCAGAAGACGGCATACGAGAT AACTCTCG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_B	CAAGCAGAAGACGGCATACGAGAT ACTATGTC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_C	CAAGCAGAAGACGGCATACGAGAT AGTAGCGT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_D	CAAGCAGAAGACGGCATACGAGAT CAGTGAGT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_E	CAAGCAGAAGACGGCATACGAGAT CGTACTCA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_F	CAAGCAGAAGACGGCATACGAGAT CTACGCAG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_G	CAAGCAGAAGACGGCATACGAGAT GGAGACTA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_H	CAAGCAGAAGACGGCATACGAGAT GTCGCTCG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_I	CAAGCAGAAGACGGCATACGAGAT GTCGTAGT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_J	CAAGCAGAAGACGGCATACGAGAT TAGCAGAC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_K	CAAGCAGAAGACGGCATACGAGAT TCATAGAC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_L	CAAGCAGAAGACGGCATACGAGAT TCGCTATA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_M	CAAGCAGAAGACGGCATACGAGAT AAGTCGAG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_N	CAAGCAGAAGACGGCATACGAGAT ATACTTCG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_O	CAAGCAGAAGACGGCATACGAGAT AGCTGCTA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_P	CAAGCAGAAGACGGCATACGAGAT CATAGAGA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_Q	CAAGCAGAAGACGGCATACGAGAT CGTAGATC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_R	CAAGCAGAAGACGGCATACGAGAT CTCGTTAC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_S	CAAGCAGAAGACGGCATACGAGAT GCGCACGT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT

V4R_MID_T	CAAGCAGAAGACGGCATACGAGAT GGTACTAT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_U	CAAGCAGAAGACGGCATACGAGAT GTATACGC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_V	CAAGCAGAAGACGGCATACGAGAT TACGAGCA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_W	CAAGCAGAAGACGGCATACGAGAT TCAGCGTT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
V4R_MID_X	CAAGCAGAAGACGGCATACGAGAT TCGCTACG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT

Table 6-3: List of corresponding primer terms

Primer term in this dissertation	Other primer termes	Binding sequence (5´-3´)
V3F	S-D-Bact-0341-b-S-17[92]	CCTACGGGAGGCAGCAG
V4F	S-D-Bact-0785-a-A-21[92]	GGACTACHVGGGTWTCTAAT

6.2 Supplementary data

Table 6-4: Complete list of all species-level classifications using the V1/V2 region. Each operational taxonomic unit (OTU) from the phylotype based analysis on species level is given with the number of reads and corresponding classification using Stirrups [78].

OTU	Number of reads	Taxonomy
Otu001	216449	Lactobacillus(100);crispatus_cluster(100);
Otu002	207056	Lactobacillus(100);iners(100);
Otu003	29095	Lactobacillus(100);gasseri_cluster(100);
Otu004	24868	Atopobium(100);vaginae(100);
Otu005	20914	Lactobacillus(100);jensenii(100);
Otu006	15499	Sneathia(100);amnii(100);
Otu007	11128	Aerococcus(100);christensenii(100);
Otu008	11101	Prevotella(100);cluster2(100);
Otu009	9290	Sneathia(100);sanguinegens(100);
Otu010	8781	Megasphaera(100);elsdenii(100);
Otu011	8733	Lachnospiraceae(100);BVAB1(100);
Otu012	7777	Enterobacteriaceae(100);cluster31(100);
Otu013	7557	Streptococcus(100);agalactiae(100);
Otu014	6130	Mycoplasma(100);hominis(100);
Otu015	5379	Prevotella(100);amnii(100);
Otu016	5165	Pseudomonas(100);gessardii(100);
Otu017	4730	Dialister(100);micraerophilus(100);
Otu018	4334	Streptococcus(100);cluster29(100);
Otu019	3758	Prevotella(100);bivia(100);
Otu020	3643	Staphylococcus(100);cluster47(100);
Otu021	3640	Veillonella(100);montpellierensis(100);
Otu022	3375	Methylobacillus(100);flagellatus_glycogenes(100);
Otu023	3200	Ureaplasma(100);cluster23(100);
Otu024	3175	Streptococcus(100);anginosus(100);
Otu025	2709	Neisseria(100);gonorrhoeae(100);
Otu026	2643	Granulicatella(100);elegans(100);

Otu027	2508	Fusobacterium(100);cluster48(100);
Otu028	2460	Lactobacillus(100);cluster17(100);
Otu029	2414	Enterobacteriaceae(100);cluster55(100);
Otu030	2259	Eggerthella(100);sinensis(100);
Otu031	1892	Prevotella(100);denticola(100);
Otu032	1793	Haemophilus(100);piscium(100);
Otu033	1758	Clostridiales(100);BVAB2(100);
Otu034	1703	Lactobacillus(100);vaginalis(100);
Otu035	1421	Porphyromonas(100);uenonis_asaccharolytica(100);
Otu036	1341	Propionibacterium(100);acnes(100);
Otu037	1233	Clostridium(100);thermoalcaliphilum(100);
Otu038	1171	Streptococcus(100);cluster21(100);
Otu039	1050	Megasphaera(100);micronuciformis(100);
Otu040	1044	Delftia(100);acidovorans_lacustris_tsuruhatensis(100);
Otu041	1026	Prevotella(100);corporis(100);
Otu042	1022	Mobiluncus(100);mulieris(100);
Otu043	1008	Gemella(100);palaticanis(100);
Otu044	950	Prevotella(100);melaninogenica_cluster(100);
Otu045	908	Parvimonas(100);micra(100);
Otu046	874	Bacillus(100);cereus(100);
Otu047	584	Clostridiales(100);BVAB3(100);
Otu048	390	Peptostreptococcus(100);anaerobius(100);
Otu049	377	Prevotella(100);intermedia(100);
Otu050	340	Leuconostoc(100);lactis(100);
Otu051	311	Prevotella(100);disiens(100);
Otu052	300	Corynebacterium(100);cluster45(100);
Otu053	299	Butyrivibrio(100);hungatei(100);
Otu054	274	Streptococcus(100);parasanguinis(100);
Otu055	254	Dialister(100);pneumosintes(100);
Otu056	249	Peptoniphilus(100);indolicus(100);
Otu057	244	Veillonella(100);atypica_dispar_parvula(100);
Otu058	238	Chryseobacterium(100);gleum(100);
Otu059	228	Acinetobacter(100);baumanii(100);

Otu060	227	Gemella(100);morbillorum_sanguinis_haemolysans(100);
Otu061	222	Anaerococcus(100);tetradius(100);
Otu062	220	Prevotella(100);loescheii(100);
Otu063	218	Gardnerella(100);vaginalis(100);
Otu064	194	Finegoldia(100);magna(100);
Otu065	184	Moryella(100);indoligenes(100);
Otu066	174	Sutterella(100);sanguinis(100);
Otu067	163	Microbacterium(100);cluster35(100);
Otu068	129	Caulobacter(100);leidyi(100);
Otu069	122	Aurantimonas(100);altamirensis(100);
Otu070	120	Taylorella(100);equigenitalis(100);
Otu071	108	Clostridium(100);methylpentosum(100);
Otu072	104	Streptococcus(100);salivarius_thermophilus_vestibularis(100);
Otu073	95	Bosea(100);cluster53(100);
Otu074	94	Mycoplasma(100);pirum(100);
Otu075	91	Enterococcus(100);faecalis(100);
Otu076	89	Haemophilus(100);parainfluenzae(100);
Otu077	85	Lactobacillus(100);cluster16(100);
Otu078	79	Anaerococcus(100);lactolyticus_cluster(100);
Otu079	78	Clostridium(100);cluster32(100);
Otu080	76	Clostridium(100);thermocellum(100);
Otu081	76	Campylobacter(100);gracilis(100);
Otu082	75	Bradyrhizobiaceae(100);cluster49(100);
Otu083	75	Dialister(100);propionicifaciens(100);
Otu084	68	Corynebacterium(100);cluster58(100);
Otu085	67	Morganella(100);morganii(100);
Otu086	64	Faecalibacterium(100);prausnitzii(100);
Otu087	62	Agromyces(100);cluster54(100);
Otu088	62	Anaerococcus(100);hydrogenalis(100);
Otu089	61	Brevibacterium(100);ravenspurgense(100);
Otu090	56	Peptoniphilus(100);lacrimalis(100);
Otu091	55	Anaerococcus(100);prevotii(100);
Otu092	54	Comamonadaceae(100);cluster57(100);

