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Progesterone-induced changes to the murine vaginal microbiota and host immunity enhance susceptibility to chlamydial infection

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Abbreviations

°C	degree Celsius
µg	microgram
µL	microliter
ATCC	American Type Cell Culture Collection
bp	base pair
BSA	bovine serum albumin
BV	bacterial vaginosis
CD	cluster of differentiation
CST	community state type
cm^2	square centimeter
CO_2	carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EB	elementary body
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FSC	Forward Scatter
FSC-A	Forward Scatter area
FSC-H	Forward Scatter height
g	gram
g	standard gravity
h	hour(s)
HCl	hydrogen chloride
HEp-2 cells	human epithelial type 2 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α	hypoxia-inducible factor-1 alpha
HIV	human immune-deficiency virus
HSV-2	herpes simplex virus-2
IFT	immunofluorescence test
IFU	inclusion forming unit

IL	interleukin
L	liter
LCA	leucocyte common antigen
LPS	lipopolysaccharide
M	mol/liter
min	minute(s)
Mio	million
mL	milliliter
mm	millimeter
mM	millimol/liter
MMP	matrix metalloprotease
MyD88	myeloid differentiation primary response 88
NaCl	sodium chloride
NGS	next-generation sequencing
NK	natural killer
ns	not significant
OTU	operational taxonomic unit
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PID	pelvic inflammatory disease
pH	negative decimal logarithm of the H^+ ion concentration
RB	reticulate body
RBC	red blood cell
rcf	relative centrifugal force
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
sec	second(s)
SPG	sucrose phosphate glucose buffer
spp.	*species pluralis*, species (plural)
SSC	Side Scatter
STD	sexually transmitted disease
TAE	Tris-acetate-EDTA
Th	T helper cell
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
UTM	universal transport medium
V	volt

Abstract

Chlamydia trachomatis is the most common sexually transmitted bacterial infection worldwide. Undetected or inadequately treated infections can lead to severe sequels, such as pelvic inflammatory disease or infertility in women. During the menstrual cycle, circulating sex hormones regulate both microbiota and immune response, shaping the vaginal environment. The distinct roles of the microbiota composition, as well as the involved immune cells, in acquisition and outcome of chlamydial infection are not yet completely understood. Therefore, a robust mouse model was used to study the hormone-microbiota-immune cell interaction. Herein, female C57BL/6 mice were vaginally infected with the murine pathogenic strain *C. muridarum*. The aim of the study was to determine the influence of progesterone on genital infection with *C. muridarum* regarding formation of pathology, vaginal microbiota and provoked immune response. Further, β-estradiol and antibiotic pretreatment were tested as potential modulators of chlamydial infection.

Progesterone-treated mice had a higher and more prolonged shedding, as well as constant development of pathology, compared to naturally cycling mice. The microbiota of untreated mice consisted mainly of Firmicutes, mostly enterococci and only a few lactobacilli. However, progesterone treatment decreased the abundance of Firmicutes/ enterococci, thereby enhancing alpha diversity of vaginal microbiota. The amount of leucocytes was significantly reduced in progesterone-treated mice prior to infection and macrophages were consistently lower compared to untreated mice. Progesterone treatment further enhanced infiltration of leucocytes to the uterine tissue seven days post infection and increased abundance of T cells 14 days post infection. Neither administration of β-estradiol nor pretreatment with antibiotics had an impact on bacterial shedding and pathology formation. However, similar to progesterone treatment, β-estradiol significantly reduced uterine leucocytes before infection, and ampicillin decreased the abundance of Firmicutes/ enterococci.

Progesterone is a potent modulator of chlamydial infections by alterations of vaginal microbiota and immune response. However, exclusive reduction in leucocytes or decrease in Firmicutes/ enterococci prior to infection did not enhance shedding or induce pathology formation. Changes in both immune response and microbiota are needed to modulate chlamydial infection. The role of progesterone should to be focused on in future studies to elucidate underlying mechanisms in the interplay of sex hormones, epithelial cells, vaginal microbiota, and involved immune cells.

Zusammenfassung

Infektionen mit *Chlamydia trachomatis* sind die häufigste sexuell übertragbare bakterielle Krankheit weltweit. Unentdeckte oder unzureichend behandelte Infektionen können bei Frauen zu schwerwiegenden Folgen wie Unterleibsentzündung oder Unfruchtbarkeit führen. Während des weiblichen Menstruationszyklus werden urogenitale Mikrobiota und Immunzellen von zirkulierenden Sexualhormonen reguliert, wodurch das vaginale Milieu beeinflusst wird. Die distinkten Rollen der mikrobiellen Zusammensetzung sowie der beteiligten Immunzellen während einer Chlamydien-Infektion sind bisher nicht vollständig verstanden. Daher wird in dieser Arbeit zur Untersuchung der Hormon-Mikrobiota-Immunzell-Interaktion ein Maus-Modell verwendet, wobei weibliche C57BL/6 Mäuse vaginal mit dem mauspathogenen Stamm *C. muridarum* infiziert wurden. Ziel dieser Studie war es den Einfluss von Progesteron auf genitale Infektionen mit *C. muridarum* hinsichtlich Pathologie-Entstehung, vaginalen Mikrobiota und der ausgelösten Immunantwort zu untersuchen. Weiterhin wurden β-Estradiol und antibiotische Vorbehandlung als Modulatoren einer Chlamydien-Infektion getestet.