Otu093	53	Atopobium(100);rimae(100);
Otu094	53	Sphingobacterium(100);spiritivorum(100);
Otu095	49	Bifidobacterium(100);breve_cluster(100);
Otu096	48	Mogibacterium(100);vescum(100);
Otu097	47	Corynebacterium(100);argentoratense(100);
Otu098	46	Porphyromonas(100);circumdentaria(100);
Otu099	44	Moraxella(100);osloensis(100);
Otu100	44	Bacteroides(100);plebeius(100);
Otu101	43	Aerococcus(100);viridans(100);
Otu102	41	Prevotella(100);copri(100);
Otu103	41	Bulleidia(100);extructa(100);
Otu104	40	Bifidobacterium(100);bifidum(100);
Otu105	39	Peptoniphilus(100);ivorii(100);
Otu106	39	Clostridium(100);lituseburense(100);
Otu107	38	Prevotella(100);buccae(100);
Otu108	38	Clostridium(100);cylindrosporum(100);
Otu109	37	Atopobium(100);parvulum(100);
Otu110	37	Actinomyces(100);turicensis(100);
Otu111	36	Dialister(100);invisus(100);
Otu112	32	Porphyromonas(100);somerae(100);
Otu113	31	Corynebacterium(100);aurimucosum_nigricans(100);
Otu114	31	Pseudomonas(100);aeruginosa(100);
Otu115	31	Fusobacterium(100);gonidiaformans_equinum(100);
Otu116	30	Eubacterium(100);rectale(100);
Otu117	30	Prevotella(100);histicola(100);
Otu118	30	Microbacterium(100);paludicola(100);
Otu119	29	Clostridiaceae(100);cluster18(100);
Otu120	29	Caulobacteraceae(100);cluster34(100);
Otu121	27	Corynebacterium(100);imitans_lipophiloflavum(100);
Otu122	26	Porphyromonas(100);endodontalis(100);
Otu123	25	Porphyromonas(100);bennonis(100);
Otu124	25	Porphyromonas(100);crevioricanis_cansulci(100);
Otu125	24	Prevotella(100);bergensis(100);

Otu126	24	Peptococcus(100);niger(100);
Otu127	22	Lactobacillus(100);crispatus_type1(100);
Otu128	22	Clostridium(100);orbiscindens(100);
Otu129	21	Sutterella(100);parvirubra(100);
Otu130	21	Enterococcus(100);cluster33(100);
Otu131	20	Peptoniphilus(100);harei(100);
Otu132	20	Agromyces(100);allii_lapidis_terreus(100);
Otu133	20	Clostridium(100);colicanis(100);
Otu134	19	Campylobacter(100);ureolyticus(100);
Otu135	19	Alloiococcus(100);otitis(100);
Otu136	18	Corynebacterium(100);thomssenii_sundsvallense(100);
Otu137	18	Clostridium(100);viride(100);
Otu138	17	Streptococcus(100);intermedius_constellatus(100);
Otu139	17	Clostridium(100);cluster46(100);
Otu140	16	Roseburia(100);cluster15(100);
Otu141	16	Propionibacterium(100);granulosum(100);
Otu142	16	Prevotella(100);pallens_aurantiaca(100);
Otu143	15	Clostridium(100);haemolyticum_novyi(100);
Otu144	15	Enterococcus(100);gallinarum(100);
Otu145	14	Lactobacillus(100);cluster14(100);
Otu146	14	Lactobacillus(100);fermentum(100);
Otu147	14	Bacteroides(100);vulgatus_dorei(100);
Otu148	14	Clostridium(100);clariflavum(100);
Otu149	14	Acidaminococcus(100);fermentans(100);
Otu150	14	Ruminococcus(100);bromii(100);
Otu151	13	Lactobacillus(100);coleohominis(100);
Otu152	13	Bacteroides(100);acidifaciens(100);
Otu153	13	Microbacterium(100);agarici_lindanitolerans(100);
Otu154	13	Alloscardovia(100);omnicolens(100);
Otu155	13	Porphyromonas(100);macacae(100);
Otu156	13	Bacteroides(100);uniformis(100);
Otu157	13	Porphyromonas(100);catoniae(100);
Otu158	13	Mobiluncus(100);curtisii(100);

Otu159	12	Roseomonas(100);cervicalis(100);
Otu160	12	Haemophilus(100);aegyptius_influenza_haemolyticus(100);
Otu161	11	Clostridium(100);symbiosum(100);
Otu162	11	Arcanobacterium(100);haemolyticum_phocae(100);
Otu163	11	Streptococcus(100);gordonii(100);
Otu164	10	Porphyromonas(100);gingivicanis(100);
Otu165	10	Oribacterium(100);sinus(100);
Otu166	10	Porphyromonas(100);gingivalis(100);
Otu167	9	Granulicatella(100);adiacens(100);
Otu168	9	Lactobacillus(100);oris_antri(100);
Otu169	9	Facklamia(100);tabacinasalis(100);
Otu170	9	Enterococcus(100);cecorum(100);
Otu171	9	Megasphaera(100);paucivorans_sueciensis(100);
Otu172	8	Clostridium(100);cluster27(100);
Otu173	8	Corynebacterium(100);kroppenstedtii(100);
Otu174	8	Dorea(100);longicatena(100);
Otu175	8	Lactobacillus(100);algidus(100);
Otu176	8	Catonella(100);morbi(100);
Otu177	8	Prevotella(100);maculosa(100);
Otu178	8	Propionibacterium(100);acidifaciens(100);
Otu179	8	Hyphomicrobium(100);sulfonivorans(100);
Otu180	8	Bacteroides(100);nordii(100);
Otu181	8	Bacteroides(100);clarus_gallinarum(100);
Otu182	7	Microbacterium(100);luticocti(100);
Otu183	7	Peptostreptococcus(100);stomatis(100);
Otu184	7	Clostridium(100);aminophilum(100);
Otu185	7	Clostridium(100);innocuum(100);
Otu186	7	Actinomyces(100);neuii(100);
Otu187	7	Mycobacterium(100);parascrofulaceum(100);
Otu188	6	Hyphomicrobium(100);vulgare(100);
Otu189	6	Clostridium(100);paradoxum(100);
Otu190	6	Helcococcus(100);ovis(100);
Otu191	6	Blautia(100);hydrogenotrophica(100);

Otu192	6	Anaerococcus(100);octavius(100);
Otu193	6	Treponema(100);pallidum(100);
Otu194	6	Actinomyces(100);nasicola(100);
Otu195	6	Clostridium(100);xylanovorans(100);
Otu196	5	Prevotella(100);brevis(100);
Otu197	5	Coprococcus(100);eutactus(100);
Otu198	5	Clostridium(100);ramosum(100);
Otu199	5	Prevotella(100);veroralis(100);
Otu200	5	Campylobacter(100);fetus_mucosalis_hyointestinalis(100);
Otu201	5	Eubacterium(100);saburreum(100);
Otu202	5	Lachnospiraceae(100);Lachnospira(100);pectinoschiza(100);
Otu203	5	Papillibacter(100);cinnamivorans(100);
Otu204	5	Bacteroides(100);coagulans(100);
Otu205	4	Prevotella(100);stercorea(100);
Otu206	4	Corynebacterium(100);testudinoris(100);
Otu207	4	Aerococcus(100);urinae(100);
Otu208	4	Blautia(100);glucerasea(100);
Otu209	4	Solobacterium(100);moorei(100);
Otu210	4	Bacteroides(100);ovatus(100);
Otu211	4	Corynebacterium(100);simulans_striatum(100);
Otu212	4	Clostridium(100);bolteae_clostridioforme(100);
Otu213	4	Facklamia(100);ignava(100);
Otu214	3	Microbacterium(100);indicum(100);
Otu215	3	Fusobacterium(100);varium(100);
Otu216	3	Lactobacillus(100);nagelii(100);
Otu217	3	Eggerthella(100);lenta(100);
Otu218	3	Allisonella(100);histaminiformans(100);
Otu219	3	Prevotella(100);oris(100);
Otu220	3	Coprococcus(100);comes(100);
Otu221	3	Enterococcus(100);aquamarinus(100);
Otu222	3	Actinomyces(100);meyeri_odontolyticus(100);
Otu223	3	Porphyromonas(100);gulae(100);
Otu224	3	Bacteroides(100);xylanisolvens(100);

Otu225	3	Corynebacterium(100);pyruviciproducens_cluster(100);
Otu226	3	Clostridium(100);cluster28(100);
Otu227	3	Clostridium(100);hathewayi(100);
Otu228	3	Blautia(100);hansenii(100);
Otu229	3	Agromyces(100);brachium(100);
Otu230	3	Corynebacterium(100);callunae(100);
Otu231	3	Lactobacillus(100);paracollinoides(100);
Otu232	2	Prevotella(100);dentasini(100);
Otu233	2	Prevotella(100);dentalis(100);
Otu234	2	Clostridium(100);uliginosum(100);
Otu235	2	Clostridium(100);disporicum_quinii(100);
Otu236	2	Bacteroides(100);coprophilus(100);
Otu237	2	Actinobaculum(100);schaalii(100);
Otu238	2	Actinomyces(100);viscosus(100);
Otu239	2	Hyphomicrobium(100);methylovorum(100);
Otu240	2	Corynebacterium(100);halotolerans(100);
Otu241	2	Clostridium(100);polysaccharolyticum(100);
Otu242	2	Weissella(100);cibaria(100);
Otu243	2	Ruminococcus(100);gnavus(100);
Otu244	2	Prevotella(100);baroniae(100);
Otu245	2	Lactobacillus(100);delbrueckii(100);
Otu246	2	Microbacterium(100);ginsengiterrae_hydrocarbonoxydans(100);
Otu247	2	Bifidobacterium(100);magnum(100);
Otu248	2	Corynebacterium(100);matruchotii(100);
Otu249	2	Atopobium(100);minutum(100);
Otu250	2	Lactobacillus(100);mucosae(100);
Otu251	2	Lactobacillus(100);pontis_frumenti(100);
Otu252	2	Corynebacterium(100);glutamicum(100);
Otu253	2	Clostridium(100);perfringens(100);
Otu254	2	Blautia(100);coccoides_producta(100);
Otu255	2	Clostridium(100);sporogenes(100);
Otu256	2	Lactobacillus(100);reuteri(100);
Otu257	2	Clostridium(100);mayombei_glycolicum(100);

Otu258	2	Actinomyces(100);oris(100);
Otu259	1	Campylobacter(100);hominis(100);
Otu260	1	Weeksella(100);virosa(100);
Otu261	1	Streptococcus(100);gallinaceus(100);
Otu262	1	Bacteroides(100);fragilis(100);
Otu263	1	Bacteroides(100);thetaiotaomicron_faecis(100);
Otu264	1	Bacteroides(100);coprocola(100);
Otu265	1	Bacteroides(100);chinchillae_satorii(100);
Otu266	1	Prevotella(100);nigrescens(100);
Otu267	1	Corynebacterium(100);mastitidis(100);
Otu268	1	Campylobacter(100);concisus(100);
Otu269	1	Streptococcus(100);orisratti(100);
Otu270	1	Prevotella(100);paludivivens(100);
Otu271	1	Clostridium(100);spiroforme(100);
Otu272	1	Propionibacterium(100);microaerophilum(100);
Otu273	1	Roseburia(100);inulinivorans(100);
Otu274	1	Enterococcus(100);asini(100);
Otu275	1	Haemophilus(100);quentini(100);
Otu276	1	Clostridium(100);aminovalericum(100);
Otu277	1	Actinomyces(100);europaeus(100);
Otu278	1	Bifidobacterium(100);longum_infantis_suis(100);
Otu279	1	Clostridium(100);stercorarium(100);
Otu280	1	Clostridium(100);proteolyticum(100);
Otu281	1	Prevotella(100);pleuritidis(100);
Otu282	1	Bacteroides(100);salanitronis(100);
Otu283	1	Actinobaculum(100);massiliense(100);
Otu284	1	Enterococcus(100);saccharolyticus(100);
Otu285	1	Haemophilus(100);parahaemolyticus_paraphrohaemolyticus(100);
Otu286	1	Prevotella(100);salivae(100);
Otu287	1	Campylobacter(100);rectus(100);
Otu288	1	Clostridium(100);fimetarium(100);
Otu289	1	Gemella(100);bergeri(100);
Otu290	1	Microbacterium(100);insulae(100);

Otu291	1	Prevotella(100);nanceiensis(100);
Otu292	1	Sporobacterium(100);olearium(100);
Otu293	1	Actinomyces(100);hyovaginalis(100);
Otu294	1	Mycoplasma(100);genitalium_pneumoniae(100);
Otu295	1	Lactobacillus(100);kalixensis(100);
Otu296	1	Porphyromonas(100);cangingivalis(100);
Otu297	1	Streptococcus(100);urinalis(100);
Otu298	1	Bacteroides(100);salyersiae(100);
Otu299	1	Prevotella(100);oulorum(100);
Otu300	1	Haemophilus(100);haemoglobinophilus(100);
Otu301	1	Clostridium(100);halophilum(100);
Otu302	1	Clostridium(100);litorale(100);
Otu303	1	Syntrophococcus(100);sucromutans(100);
Otu304	1	Clostridium(100);thermobutyricum_thermopalmarium(100);
Otu305	1	Clostridium(100);felsineum(100);
Otu306	1	Bacteroides(100);graminisolvens(100);
Otu307	1	Propionimicrobium(100);lymphophilum(100);

Table 6-5: Complete list of all species-level classifications using the V3/V4 region. Each operational taxonomic unit (OTU) from the phylotype based analysis on species level is given with the number of reads and corresponding classification using Stirrups [78].

OTU	Number of Reads	Taxonomy
Otu001	222996	Lactobacillus(100);crispatus_cluster(100);
Otu002	156842	Lactobacillus(100);iners(100);
Otu003	43899	Gardnerella(100);vaginalis(100);
Otu004	26023	Lactobacillus(100);gasseri_cluster(100);
Otu005	18633	Prevotella(100);cluster2(100);
Otu006	14815	Atopobium(100);vaginae(100);
Otu007	8057	Prevotella(100);amnii(100);
Otu008	8019	Enterobacteriaceae(100);cluster31(100);
Otu009	7879	Sneathia(100);amnii(100);
Otu010	7522	Lachnospiraceae(100);BVAB1(100);
Otu011	7056	Sneathia(100);sanguinegens(100);
Otu012	6600	Bifidobacterium(100);breve_cluster(100);
Otu013	6510	Veillonella(100);montpellierensis(100);
Otu014	6037	Dialister(100);micraerophilus(100);
Otu015	5686	Prevotella(100);bivia(100);
Otu016	4730	Streptococcus(100);agalactiae(100);
Otu017	4165	Streptococcus(100);cluster29(100);
Otu018	3706	Staphylococcus(100);cluster47(100);
Otu019	3225	Aerococcus(100);christensenii(100);
Otu020	2922	Bifidobacterium(100);bifidum(100);
Otu021	2790	Neisseria(100);gonorrhoeae(100);
Otu022	2784	Ureaplasma(100);cluster23(100);
Otu023	2654	Enterobacteriaceae(100);cluster55(100);
Otu024	2471	Lactobacillus(100);cluster17(100);
Otu025	2232	Fusobacterium(100);cluster48(100);
Otu026	1971	Porphyromonas(100);uenonis_asaccharolytica(100);
Otu027	1940	Acinetobacter(100);baumanii(100);
Otu028	1920	Lactobacillus(100);vaginalis(100);