Progesteron-behandelte Mäuse hatten im Vergleich zu natürlich zirkulierenden Mäusen eine höhere und länger anhaltende Bakterienlast sowie regelmäßige Entstehung von Pathologien. Die Mikrobiota von unbehandelten Mäusen bestanden vorwiegend aus Firmicutes, wobei überwiegend Enterokokken und nur wenige Laktobazillen entdeckt wurden. Behandlung der Mäuse mit Progesteron führte zu einer Verminderung an Firmicutes/ Enterokokken, wodurch die Alpha Diversität der vaginalen Mikrobiota erhöht wurde. In Progesteron-behandelten Mäusen war die Menge an Leukozyten vor der Infektion signifikant vermindert und Makrophagen waren durchgängig reduziert im Vergleich zu unbehandelten Mäusen. Weiterhin führte Progesteron zu einer erhöhten Infiltration von Leukozyten in das Uterusgewebe 7 Tage nach Infektion und zu vermehrtem Vorkommen von T Zellen 14 Tage nach Infektion. Weder die Verabreichung von β-Estradiol, noch eine antibiotische Vorbehandlung der Mäuse hatte einen Einfluss auf die Bakterienlast und Entstehung von Pathologien. Ähnlich jedoch zur Progesteron-Behandlung reduzierte β-Estradiol signifikant Leukozyten im Uterus vor der Infektion und Ampicillin verminderte die Abundanz von Firmicutes/ Enterokokken.

Progesteron ist ein potenter Modulator von Chlamydien-Infektionen durch Veränderungen der vaginalen Mikrobiota und der Immunantwort. Allerdings führte die ausschließliche Reduktion von Leukozyten oder die Verminderung von Firmicutes/ Enterokokken vor einer Infektion zu keiner Erhöhung der Bakterienlast oder Änderung der Entstehung von

Pathologien. Offensichtlich sind die durch Progesteron induzierten Veränderungen sowohl in der Immunzellantwort als auch der vaginalen Mikrobiota notwendig, um eine Chlamydien-Infektion zu modulieren. Zukünftigen Studien werden sich auf die Rolle von Progesteron konzentrieren um die zugrundeliegenden Mechanismen des Wechselspiels zwischen Sexualhormonen, Epithelzellen, vaginalen Mikrobiota und beteiligten Immunzellen aufzuklären.

1. Introduction

1.1 Sexually transmitted infections with *Chlamydia trachomatis*

Chlamydia trachomatis causes genital infections in men and women and is the most common bacterial sexually transmitted disease (STD) worldwide. In Europe, roughly 400,000 cases of infections with *C. trachomatis* were reported in 2016 [ECDC, 2018]. Of those, more cases were reported for women than for men, while a large proportion of cases was among 15-24 year-olds. However, rates differ drastically between countries. This difference can be attributed to some countries having active screening or opportunistic testing, whereas others do not report to the European Centre for Disease Prevention and Control [ECDC, 2018]. The number of actual cases is therefore unknown, but estimated to be high.

1.1.1 The pathogen *Chlamydia trachomatis*

C. trachomatis is a Gram-negative obligate intracellular bacterium that has a unique biphasic lifecycle. *Chlamydiae* exist in two distinct forms: the elementary bodies (EBs) and reticulate bodies (RBs). Infectious EBs adhere to the surface of mostly epithelial cells. After internalization into the host cell, chlamydial EBs differentiate into metabolically highly active, but non-infectious, RBs within a special intracellular membrane-limited compartment called inclusion. RBs replicate within the inclusion by binary fission [Moulder, 1991] and redifferentiate back to EBs. The infectious cycle ends with cell lysis, releasing EBs, and begins again 24-48 h post infection with EB attachment to adjacent epithelial cells [Wyrick, 2000]. *In vitro* and *in vivo* models of chlamydial persistence describe aberrant bodies, which can be induced by β-lactam antibiotics, iron chelators, and interferon-γ (IFN-γ) [Mpiga *et al.*, 2006]. Aberrant bodies differ in their morphology and are in a viable but non-cultivable growth stage [Hogan *et al.*, 2004]. The chlamydial genome comprises roughly 1.1 Mio base pairs (bp) [Stephens *et al.*, 1998], leading to a truncation or, in some cases, an absence of many pathways, causing dependence on host cell metabolism and energy supply [Omsland *et al.*, 2014]. *Chlamydiae* possess specialized transport systems to import metabolites from the host, such as amino acids, vitamins, and cofactors, which they are auxotrophic for [Stephens *et al.*, 1998]. Of clinical relevance is the amino acid tryptophan, which *Chlamydiae* cannot synthesize themselves. If host IFN-γ concentration is high in response to infection, host indoleamine 2,3-dioxygenase (IDO) is induced and leads to a degradation of intracellular tryptophan, directly affecting chlamydial growth [Aiyar *et al.*, 2014]. Instead, some chlamydial strains are able to metabolize indole, another amino acid, to tryptophan via tryptophan synthase [Byrne *et al.*, 2012].

C. trachomatis belongs to the *Chlamydiaceae* and can be subdivided into different serovar, all causing disease. Trachoma, a common chronic recurrent disease of the conjunctiva and cornea, is caused by *C. trachomatis* serovar A-C. Sexually transmitted *C. trachomatis* serovar D-K and L1-L3 cause either genital infection or lymphogranuloma venereum, respectively.

1.1.2 Clinical aspects: treatment and sequels

Most infections with *C. trachomatis* proceed asymptomatic in men as well as in women. If infections remain untreated, the risk of transmission increases, which can be a reason for recurrent *C. trachomatis* infections [Batteiger *et al.*, 2010]. Uncomplicated infections have a natural resolution and last an average of 1.36 years if untreated [Crofts *et al.*, 2015]. Generally, the rate of spontaneous resolution increases with elapsed time from initial testing. Approximately 50% of infections are resolved within one year [Geisler, 2010]. Data on the persistence of months to years is an indication for the development of partial protective immunity to *C. trachomatis* [Molano *et al.*, 2005, Geisler, 2010, O'Connell *et al.*, 2016]. Clearance is attributed to host immune responses, yet the mechanisms and involved immune cells remain poorly understood.