Otu029	1889	Mycoplasma(100);hominis(100);
Otu030	1871	Clostridiales(100);BVAB2(100);
Otu031	1852	Prevotella(100);melaninogenica_cluster(100);
Otu032	1696	Parvimonas(100);micra(100);
Otu033	1683	Streptococcus(100);anginosus(100);
Otu034	1461	Prevotella(100);loescheii(100);
Otu035	1354	Granulicatella(100);elegans(100);
Otu036	1332	Gemella(100);palaticanis(100);
Otu037	1257	Delftia(100);acidovorans_lacustris_tsuruhatensis(100);
Otu038	1216	Scardovia(100);wiggsiae(100);
Otu039	1193	Prevotella(100);multiformis(100);
Otu040	1083	Prevotella(100);disiens(100);
Otu041	1071	Enterococcus(100);faecalis(100);
Otu042	995	Clostridium(100);cluster32(100);
Otu043	929	Bifidobacterium(100);longum_infantis_suis(100);
Otu044	788	Streptococcus(100);alactolyticus(100);
Otu045	780	Dialister(100);propionicifaciens(100);
Otu046	750	Corynebacterium(100);cluster45(100);
Otu047	702	Peptostreptococcus(100);anaerobius(100);
Otu048	676	Pseudomonas(100);gessardii(100);
Otu049	668	Chlamydia(100);trachomatis(100);
Otu050	653	Bacteroides(100);thetaiotaomicron_faecis(100);
Otu051	635	Lactobacillus(100);kalixensis(100);
Otu052	603	Peptoniphilus(100);ivorii(100);
Otu053	592	Gemella(100);morbillorum_sanguinis_haemolysans(100);
Otu054	587	Lachnospira(100);pectinoschiza(100);
Otu055	581	Dialister(100);invisus(100);
Otu056	577	Eggerthella(100);sinensis(100);
Otu057	576	Anaerococcus(100);lactolyticus_cluster(100);
Otu058	563	Clostridiales(100);BVAB3(100);
Otu059	545	Finegoldia(100);magna(100);
Otu060	525	Megasphaera(100);micronuciformis(100);
Otu061	509	Campylobacter(100);gracilis(100);

Otu062	503	Anaerococcus(100);hydrogenalis(100);
Otu063	495	Prevotella(100);histicola(100);
Otu064	486	Clostridium(100);halophilum(100);
Otu065	483	Alloscardovia(100);omnicolens(100);
Otu066	478	Peptoniphilus(100);indolicus(100);
Otu067	454	Haemophilus(100);aegyptius_influenza_haemolyticus(100);
Otu068	437	Aurantimonas(100);altamirensis(100);
Otu069	427	Porphyromonas(100);crevioricanis_cansulci(100);
Otu070	395	Veillonella(100);atypica_dispar_parvula(100);
Otu071	365	Clostridium(100);aminophilum(100);
Otu072	362	Porphyromonas(100);macacae(100);
Otu073	353	Bifidobacterium(100);dentium(100);
Otu074	339	Moraxella(100);osloensis(100);
Otu075	332	Anaerococcus(100);tetradius(100);
Otu076	327	Haemophilus(100);parainfluenzae(100);
Otu077	284	Mobiluncus(100);mulieris(100);
Otu078	283	Porphyromonas(100);somerae(100);
Otu079	260	Chryseobacterium(100);gleum(100);
Otu080	259	Enterococcus(100);cluster33(100);
Otu081	251	Pseudomonas(100);aeruginosa(100);
Otu082	229	Prevotella(100);intermedia(100);
Otu083	223	Caulobacteraceae(100);cluster34(100);
Otu084	220	Microbacterium(100);cluster35(100);
Otu085	200	Dialister(100);pneumosintes(100);
Otu086	192	Mycoplasma(100);iowae(100);
Otu087	186	Methylobacillus(100);flagellatus_glycogenes(100);
Otu088	179	Peptoniphilus(100);lacrimalis(100);
Otu089	174	Lactobacillus(100);jensenii(100);
Otu090	172	Streptococcus(100);sinensis(100);
Otu091	171	Moryella(100);indoligenes(100);
Otu092	171	Roseomonas(100);cervicalis(100);
Otu093	170	Porphyromonas(100);endodontalis(100);
Otu094	161	Clostridium(100);cellulosi(100);

Otu095	154	Streptococcus(100);parasanguinis(100);
Otu096	146	Bosea(100);cluster53(100);
Otu097	141	Helcococcus(100);ovis(100);
Otu098	139	Prevotella(100);corporis(100);
Otu099	137	Prevotella(100);salivae(100);
Otu100	135	Propionibacterium(100);acnes(100);
Otu101	134	Caulobacter(100);leidyi(100);
Otu102	133	Prevotella(100);shahii(100);
Otu103	125	Campylobacter(100);ureolyticus(100);
Otu104	125	Fusobacterium(100);gonidiaformans_equinum(100);
Otu105	123	Clostridium(100);viride(100);
Otu106	118	Prevotella(100);veroralis(100);
Otu107	118	Porphyromonas(100);bennonis(100);
Otu108	108	Anaerococcus(100);octavius(100);
Otu109	104	Haemophilus(100);piscium(100);
Otu110	102	Bacteroides(100);nordii(100);
Otu111	102	Mycobacterium(100);parascrofulaceum(100);
Otu112	101	Sutterella(100);sanguinis(100);
Otu113	95	Lactobacillus(100);pontis_frumenti(100);
Otu114	94	Granulicatella(100);adiacens(100);
Otu115	91	Clostridium(100);symbiosum(100);
Otu116	87	Bacteroides(100);coagulans(100);
Otu117	86	Bradyrhizobiaceae(100);cluster49(100);
Otu118	84	Morganella(100);morganii(100);
Otu119	83	Atopobium(100);minutum(100);
Otu120	81	Lactobacillus(100);fermentum(100);
Otu121	81	Prevotella(100);bergensis(100);
Otu122	79	Prevotella(100);dentalis(100);
Otu123	78	Streptococcus(100);salivarius_thermophilus_vestibularis(100);
Otu124	74	Clostridium(100);colicanis(100);
Otu125	73	Treponema(100);pallidum(100);
Otu126	72	Comamonadaceae(100);cluster57(100);
Otu127	71	Atopobium(100);parvulum(100);

Otu128	70	Corynebacterium(100);aurimucosum_nigricans(100);
Otu129	67	Atopobium(100);rimae(100);
Otu130	65	Lactobacillus(100);amylolyticus_hamsteri(100);
Otu131	64	Catonella(100);morbi(100);
Otu132	61	Sphingobacterium(100);spiritivorum(100);
Otu133	58	Actinomyces(100);turicensis(100);
Otu134	57	Faecalibacterium(100);prausnitzii(100);
Otu135	55	Lactobacillus(100);delbrueckii(100);
Otu136	49	Peptococcus(100);niger(100);
Otu137	49	Mobiluncus(100);curtisii(100);
Otu138	45	Bifidobacterium(100);scardovii(100);
Otu139	44	Clostridium(100);caenicola(100);
Otu140	43	Porphyromonas(100);gingivalis(100);
Otu141	43	Lactobacillus(100);cluster14(100);
Otu142	43	Clostridium(100);litorale(100);
Otu143	41	Enterococcus(100);gallinarum(100);
Otu144	40	Bifidobacterium(100);catenulatum_pseudocatenulatum(100);
Otu145	38	Actinomyces(100);hongkongensis(100);
Otu146	38	Porphyromonas(100);gingivicanis(100);
Otu147	38	Varibaculum(100);cambriense(100);
Otu148	37	Prevotella(100);stercorea(100);
Otu149	36	Streptococcus(100);cluster21(100);
Otu150	34	Bulleidia(100);extructa(100);
Otu151	33	Lactobacillus(100);kefirgranum_kefiranofaciens(100);
Otu152	33	Actinomyces(100);neuii(100);
Otu153	32	Peptoniphilus(100);harei(100);
Otu154	31	Corynebacterium(100);cluster58(100);
Otu155	31	Bacillus(100);cereus(100);
Otu156	30	Lactobacillus(100);salivarius(100);
Otu157	30	Mycoplasma(100);genitalium_pneumoniae(100);
Otu158	30	Clostridium(100);cellobioparum_termitidis(100);
Otu159	29	Actinomyces(100);meyeri_odontolyticus(100);
Otu160	29	Corynebacterium(100);imitans_lipophiloflavum(100);

Otu161	28	Bacteroides(100);graminisolvens(100);
Otu162	26	Bacteroides(100);vulgatus_dorei(100);
Otu163	25	Lactobacillus(100);mucosae(100);
Otu164	23	Blautia(100);coccoides_producta(100);
Otu165	22	Sutterella(100);parvirubra(100);
Otu166	22	Facklamia(100);miroungae(100);
Otu167	22	Clostridium(100);cluster27(100);
Otu168	22	Corynebacterium(100);simulans_striatum(100);
Otu169	21	Mogibacterium(100);vescum(100);
Otu170	21	Fusobacterium(100);russii(100);
Otu171	20	Lactobacillus(100);secaliphilus(100);
Otu172	20	Lactobacillus(100);cluster16(100);
Otu173	19	Corynebacterium(100);glutamicum(100);
Otu174	19	Aerococcus(100);sanguinicola(100);
Otu175	19	Pasteurellaceae(100);cluster56(100);
Otu176	18	Prevotella(100);nigrescens(100);
Otu177	18	Corynebacterium(100);pyruviciproducens_cluster(100);
Otu178	18	Bacteroides(100);massiliensis(100);
Otu179	17	Corynebacterium(100);mycetoides(100);
Otu180	17	Clostridium(100);orbiscindens(100);
Otu181	17	Anaerococcus(100);prevotii(100);
Otu182	17	Aerococcus(100);viridans(100);
Otu183	17	Megasphaera(100);paucivorans_sueciensis(100);
Otu184	17	Clostridium(100);thermocellum(100);
Otu185	16	Acidaminococcus(100);fermentans(100);
Otu186	16	Lactobacillus(100);animalis_apodemi_murinus(100);
Otu187	16	Megasphaera(100);elsdenii(100);
Otu188	15	Campylobacter(100);rectus(100);
Otu189	15	Corynebacterium(100);durum(100);
Otu190	15	Campylobacter(100);hominis(100);
Otu191	14	Microbacterium(100);ginsengiterrae_hydrocarbonoxydans(100);
Otu192	14	Lactobacillus(100);crispatus_type1(100);
Otu193	13	Actinobaculum(100);schaalii(100);

Otu194	13	Peptostreptococcus(100);stomatis(100);
Otu195	13	Streptococcus(100);intermedius_constellatus(100);
Otu196	12	Actinomyces(100);europaeus(100);
Otu197	12	Brevibacterium(100);ravenspurgense(100);
Otu198	12	Microbacterium(100);mitrae(100);
Otu199	12	Lactobacillus(100);crustorum_mindensis_farciminis(100);
Otu200	12	Haemophilus(100);quentini(100);
Otu201	12	Weeksella(100);virosa(100);
Otu202	11	Lactobacillus(100);reuteri(100);
Otu203	11	Agromyces(100);cluster54(100);
Otu204	11	Arcanobacterium(100);hippocoleae(100);
Otu205	10	Prevotella(100);nanceiensis(100);
Otu206	9	Butyrivibrio(100);hungatei(100);
Otu207	9	Yersinia(100);intermedia_kristensenii(100);
Otu208	9	Dorea(100);longicatena(100);
Otu209	9	Eggerthella(100);lenta(100);
Otu210	9	Bacteroides(100);coprophilus(100);
Otu211	9	Prevotella(100);denticola(100);
Otu212	9	Corynebacterium(100);thomssenii_sundsvallense(100);
Otu213	9	Aerococcus(100);urinae(100);
Otu214	8	Prevotella(100);paludivivens(100);
Otu215	8	Clostridiaceae(100);cluster18(100);
Otu216	8	Methylophilus(100);cluster11(100);
Otu217	8	Microbacterium(100);indicum(100);
Otu218	8	Porphyromonas(100);gulae(100);
Otu219	8	Prevotella(100);oris(100);
Otu220	8	Arcanobacterium(100);haemolyticum_phocae(100);
Otu221	8	Clostridium(100);clariflavum(100);
Otu222	8	Butyrivibrio(100);proteoclasticus(100);
Otu223	7	Pseudobutyrivibrio(100);ruminis(100);
Otu224	7	Roseburia(100);inulinivorans(100);
Otu225	7	Lactobacillus(100);rossiae(100);
Otu226	7	Prevotella(100);oralis(100);

Otu227	7	Enterococcus(100);canintestini_dispar(100);
Otu228	7	Actinobaculum(100);massiliense(100);
Otu229	7	Ruminococcus(100);torques(100);
Otu230	7	Corynebacterium(100);kroppenstedtii(100);
Otu231	7	Lactobacillus(100);oris_antri(100);
Otu232	7	Leuconostoc(100);lactis(100);
Otu233	6	Actinomyces(100);radicidentis(100);
Otu234	6	Bacteroides(100);stercoris(100);
Otu235	6	Clostridium(100);putrefaciens_algidicarnis(100);
Otu236	6	Oribacterium(100);sinus(100);
Otu237	6	Clostridium(100);populeti(100);
Otu238	6	Bacteroides(100);xylanisolvens(100);
Otu239	6	Blautia(100);glucerasea(100);
Otu240	6	Prevotella(100);pallens_aurantiaca(100);
Otu241	6	Bacteroides(100);coprocola(100);
Otu242	6	Actinomyces(100);dentalis(100);
Otu243	6	Clostridium(100);collagenovorans(100);
Otu244	5	Porphyromonas(100);catoniae(100);
Otu245	5	Enterococcus(100);saccharolyticus(100);
Otu246	5	Propionimicrobium(100);lymphophilum(100);
Otu247	5	Streptococcus(100);ovis(100);
Otu248	5	Lactobacillus(100);agilis(100);
Otu249	5	Bacteroides(100);pyogenes(100);
Otu250	5	Clostridium(100);ramosum(100);
Otu251	5	Bacteroides(100);coprosuis(100);
Otu252	5	Bacteroides(100);barnesiae(100);
Otu253	5	Streptococcus(100);dentapri(100);
Otu254	5	Clostridium(100);bolteae_clostridioforme(100);
Otu255	5	Acetitomaculum(100);ruminis(100);
Otu256	5	Bacteroides(100);fluxus(100);
Otu257	5	Ruminococcus(100);bromii(100);
Otu258	4	Weissella(100);cibaria(100);
Otu259	4	Actinomyces(100);naeslundii(100);