If undetected or inadequately treated, the pathogen is able to ascend the urogenital tract and cause chronic inflammation, leading to pelvic inflammatory disease (PID) in roughly 15% of untreated chlamydial infections (reviewed in [Brunham *et al.*, 2015]). PID is an infection-induced inflammation of the upper genital tract of women, affecting the ovaries, fallopian tubes and uterus (reviewed in [Brunham *et al.*, 2015]). Manifestation of PID might lead to tubal scarring and dysfunction, thereby increasing the risk of chronic pelvic pain, ectopic pregnancy, and infertility (reviewed in [Mitchell *et al.*, 2013]). Indications of a symptomatic infection with *C. trachomatis* are dysuria, mucopurulent endocervical discharge, itching and burning, easily induced endocervical bleeding (e.g. between periods), and different loci of pain [Marrazzo *et al.*, 2002]. Suspected infections with *C. trachomatis* are confirmed using highly specific and sensitive nucleic acid amplification tests on vaginal swab material [Papp *et al.*, 2014].

Diagnosed infections are treated with doxycycline (100 mg orally twice a day for seven days) or azithromycin (1 g orally in a single dose) [Workowski *et al.*, 2015], with cure rates of 98% and 97% [Lau *et al.*, 2002], respectively. During pregnancy, doxycycline is contraindicated, and therefore the alternative treatment regimen of 500 mg amoxicillin three times a day for 7 days is recommended alongside regular azithromycin usage [Workowski *et al.*, 2015]. To prevent reinfection, it is important to identify, test and treat the patient’s sexual partners as soon as possible [Batteiger *et al.*, 2010]. However, up to 8% treatment

failures have been reported [Horner, 2006]. Infections are typically resolved by antibiotic treatment; however pre-existing inflammatory-mediated tissue damage cannot be ameliorated [O'Connell *et al.*, 2016].

1.1.3 The impact of vaginal microbiota on female health and disease

The vagina is colonized by bacteria and an intact microbial community is crucial for urogenital health. Commensal vaginal microbiota is mainly composed of lactobacilli and many anaerobic bacteria [Ravel *et al.*, 2011], as the vaginal environment is hypoxic compared to normal air [Juul *et al.*, 2007]. Presently, bacteria are assessed by next-generation sequencing (NGS), which is an amplification of the partial 16S rRNA gene and, more specifically, its hypervariable regions [Robinson *et al.*, 2016]. The obtained data on the composition of urogenital microbiota allows insights into the interplay of commensal microbiota and sexually transmitted pathogens.

A first characterization of the composition of urogenital microbiota of reproductive-age women was published by Ravel *et al.* in 2011 [Ravel *et al.*, 2011]. Using 454-pyrosequencing, they showed variations in colonization within participants. Clustering in so-called community groups is based on the dominance of a single species, which are mainly *Lactobacillus* spp. Not being dominated by a single species, a diverse type is described. Using a different set of primers, Nunn *et al.* detected another community with dominance in *Gardnerella vaginalis* [Nunn *et al.*, 2015]. There is a debate concerning ethnicity as females with Hispanic or black ancestry often have a diverse community compared to Asian and white women [Ravel *et al.*, 2011, Gajer *et al.*, 2012, Borgdorff *et al.*, 2017]; nonetheless, behavioral confounders cannot be excluded. However, a healthy vaginal microbiota is mainly associated with a high abundance of lactobacilli. *Lactobacillus* spp. are producers of lactic acid, maintaining the vaginal pH at roughly 4, and are the first line of defense against invading pathogens and pivotal for female health [Redondo-Lopez *et al.*, 1990]. Commensal colonization is varying in dependence of the dominating *Lactobacillus* spp. [Boris *et al.*, 2000, Tachedjian *et al.*, 2017], as they have been shown to differentially influence the pH [Ravel *et al.*, 2011]. However, the role of lactobacilli products, such as hydrogen peroxide and bacteriocin-like substances, on vaginal microbiota is controversially discussed [Boris *et al.*, 2000].

The urogenital microbiota has been shown to be dynamic and undergoes rapid changes from one community state type (CST) to another. Instability more frequently occurs in the diverse CST; however, as of yet there is no predictive tool for upcoming fluctuations [Gajer *et al.*, 2012]. The menstrual cycle also appears to play a crucial role as CST transitions occur repeatedly in unstable microbiota [Chaban *et al.*, 2014]. The impact of contraceptives

on the vaginal microbiota is discussed and results remain inconsistent. Achilles *et al.* showed that copper intrauterine devices could cause an increase in bacterial vaginosis (BV) associated bacteria, whereas hormonal contraception had no influence on vaginal microbiota [Achilles *et al.*, 2018]. Further factors influencing urogenital microbiota stability are socioeconomic behavior [Brotman *et al.*, 2014], sexual behavior [Schwebke *et al.*, 1999, Stock *et al.*, 2001, O'Connell *et al.*, 2009, Brotman *et al.*, 2010, Cools *et al.*, 2016, Norris Turner *et al.*, 2016], and hygiene [Fashemi *et al.*, 2013]. Interestingly, sexual partners remain a key factor to consider, as the penile microbiome might serve as a reservoir for bacteria associated with BV [Liu *et al.*, 2015]. BV is a common vaginal syndrome caused by an imbalance in urogenital microbiota, leading to discharge, itching, burning and inflammation. It is clinically well-characterized and regularly diagnosed using the Nugent Score [Nugent *et al.*, 1991]. There is a high variability in the composition of urogenital microbiota among BV patients [Srinivasan *et al.*, 2010, Ravel *et al.*, 2013], implying the lack of a clear definition of a microbial composition being indicative for BV based on NGS-analysis. Thereby, phenotypes among BV patients are described rather than one clear composition. It was shown that in reproductive-age women with dysbiosis, characterized by increased abundance of *G. vaginalis* and *Bifidobacterium breve*, a pro-inflammatory process is triggered [Campisciano *et al.*, 2018]. Further, the stable state level of T helper cell (Th)-2 present might influence clinical manifestations in the patients, leading to the question of the utility of vaginal immunological markers as tools for the detection of alterations within the microbiota [Campisciano *et al.*, 2018].