Otu260	4	Prevotella(100);dentasini(100);
Otu261	4	Coprococcus(100);eutactus(100);
Otu262	4	Prevotella(100);maculosa(100);
Otu263	4	Prevotella(100);copri(100);
Otu264	4	Actinomyces(100);oris(100);
Otu265	4	Haemophilus(100);parahaemolyticus_paraphrohaemolyticus(100);
Otu266	3	Lactobacillus(100);hayakitensis(100);
Otu267	3	Microbacterium(100);ulmi(100);
Otu268	3	Lactobacillus(100);equi(100);
Otu269	3	Clostridium(100);spiroforme(100);
Otu270	3	Clostridium(100);polysaccharolyticum(100);
Otu271	3	Propionibacterium(100);cyclohexanicum(100);
Otu272	3	Lactobacillus(100);acetotolerans(100);
Otu273	3	Clostridium(100);ljungdahlii(100);
Otu274	3	Clostridium(100);alkalicellulosi(100);
Otu275	3	Clostridium(100);tetanomorphum(100);
Otu276	3	Bacteroides(100);acidifaciens(100);
Otu277	3	Clostridium(100);acidisoli_akagii(100);
Otu278	3	Lactobacillus(100);intestinalis(100);
Otu279	3	Mycoplasma(100);spermatophilum(100);
Otu280	3	Streptococcus(100);hyointestinalis(100);
Otu281	3	Lactobacillus(100);algidus(100);
Otu282	3	Bacteroides(100);uniformis(100);
Otu283	3	Prevotella(100);falsenii(100);
Otu284	3	Facklamia(100);tabacinasalis(100);
Otu285	3	Lactobacillus(100);thailandensis_pantheris(100);
Otu286	2	Propionibacterium(100);freudenreichii(100);
Otu287	2	Lactobacillus(100);cluster26(100);
Otu288	2	Lactobacillus(100);concavus(100);
Otu289	2	Prevotella(100);baroniae(100);
Otu290	2	Corynebacterium(100);testudinoris(100);
Otu291	2	Campylobacter(100);sputorum(100);
Otu292	2	Lactobacillus(100);sharpeae(100);

Otu293	2	Bacteroides(100);propionicifaciens(100);
Otu294	2	Clostridium(100);uliginosum(100);
Otu295	2	Actinomyces(100);radingae(100);
Otu296	2	Eubacterium(100);saburreum(100);
Otu297	2	Anaerostipes(100);butyraticus(100);
Otu298	2	Allisonella(100);histaminiformans(100);
Otu299	2	Facklamia(100);ignava(100);
Otu300	2	Sporobacterium(100);olearium(100);
Otu301	2	Mycoplasma(100);iners(100);
Otu302	2	Actinomyces(100);massiliensis(100);
Otu303	2	Clostridium(100);hylemonae(100);
Otu304	2	Prevotella(100);ruminicola(100);
Otu305	2	Actinomyces(100);viscosus(100);
Otu306	2	Bacteroides(100);helcogenes(100);
Otu307	2	Lactobacillus(100);satsumensis_oeni(100);
Otu308	2	Ruminococcus(100);gnavus(100);
Otu309	2	Propionibacterium(100);granulosum(100);
Otu310	2	Actinomyces(100);bowdenii(100);
Otu311	2	Clostridium(100);thermoalcaliphilum(100);
Otu312	1	Clostridium(100);hathewayi(100);
Otu313	1	Lactobacillus(100);amylotrophicus(100);
Otu314	1	Lactobacillus(100);sanfranciscensis(100);
Otu315	1	Clostridium(100);paradoxum(100);
Otu316	1	Bifidobacterium(100);adolescentis(100);
Otu317	1	Clostridium(100);cluster46(100);
Otu318	1	Actinobaculum(100);suis(100);
Otu319	1	Clostridium(100);oroticum(100);
Otu320	1	Haemophilus(100);influenzae-murium(100);
Otu321	1	Corynebacterium(100);atypicum(100);
Otu322	1	Propionibacterium(100);thoenii(100);
Otu323	1	Lactobacillus(100);perolens(100);
Otu324	1	Lactobacillus(100);cluster22(100);
Otu325	1	Lactobacillus(100);ruminis(100);

Otu326	1	Agromyces(100);allii_lapidis_terreus(100);
Otu327	1	Streptococcus(100);plurextorum(100);
Otu328	1	Coprococcus(100);catus(100);
Otu329	1	Prevotella(100);bryantii(100);
Otu330	1	Lactobacillus(100);vini(100);
Otu331	1	Clostridium(100);haemolyticum_novyi(100);
Otu332	1	Enterococcus(100);cluster24(100);
Otu333	1	Bacteroides(100);finegoldii(100);
Otu334	1	Lactobacillus(100);gastricus(100);
Otu335	1	Caulobacteraceae(100);bacteroides_variabilis(100);
Otu336	1	Haemophilus(100);haemoglobinophilus(100);
Otu337	1	Bacteroides(100);fragilis(100);
Otu338	1	Bifidobacterium(100);psychroaerophilum(100);
Otu339	1	Streptococcus(100);ictaluri_iniae_parauberis(100);
Otu340	1	Actinomyces(100);timonensis_denticolens(100);
Otu341	1	Campylobacter(100);cluster30(100);
Otu342	1	Prevotella(100);albensis(100);
Otu343	1	Clostridium(100);aurantibutyricum_acetobutylicum(100);
Otu344	1	Bacteroides(100);ovatus(100);
Otu345	1	Clostridium(100);cadaveris(100);
Otu346	1	Bacteroides(100);plebeius(100);
Otu347	1	Clostridium(100);methylpentosum(100);
Otu348	1	Microbacterium(100);soli(100);
Otu349	1	Lactobacillus(100);farraginis(100);
Otu350	1	Lactobacillus(100);kunkeei(100);
Otu351	1	Campylobacter(100);showae(100);
Otu352	1	Lactobacillus(100);composti(100);
Otu353	1	Eubacterium(100);rectale(100);
Otu354	1	Porphyromonas(100);circumdentaria(100);
Otu355	1	Prevotella(100);oulorum(100);
Otu356	1	Corynebacterium(100);callunae(100);
Otu357	1	Clostridium(100);argentinese_schirmacherense(100);
Otu358	1	Clostridium(100);cylindrosporum(100);

Otu359	1	Lactobacillus(100);bifermentans(100);
Otu360	1	Campylobacter(100);concisus(100);
Otu361	1	Roseburia(100);cluster15(100);
Otu362	1	Microbacterium(100);hatanonis(100);
Otu363	1	Corynebacterium(100);efficiens(100);

6.3 Information sheet for study participants

Aufklärungsbogen

Studie: **Bedeutung der mikrobiellen Flora (Mikrobiom) für die Entstehung tubarer Infertilität**

Sehr geehrte Studienteilnehmerin,

hiermit möchten wir Sie zusätzlich zum persönlichen Gespräch über das geplante Vorhaben informieren. Bitte lesen Sie diese Patienteninformation sorgfältig durch. Ihr Arzt wird mit Ihnen auch direkt über die Studie sprechen. Bitte fragen Sie Ihren Arzt, wenn Sie etwas nicht verstehen oder wenn Sie zusätzlich etwas wissen möchten. Sollten Sie weitere Fragen bezüglich der Studie haben, wenden Sie sich bitte an den aufklärenden Arzt (Fr. Dr. R. Speer) oder direkt an die Leiter der Studie (Prof. Dr. Jan Rupp, PD Dr. M. K. Bohlmann).

Informationen zur Studie:

Chronische Eileiterentzündungen sind zumeist im geschlechtsreifen Alter vorkommende Erkrankungen, die häufig durch bakterielle Infektionserreger verursacht werden. In 20- 30% der Fälle kann eine solche Entzündung zur Sterilität der betroffenen Frauen und ungewollter Kinderlosigkeit führen. Nahezu 50% der infektiösen Eileiterentzündungen werden durch Chlamydien hervorgerufen, zumeist ohne dass die Infektion von den Frauen bemerkt wird. Zur Ursachenaufklärung führen wir nun Untersuchungen durch, die erklären sollen, welche Bedingungen die Entstehung chronischer Eileiterentzündungen begünstigen. Wir erhoffen uns dadurch neue Erkenntnisse für die Behandlung der Erkrankung.

Die Teilnahme an dieser Studie ist freiwillig. Als Teilnehmerin an dieser Studie bitten wir Sie darum, dass wir einmalig entsprechende Abstriche vom Gebärmutterhals und 20 ml Blut für weitergehende Untersuchungen auf abgelaufene Infektionen mit Chlamydien entnehmen dürfen. Ausserdem möchten wir Sie bitten, den anonymisierten Fragebogen zu Lebensgewohnheiten und bisherigen Erkrankungen möglichst wahrheitsgetreu auszufüllen.

Es entstehen für Sie durch die Teilnahme an der Studie keine Folgeuntersuchungen oder zusätzliche zeitliche oder finanzielle Belastungen.

Sie können jederzeit ohne Angabe von Gründen die Teilnahme an der Studie beenden, ohne dass Ihnen dadurch Nachteile im Hinblick auf die Behandlung oder Ihr Verhältnis zu Ihrer behandelnden Ärztin bzw. Arzt entstehen. Nach Beendigung Ihrer Teilnahme werden keine weiteren Daten von Ihnen erhoben. Ihre bisherigen Daten werden unwiderruflich anonymisiert, d.h. Sie können nicht mehr anhand der Daten identifiziert werden.

Datenschutzrechtliche Informationen:

Für die Datenverarbeitung verantwortlich sind die Leiter der Studie:

Prof. Dr. med. Jan Rupp
Institut für Med. Mikrobiologie und Hygiene
Infektionsambulanz/ Med. Klinik III
UK-SH/ Campus Lübeck
Email: Jan.Rupp@uk-sh.de
Tel: 0451 500- 4409

PD Dr. med. Michael K. Bohlmann
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Email: Michael.Bohlmann@uk-sh.de
Tel: 0451 500- 2134

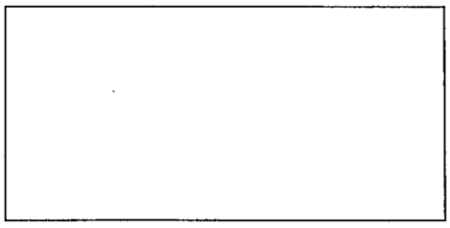
Die Datenerhebung erfolgt zum Zweck des oben genannten Studienziels/ Forschungsvorhabens. Es werden Daten über das Lebensalter, den bisherigen Krankheitsverlauf, sowie über den den Fragebogen Daten zur Lebenssituation und vorangegangene Erkrankungen erhoben. Ihre Daten werden in pseudonymisierter Form, d.h. ohne direkten Bezug zu Ihrem Namen, elektronisch gespeichert und ausgewertet. Die Bestimmungen des Datenschutzgesetzes werden eingehalten. Zugriff auf Ihre Daten haben nur Mitarbeiter der Studie. Diese Personen sind zur Verschwiegenheit verpflichtet. Die Daten sind vor fremden Zugriff geschützt. Die personenbezogenen Daten werden nach Erreichen des Studienziels/am Ende des Forschungsvorhaben, spätestens jedoch nach 5 Jahren anonymisiert, soweit gesetzliche Vorgaben nicht längere Archivierungspflichten vorsehen. Die gewonnenen Materialien (Abstrichproben, Blutprobe) werden in pseudonymisierter Form gelagert und nach einem Zeitraum von 5 Jahren vernichtet.

6.4 Declaration of consent

Einverständniserklärung

(zum Verbleib beim Prüfarzt)

zur Studie: **Bedeutung der mikrobiellen Flora (Mikrobiom) für die Entstehung tubarer Infertilität**



Ich bin heute von einem Arzt/ einer Ärztin über die Studie

"Bedeutung der mikrobiellen Flora (Mikrobiom)

für die Entstehung tubarer Infertilität"

informiert worden. Ich hatte Gelegenheit alle meine Fragen zu stellen. Diese wurden zufriedenstellend und vollständig beantwortet. Ich habe die schriftliche Patienteninformation zur oben genannten Studie erhalten, gelesen und verstanden. Ich wurde ausführlich – mündlich und schriftlich – über das Ziel und den Verlauf der Studie, meine Rechte und Pflichten, den mir zustehenden Versicherungsschutz und die Freiwilligkeit der Teilnahme aufgeklärt.

Einwilligung zur Teilnahme

Ich erkläre hiermit meine Teilnahme an der oben genannten Studie. Ich wurde darauf hingewiesen, dass meine Teilnahme freiwillig ist und dass ich das Recht habe, diese jederzeit ohne Angabe von Gründen zu beenden, ohne dass mir dadurch Nachteile entstehen.

Ferner bestätige ich, daß ich mit der wissenschaftlichen Auswertung meiner anonymisierten, personenbezogenen Daten einverstanden bin. Mir ist bekannt, daß ich jederzeit ohne Angabe von Gründen meine Zustimmung widerrufen kann. **Ich wurde über meine Datenschutzrechte informiert. Mit der Erhebung, Verarbeitung und Speicherung meiner Daten, sowie der Übermittlung im Rahmen der Studie bin ich einverstanden.**

Lübeck, den

Unterschrift des Probanden:

Unterschrift des aufklärenden Arztes:

6.5 Questionnaire

Studie: Bedeutung der mikrobiellen Flora für die Entstehung tubarer Infertilität

Datum: .....

ID: .....

Fragebogen zur Studie:

„Bedeutung der mikrobiellen Flora (Mikrobiom) für die Entstehung tubarer Infertilität"

Sehr geehrte Studienteilnehmerin,

vielen Dank für Ihr Interesse und Ihre Teilnahme an unserer Studie.
Ergänzend zu den Abstrichen möchten wir mit diesem Fragebogen einige Informationen gewinnen, die bei der Interpretation der Ergebnisse von Bedeutung sein können.
Wir bitten Sie daher, sich einen Moment Zeit zu nehmen und diesen Bogen auszufüllen. Sollten dabei Fragen aufkommen (z.B. zu Medikamenten oder Diagnosen), helfen Ihnen die Arzthelferinnen gerne weiter. Alle Angaben sind freiwillig und werden anonym behandelt. Falls Sie eine der Fragen nicht beantworten möchten, lassen Sie diese aus.

Den ausgefüllten Fragebogen werfen Sie bitte in den dafür vorgesehenen Briefkasten.

Vielen Dank!

1. Demographische Angaben

Alter in Jahren: .....

Nationalität: .....

Gewicht in kg: .....

Größe in cm: .....

Höchster Abschluss: ☐ keiner ☐ Hauptschulabschluss ☐ Realschulabschluss ☐ Hochschulreife ☐ Hochschulabschluss

2. Medikamentenanamnese

	ja	nein	weiß nicht
Nehmen Sie Medikamente ein?	☐	☐	☐

Wenn ja, welche? .....

.....

.....

Studie: Bedeutung der mikrobiellen Flora für die Entstehung tubarer Infertilität

	ja	nein	weiß nicht
Nehmen Sie zurzeit ein Antibiotikum ein?	☐	☐	☐
Wenn ja, welches?			
Seit wann?			
Weshalb?			