The reasons for acquisition of STDs are still not properly characterized. Therefore, vaginal microbiota is extensively studied to find indicators for interplay with pathogens. For viral infections with herpes simplex virus-2 (HSV-2) and human immuno-deficiency virus (HIV), it has already been shown that a BV-like microenvironment is favorable [Martin *et al.*, 1999, Cherpes *et al.*, 2003]. Lactobacilli, mainly *L. crispatus*, decrease the viability of bacterial infection with *Neisseria gonorrhoeae* [Breshears *et al.*, 2015, Foschi *et al.*, 2017], and have high inhibitory capacity against *Escherichia coli* [Ghartey *et al.*, 2014]. BV is additionally predictive for infections with *N. gonorrhoeae* and *C. trachomatis* upon intercourse with infected partners [Wiesenfeld *et al.*, 2003]. Further inspection of NGS-based correlations of the composition of microbiota and *C. trachomatis* infections is required for better prediction and understanding of the acquisition of STDs. Filardo *et al.* showed in a pilot study that asymptomatic infection with *C. trachomatis* is related to decreased lactobacilli and an enhancement of anaerobic bacteria as *G. vaginalis*, *Prevotella amnii*, *P. timonensis* and *Leptotrichia amnionii* [Filardo *et al.*, 2017]. Another study showed a significant association between the risk of acquiring an infection and a *L. iners* dominated CST [van Houdt *et al.*, 2018], indicating specific signatures for prediction of host predisposition to STDs. Females

highly likely of a recent exposition to *C. trachomatis*-infected sexual partners have cervicovaginal microbiota dominated by *L. iners* or diverse anaerobic bacteria rather than *L. crispatus* [van der Veer *et al.*, 2017, Tamarelle *et al.*, 2018]. Accordingly, a *L. iners*-dominated CST is found to be associated with an increased risk for *C. trachomatis* infection [van Houdt *et al.*, 2018]. Women with an asymptomatic co-infection of HPV and *C. trachomatis* have a significantly higher diversity of their cervical microbiota compared to controls. In this study, *C. trachomatis* occurrence is associated with abundance of *Aerococcus christensenii* [Di Pietro *et al.*, 2018].

The composition of the vaginal microbiota is crucial if considering the abundance of anaerobic bacteria, for example *Prevotella* spp., *Peptostreptococcus* spp., *Peptoniphilus* spp., and *Porphyromonas* spp., which are indole producers [Ziklo *et al.*, 2016a, Ziklo *et al.*, 2016b]. *C. trachomatis* is dependent on tryptophan from the host; however during host defense against invading pathogens, IFN-γ levels are increased, leading to IDO1 induction and thereby depleting host cell tryptophan pools (Figure 1A). Persistent infections might occur as a consequence of permanently depleted tryptophan, which is intracellularly converted to kynurenine (Figure 1B). However, if urogenital microbiota is in a state of dysbiosis, the abundance of anaerobic, indole producing bacteria is enhanced, leading to elevated indole concentration in the vagina. Some *C. trachomatis* spp. are able to metabolize indole to tryptophan, which might in turn lead to chlamydial recovery (Figure 1 C).

The composition of urogenital microbiota plays an important role in the acquisition of STDs. Abundance of *L. iners* and a diverse CST are associated with the occurrence of *C. trachomatis* infection [van der Veer *et al.*, 2017, van Houdt *et al.*, 2018]. However, ethically, it is not reasonable to infect patients with *C. trachomatis*; which presents a challenge for studies, as the microbial composition at infection cannot be characterized. Due to the dynamics and natural fluctuations, yet only estimates and associations were concluded from published studies (e.g. [Ravel *et al.*, 2013]). Therefore, an animal model of chlamydial infection is advantageous to elucidate the underlying mechanisms to characterize host factors with an influence on acquisition of *C. trachomatis* infection.

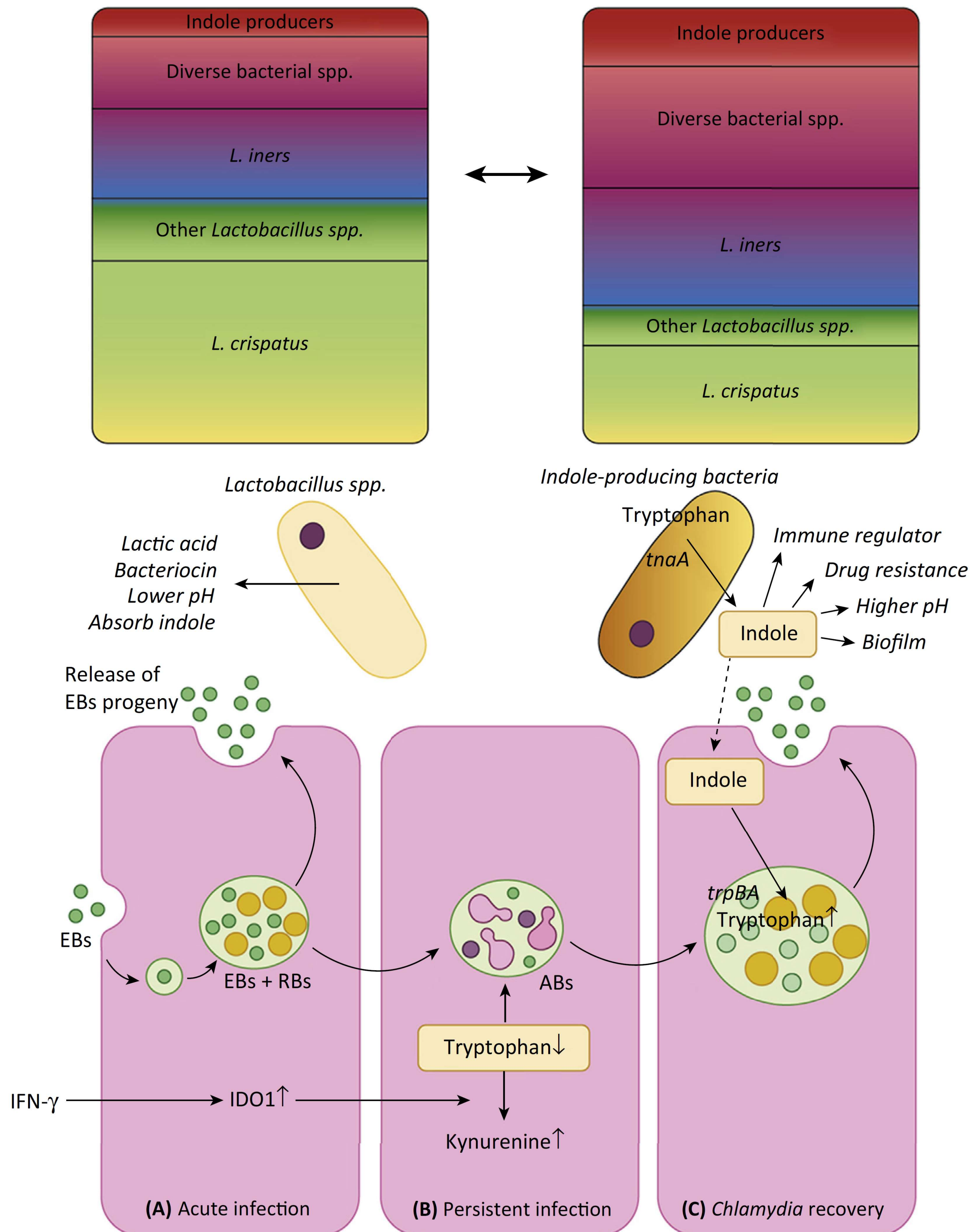

Figure 1: *C. trachomatis*-IFNγ-microbiota axis. Healthy microbiota, dominated by *Lactobacillus* spp., mainly *L. crispatus*, secretes lactic acid, leading to a low pH. Simultaneously, bacteriocins are released and indole is absorbed. In a dysbiosed state, the ratio of *L. crispatus* to anaerobic and indole producing bacteria shifts. **(A)** Acute infection with *C. trachomatis* leads to IFN-γ secretion from the host. Intracellular IDO1 is enhanced and thereby tryptophan is depleted. **(B)** *Chlamydiae* switch to a persistent state of infection if tryptophan levels are low and kynurenine levels are high. **(C)** In the case of microbial dysbiosis, there is a shift towards a diverse CST, which is associated with an increase of anaerobic bacteria and enhanced indole production. *Chlamydiae* are able to metabolize indole to tryptophan, thereby recovering from persistence to acute infection. (from [Ziklo *et al.*, 2016a])

1.2 Experimental design of chlamydial infections

For studies of chlamydial infection, different animal models have been established in the past 50 years, including monkeys, guinea pigs, rats and mice [De Clercq *et al.*, 2013]. The experimental setup for genital chlamydial infections differs among the animal species, which is an essential consideration during the planning process. Mice are comparably inexpensive, convenient to house, reproduce relatively quickly, and show many anatomic, physiologic and genomic similarities to humans. These qualities make mice a suitable model organism for translational studies. Furthermore, it is straightforward to genetically modify mice for various purposes, including complex biological phenomena or diseases. Since the 1980s, mice have been utilized for genital chlamydial infections with either human strains *C. trachomatis* serovar D-L (e.g. [Tuffrey *et al.*, 1986b]), or the murine pathogen *C. muridarum* [Barron *et al.*, 1981].

1.2.1 Development and characterization of a mouse infection model

The probability of appropriate assumptions of the revealed link and immune responses caused by *C. muridarum* in the mouse are likely to be predictive of the interaction of *C. trachomatis* and humans. The genomes of *C. trachomatis* and *C. muridarum* are of comparable size, gene content and order, but differ in the plasticity zone [Stephens *et al.*, 1998, Read *et al.*, 2000]. Therefore, both strains presumably share virulence and pathogenic mechanisms, as well as the elicited immune response [Morrison *et al.*, 2002]. *C. muridarum* originates from asymptomatic albino Swiss mice's throat washings, and was therefore formerly named *C. trachomatis* mouse pneumonitis (MoPn) [Nigg *et al.*, 1944].

In 1981, Barron *et al.* were the first to show that *C. trachomatis* MoPn is able to infect the genital tract of Swiss-Webster mice. Intravaginally, the infection rate is approximately 20% of all infected mice. This low rate is ascribed to the menstrual cycle, as the authors did not stage the mice [Barron *et al.*, 1981]. Following this model, *C. trachomatis* MoPn was injected into the ovarian bursa to induce salpingitis. Swiss white mice develop gross pathology, the so-called hydrosalpinx, which is a distal occlusion of the fallopian tube, filled with clear liquid [Swenson *et al.*, 1983]. Furthermore, *Chlamydiae* are recovered from tissue up to 21 days post infection, and there is evidence suggesting a spread "backwards", as Swenson *et al.* also recover *Chlamydiae* from uterus and vagina [Swenson *et al.*, 1983]. Intravaginal infection of mice with *C. trachomatis* MoPn leads to a reduction in the quantity of pregnant mice, as well as a reduction of number of embryos [de la Maza *et al.*, 1994]. The same results have been shown to be dose dependent and determined to the phase of estrous cycle, as infection during the luteal phase promotes infertility [Pal *et al.*, 1998].