3. Organanamnese

Haben Sie aktuell eine der folgenden Erkrankungen? Wenn ja, welche?	ja	nein	weiß nicht
Erkrankung der Geschlechtsorgane:	☐	☐	☐
Erkrankung des Herz-Kreislauf-Systems:	☐	☐	☐
Erkrankung der Atemwege:	☐	☐	☐
Erkrankung des Verdauungstraktes:	☐	☐	☐
Erkrankung der Niere und Harnwege:	☐	☐	☐
Erkrankung des Nervensystems:	☐	☐	☐
Erkrankung der Knochen und Gelenke:	☐	☐	☐
Erkrankung der Haut:	☐	☐	☐
Erkrankung des Hormonhaushaltes *(z.B. Diabetes mellitus,Schilddrüsen-Über oder Unterfunktion)*:	☐	☐	☐
Erkrankung des Immunsystems *(z.B. Allergien, Asthma, rheumatische Erkrankungen, Myasthenia gravis, Multiple Sklerose, Colitis ulcerosa, M. Crohn, M. Basedow, M. Hashimoto, Sarkoidose, Zöliakie)*:	☐	☐	☐
andere Erkrankungen:	☐	☐	☐

udie: Bedeutung der mikrobiellen Flora für die Entstehung tubarer Infertilität

4. Angaben zum Tabak-, Alkohol- und Drogenkonsum		
	ja	nein
Rauchen Sie? Wenn ja, wie viele Zigaretten pro Tag? ..... Zigaretten pro Tag	☐	☐
Wie häufig trinken Sie Alkohol? An ..... Tagen pro Woche		
Nehmen Sie regelmäßig (mind. 1 x pro Woche) illegale Drogen? Wenn ja, welche und wie häufig?	☐	☐

5. Gynäkologische Anamnese		
Wie alt waren Sie bei Ihrer ersten Regelblutung?	..... Jahre	☐ weiß nicht
Wie alt waren Sie bei Ihrem ersten Geschlechtsverkehr?	..... Jahre	☐ weiß nicht
Wie häufig haben Sie etwa Geschlechtsverkehr?	..... Mal pro Monat	
Betreiben Sie Empfängnisverhütung?	☐ ja	☐ nein
Wenn ja, in welcher Form?		
Mit wie viel verschiedenen Personen hatten Sie in den letzten 12 Monaten sexuellen Kontakt (analer, oraler oder vaginaler Geschlechtsverkehr)? Mit ..... Personen		
Wie häufig haben Sie in den letzten 12 Monaten Kondome verwendet? Bitte markieren Sie die Antwort mit einem Kreuz auf der Skala zwischen nie und immer: nie — immer		

Studie: Bedeutung der mikrobiellen Flora für die Entstehung tubarer Infertilität

Gehen Sie regelmäßig zum Frauenarzt? ☐ ja, alle ..... Monate ☐ nein			
Wurde bei Ihnen schon einmal eine Infektionskrankheit diagnostiziert? ☐ ja ☐ nein ☐ weiß nicht			
Wenn ja, welche und in welchem Jahr?	ja: bitte das Jahr angeben	nein	weiß nicht
Humanes Papilloma-Virus (HPV) bzw. ein auffälliger PAP-Abstrich	☐ ja:	☐	☐
Gonokokken (Tripper)	☐ ja:	☐	☐
Chlamydien	☐ ja:	☐	☐
Herpes genitalis	☐ ja:	☐	☐
Lues (Syphilis)	☐ ja:	☐	☐
Genitale Pilzinfektion	☐ ja:	☐	☐
HIV	☐ ja:	☐	☐
Hepatitis (B oder C)	☐ ja:	☐	☐
Andere:	☐ ja:		
.....	☐ ja:		
Haben oder hatten Sie unangenehm riechenden Scheidenausfluss? Wenn ja, in welchem Jahr? ☐ ja: ..... ☐ nein ☐ weiß nicht			
Haben oder hatten Sie eine bakterielle Vaginose? Wenn ja, in welchem Jahr? ☐ ja: ..... ☐ nein ☐ weiß nicht			

udie: Bedeutung der mikrobiellen Flora für die Entstehung tubarer Infertilität

Haben/hatten Sie Kinder? ☐ ja ☐ nein Wenn ja, wann sind diese geboren? ....
Hatten Sie Fehlgeburten? ☐ ja ☐ nein Wenn ja, in welchem Jahr? ..... Wenn ja, in welcher Schwangerschaftswoche? .....
Hatten Sie Eileiter-Schwangerschaften? ☐ ja ☐ nein ☐ weiß nicht Wenn ja, in welchem Jahr? .....
Hatten Sie Schwangerschaftsabbrüche? ☐ ja ☐ nein Wenn ja, in welchem Jahr? .....

Vielen Dank für das Ausfüllen des Fragebogens!

Für Rückfragen stehen wir Ihnen gern zur Verfügung:

Prof. Dr. med. Jan Rupp
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Email: Michael.bohlmann@uk-sh.de
Tel: 0451 500- 2134

6.6 Publications

- Fuchs B, Wang W, Graspeuntner S, Li Y, Insua S, Herbst EM, Dirksen P, Böhm AM, Hemmrich G, Sommer F, Domazet-Lošo T, Klostermeier UC, Anton-Erxleben F, Rosenstiel P, Bosch TCG, Khalturin K (2014) Regulation of Polyp-to-Jellyfish Transition in *Aurelia aurita. Current Biology* 24(3):263-273.

- Shima K, Coopmeiners J, Graspeuntner S, Dahlhoff K, Rupp J (2016) Impact of micro-environmental changes on respiratory tract infections with intracellular bacteria. *FEBS Lett* doi:10.1002/1873-3468.12353*

  Giles DA, Moreno-Fernandez ME, Stankiewicz TE, Graspeuntner S, Cappelletti M, Wu D, Mukherjee R, Chan CC, Klarquist J, Softic S, Stemmer K, Karns R, Iwakura Y, Kahn RC, Steinbrecher K, Karp CL, Reynaud D, Shanmukhappa SK, Haslam D, Sina S, Rupp R, Hogan SP, Divanovic S (2016) Thermoneutral housing leads to informative humanization of non-alcoholic fatty liver disease in mice. *In revision*.*

- Graspeuntner S, Bohlmann MK, Gillmann K, Speer R, Künzel S, Mark H, Hoellen F, Lettau R, Griesinger G, König IR, Baines JF, Rupp J (2016) A microbiome-based strategy for the diagnosis of infectious infertility. *Submitted*.**

- *: Publication derived from work during my PhD phase
- **: Publication directly related to this PhD thesis

6.7 Conference contributions

Poster: Graspeuntner S, Gillmann K, Speer R, Bohlmann MK, König IR, Baines JF, Rupp J (2013) The microbial diversity of the female urogenital tract in the context of STIs and infertility. (2013) *65. Jahreskongress der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Rostock.***

Talk: Graspeuntner S, Gillmann K, Speer R, Bohlmann MK, König IR, Baines JF, Rupp J (2014) The microbial diversity of the female urogenital tract in the context of STIs and female infertility. *12. Chlamydienworkshop, Berlin.***

Talk: Graspeuntner S, Gillmann K, Speer R, Bohlmann MK, König IR, Baines JF, Rupp J (2014) The microbial diversity of the female urogenital tract in the context of STIs and female infertility. *66. Jahreskongress der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Dresden.***

Poster: Graspeuntner S, Gillmann K, Speer R, Bohlmann MK, König IR, Baines JF, Rupp J (2015) The microbial diversity of the female urogenital tract in the context of STIs and female infertility. *International Cluster Symposium, Cluster of Excellence "Inflammation at Interfaces", Kiel.***

Talk: Graspeuntner S, Ledig S, Kaufhold I, Pfarr K, Hörauf A, Rupp J (2015) High efficacy of a novel bacterial DNA dependent RNA polymerase inhibitor against various chlamydial species. *13. Chlamydienworkshop, Vienna.**

Talk: Graspeuntner S, Bohlmann MK, Gillmann K, Speer R, Künzel S, Mark H, Hoellen F, Lettau R, Griesinger G, König IR, Baines JF, Rupp J (2016) A microbiome-based strategy for the diagnosis of infectious infertility*. 68. Jahreskongress der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Ulm.***

- *: Conference contributions derived from work during my PhD phase
- **: Conference contributions directly related to this PhD thesis

7 Curriculum vitae

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