Generally, different factors need to be considered in planning mouse infection experiments. The choice of mouse strain and chlamydial serovar is of great importance for the success and outcome of the genital infection. In Th2 prone BALB/c mice, the shedding of *C. trachomatis* serovar D is significantly higher compared to Th1-inherited C57BL/6 mice, but has a shorter median duration of 12 days, in comparison to 42 days in Th1 prone mice [Lyons *et al.*, 2005]. *C. trachomatis* serovar E is capable of infertility induction in C3H mice, whereas TO mice appear resistant [Tuffrey *et al.*, 1986a]. Further, the age of the mice is an important factor for the success of chlamydial infection. Younger animals had a higher percentage of positive *C. trachomatis* MoPn culture from vaginal swabs, increased numbers of inclusion forming units (IFUs) recovered per mouse, as well as prolonged shedding as old mice [Pal *et al.*, 2001]. Chlamydial strains share different properties concerning infectious success and outcome. The two most commonly used strains are MoPn NiggII and MoPn Weiss, which show different characteristics in regard of growth properties *in vitro* and virulence *in vivo*; MoPn NiggII is less virulent compared to MoPn Weiss [Ramsey *et al.*, 2009]. Virulence is furthermore dependent on the presence of the bacterial plasmid, which most strains of *C. trachomatis* possess. For *C. trachomatis* serovar E and F, it has been shown that the plasmid-free strains display significantly decreased infectivity and virulence compared to plasmid-containing strains [Sigar *et al.*, 2014]. Similar results were obtained with a plasmid-deficient *C. muridarum* strain, which is able to infect the murine genital tract but fails to develop pathology at the ovaries [O'Connell *et al.*, 2007]. Commonly, the induction of pathologies is difficult using *C. trachomatis* serovar D for vaginal infection in mice. Tuffrey *et al.* could induce salpingitis with human strains of *C. trachomatis* in progesterone-treated mice if inoculated intrabursally or intrauterine; however, results are different in dependence on the used mouse strains and bacterial strains [Tuffrey *et al.*, 1986b]. Still, it is difficult to establish an infection with *C. trachomatis* serovar D via the vaginal route, and it is not yet possible without prior progesterone treatment in CBA mice [Tuffrey *et al.*, 1981]. A supposed reason is that low levels of progesterone lead to inadequate contact between organisms and target cells as progesterone likely prevents the loss of target epithelial cells during the 4 to 5 days of the menstrual cycle [Tuffrey *et al.*, 1981]. The nonsurgical embryo transfer device (NSET) was originally developed to facilitate the transfer of embryos to the uterus horn and to omit surgical procedures [Green *et al.*, 2009]. However, it was used by Gondek *et al.* to directly locate *C. trachomatis* serovar L2 in the uterus horns, called the intrauterine or transcervical inoculation method [Gondek *et al.*, 2012]. As a result, they could show that intrauterine infection with *C. trachomatis* serovar L2 leads to comparable numbers in bacterial shedding as *C. muridarum*, development of major nodes of inflammation, which ascended the length of the uterus horns, and histologically detectable severe oophoritis [Gondek *et al.*, 2012].

A robust model for genital chlamydial infection has been developed in the past years by several research groups. An important aspect is the administration of progesterone, as without estrous cycle normalization by high long-lasting progesterone levels, an infection will be unsuccessful [Tuffrey *et al.*, 1981]. As not only hormones have an impact on the success of infection, the immune response towards infection is studied to elucidate underlying mechanisms in immunity between mouse strains towards different chlamydial strains, which are frequently studied using various approaches.

1.2.2 Host immune response towards chlamydial infections

The outcome of chlamydial infection is not only dependent on the chlamydial species, but also on the choice of the mouse strain. Outbred mice’s reproduction is strictly controlled, for example by matching breeding schemata in order to retain a constant heterozygosity. In contrast, the broad homozygosity after 20 generations of inbreeding is characteristic for inbred mice. In many approaches, inbred mice are used to exclude variations caused by the genetic background [Festing, 2014]. The resolution of the infection with little to no pathology formation is therefore dependent on the kind and quantity of the immune response, as it is the evoked immune response, and not the pathogen that leads to pathology development.

C57BL/6 mice are prone towards a Th1 immune response. If infected with *C. trachomatis* serovar E or *C. muridarum*, there is a mouse strain independent (C57BL/6 and C3H) immune response predominated by Th1 [Darville *et al.*, 1997]. Human isolates of *C. trachomatis* elicit Th1 and Th17 prone immune responses after vaginal challenge [Vicetti Miguel *et al.*, 2016]. However, shortly after the resolution of infection with *C. trachomatis* serovar D, different mouse strains express diverse immunoglobulin isotype profiles. While the isotype profile of C57BL/6 mice consists mostly of IgG2b and minor IgM, BALB/c mice show no IgM but equal proportions of IgG2a and IgG3. BALB/c mice display an IgG2a to IgG1ratio of 2.5:1 and more often expressed IgA [Lyons *et al.*, 2005]. Occurrence of *Chlamydia*-specific IgG and IgA does not reduce the susceptibility of CBA mice to infection, but rather accelerates clearance [Tuffrey *et al.*, 1984]. There is no yet a solution for a vaccine-based strategy for the prevention of chlamydial infection. Presently, the protection against *Chlamydia* spp. is not termed Th1 driven, but rather “multifunctional Th1”, as Th2 and Th17 driven responses are detected as well [Johnson *et al.*, 2016]. Belonging to this generalized Th1 response are different receptors, cytokines, chemokines and immune cells, whose distinct roles will be further explained in the next paragraphs.

Invading pathogens are recognized by toll-like receptors (TLRs), which are differentially expressed on innate immune cells and activate the immune response. TLRs are pattern recognition receptors (PRRs), and TLR1-13 are expressed on various cells; if activated, innate immunity pathways are induced. They play different roles in recognition of viruses, fungi, parasites, and bacteria. Bacterial pathogen-associated molecular patterns (PAMPs) are either lipoproteins, DNA, or RNA, which are recognized by different TLRs [Kawai *et al.*, 2011]. In infected mice, *C. trachomatis* extracellular EBs are recognized by TLR2 on phagocytic, as well as epithelial, cells [Witkin *et al.*, 2017]. TLR2 is crucial for immune response during *C. muridarum* infection since it promotes the production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), as well as formation of characteristic pathologies, namely hydrosalpinx [O'Connell *et al.*, 2007]. The course of infection in mice deficient in TLR2 was similar to wild type controls; however, pathology was significantly reduced. TLR4, in contrast, had no further influence on the course and outcome of chlamydial infection *in vivo* [Darville *et al.*, 2003]. As a downstream target of TLR2, there is the adapter molecule myeloid differentiation primary response 88 (MyD88), which is a critical signaling adaptor molecule for many PRRs. The role of MyD88 is highly dependent on infection type and condition. The absence of MyD88 leads to a prolonged infection and enhanced pathology formation [Chen *et al.*, 2010]. Downstream of the activation of signaling molecules, cytokines and chemokines are produced and secreted to further initiate the immune response towards infection.

Intracellular signaling cascades lead to the production of cytokines and chemokines. O'Connell *et al*. proved that the viability of *Chlamydia* is crucial for a robust immune response since UV-inactivated pathogens are not able to induce interleukin (IL)-8 secretion [O'Connell *et al.*, 2006]. Therefore, the production of cytokines is dependent on viable pathogens and their ability of replication [O'Connell *et al.*, 2006]. IL-1β is important for chlamydial clearance, as IL-1β knock-out mice display a decreased rate of hydrosalpinx and delayed clearance of the pathogen [Prantner *et al.*, 2009]. Further, caspase 1 knock-out mice show a regular course of infection but reduced oviduct pathology due to impaired IL-1β secretion [Cheng *et al.*, 2008]. Also important in the acute phase reaction of inflammation is TNF-α, which is a pro-inflammatory cytokine that is produced by monocytes, macrophages, and $CD8^+$ T cells. TNF-α is significantly increased in secretions from genital tracts of C57BL/6 mice compared to C3H/Hen mice after chlamydial infection. Authors concluded early eradication of the pathogen in C57BL/6J mice by increased TNF-α levels may be beneficial to the host by faster eradication of the pathogen; thereby preventing infection of the oviduct, and thus pathology formation [Darville *et al.*, 1997]. In contrast, another study showed reduced pathology formation if either perforin or TNF-α are knocked out. Therefore, it is suggest that $CD8^+$ T cell secreted TNF-α plays a crucial role in

Chlamydia induced pathology [Murthy *et al.*, 2011]. The role of TNF-α in mice appears two sided; it promotes fast eradication as well as pathology formation concurrently. In contrast, IFN-γ has a clear function in immunity against chlamydial infection. The IFN-γ response during chlamydial infection differs between mice and humans. *C. trachomatis* is susceptible to IFN-γ, whereas *C. muridarum* has developed an evasion strategy to this immune response [Coers *et al.*, 2008]. IFN-γ induces IDO in human epithelial cells and p47 GTPases in murine epithelial cells. Due to co-evolution, both strains are able to escape the host IFN-γ response. However, human *C. trachomatis* isolates are susceptible to mouse immune response [Nelson *et al.*, 2005]. *C. trachomatis* is able to propagate, if transcervically infected to IFN-γ knock-out mice, showing the huge impact of IFN-γ on the human serovar [Gondek *et al.*, 2012]. However, the growth restriction of *C. trachomatis* in wild type mice start prior to T cell peak infiltration, and therefore leads to suggestion of another source of IFN-γ, such as natural killer (NK) cells [Gondek *et al.*, 2012]. During infection with *Chlamydia*, IFN-γ producing $CD4^+$ Th1 cells are migrating to the site of infection and are the dominant cell population in the genital tract. NK cells are part of the early response (within 12 h) to chlamydial infection. They have been shown to play a significant role in the development of $CD4^+$ Th1 response and control of chlamydial infection, as depletion of NK cells leads to exacerbated course of infection and a Th2 prone response [Tseng *et al.*, 1998]. Chlamydial infection is not restricted to urogenital tissue. *C. muridarum* is able to disseminate to other tissues, and in IFN-γ knock-out mice this reaction is enhanced and prolonged [Cotter *et al.*, 1997]. IFN-γ induces different responses in humans and mice, making it difficult to infect mice with the human serovar, as they are prone to avoid IFN-γ response. Cytokines display a wide range of effects upon chlamydial infection in mice, some being controversially discussed. Besides cytokines, chemokines are secreted to attract further effector immune cells to the genital mucosa. There have been several attempts to elucidate specific chemokine receptors that are necessary for T cells to migrate to the mucosa. For *C. trachomatis* serovar L2, CXCR3 and CCR5 play a pivotal role, as a lack of these receptors leads to an impairment of the protective capacity [Olive *et al.*, 2011]. Cytokines and chemokines are humoral components, and as such are involved in the clearance of the pathogen; however they may also be considered in pathology development. They are secreted by immune cells, which are important mediators of inflammation in response to chlamydial infection.

Neutrophils are part of the innate immune system. Following chlamydial infection, neutrophils migrate to inflamed oviduct, which correlates with the development of characteristic pathologies, after the infection is resolved [Darville *et al.*, 2001, Shah *et al.*, 2005]. Neutrophils release matrix metalloproteases (MMP). MMP9 is involved in tubal scarring and fibrosis after chlamydial infection [Ramsey *et al.*, 2005]. Vaginal tissue of hysterectomized mice contains neutrophil infiltrates after infection with *C. muridarum* but not *C. trachomatis*, which correlates with the induction of pathology formation in healthy mice [Yang *et al.*, 2017]. Due to phagocytic internalization of *C. trachomatis*, discrete antigens remain on the cell surface leading to a humoral response via T cell and B cell activation (reviewed in [Witkin *et al.*, 2017]). Morrison *et al*. compared results on adaptive immunity of mice and humans, showing similar factors affecting histopathology and protective immunity. $CD4^+$ T cells are important in immunity, as well as the induction of $CD8^+$ T cells ensuing infection with a still-doubted role *in vivo*; while the function of B cells and antibody in protective immunity is undefined [Morrison *et al.*, 2002]. As of yet, there have been few extensive studies of the role of B cells in chlamydial infection. Studies suggest that protective immunity differs drastically between primary and secondary infection. B cells appear to have a protective role, as mice deficient in B cells are more susceptible to secondary infection (reviewed in [Li *et al.*, 2015]). Thus, clearance of primary infection seems to rely on CD4 T cells; whereas for secondary infection, both T cells and B cells are efficient. However, expansion of CD4 T cells is dependent on B cell abundance during primary infection [Li *et al.*, 2015].

As a new concept of interplay of immunity, Johnson and Rank published a review on protective tissue-resident memory T cells and the formation of *Chlamydia*-memory lymphocyte cluster. These clusters, composed of B cells, $CD4^+$ T cells and a few macrophages, may develop after resolved infection, and persist in the uterine and fallopian tube tissue. Thereby, a protective immunity shall emerge as newly invading *Chlamydia* would not have adequate time for replication [Johnson *et al.*, 2016].

Concluding, the immediate and long-lasting immune responses towards infections with *C. muridarum* and *C. trachomatis* are still not completely understood. Different mouse strains, chlamydial species, and experimental setups lead to diverse results. However, T cells play a pivotal role in the clearance of infections, B cells protect against secondary infection, and innate mechanisms appear to be crucial for pathology formation. Most studies were conducted in progesterone-treated animals; however, the immune priming effect of circulating sex hormones has been neglected in these studies.

1.3 The impact of female sex hormones on infectious diseases

The female genital tract is associated with the immune system, as it is a crucial site regarding pathogen invasion. During the menstrual cycle, the amount and activity of immune cells vary (reviewed in [Beagley *et al.*, 2003]). Therefore, in studies in females and other mammals, it is important to consider the impact of hormonal fluctuations during the menstrual cycle/ estrous cycle, as well as the impact of exogenous hormones on immunity.

1.3.1 The estrous cycle of rodents

The menstrual cycle of reproductive women is regulated on a hormonal and cellular level. It begins at day 1 with the menses and lasts 26-35 days in total (reviewed in [Mihm *et al.*, 2011]). This roughly 4-week cycle can be divided into two phases: the follicular phase, characterized by high estrogen levels, and the luteal phase, characterized by increased progesterone levels. The hormonal constitution has major impact on the acquisition not only of STDs, but also on other diseases (reviewed in [Oertelt-Prigione, 2012]). In contrast, the estrous cycle of mice and other rodents has duration of 4-5 days; however, it is also shaped by variations within hormone levels (Figure 2) and subdivided into proestrous, estrous, metestrous and diestrous. Besides the shorter duration, the estrous cycle in rodents differs from human menstrual cycle due to mice lacking menses. The murine estrous cycle can be easily determined by two methods: vaginal smears and vaginal appearance. To prepare vaginal smears, vaginal washes, swabs or scrapes can be used. Material is applied on a slide and the cell composition is analyzed using a microscope [Caligioni, 2009]. A less stressful method is the determination of the stage of estrous cycle by the appearance of the vagina [Byers *et al.*, 2012]. Therefore, criteria as vaginal swelling, color and moistness of tissues, size of the vaginal opening, and occurrence of cellular debris in the vagina were considered, decreasing the risk of mechanical manipulation [Champlin *et al.*, 1973].

The proestrous phase in mice is comparable to the human follicular phase and is characterized by pre-ovulatory enhancement of 17β-estradiol plasma concentration, a slight increase in prolactin, and occurrence of majorly nucleated epithelial cells (Figure 2 A, B). Estradiol belongs to the family of estrogens and has a high level in non-pregnant as well as pregnant women. The estrogen receptor is expressed on many different cells, such as lymphocytes and macrophages. In low doses, estradiol enhances the secretion of pro-inflammatory cytokines and a Th1 response, while in high concentrations it decreases those cytokines and induces a Th2 directed response and humoral immunity (reviewed in [Robinson *et al.*, 2012]).

Diestrous in murine estrous cycle is defined by a high level of progesterone, great abundance of leucocytes in vaginal smears, and corresponds to the human luteal phase (Figure 2 A, C) [McLean *et al.*, 2012]. In non-pregnant females, progesterone is produced by the corpus lutea in the ovaries, and in pregnant women by the placenta. The progesterone receptor was identified on epithelial cells, eosinophils, macrophages, and lymphocytes. Additionally, progesterone binds to glucocorticoid receptors, which are widespread on immune cells and may be regarded as a substitute mechanism for changes in the immune function caused by progesterone (reviewed in [Robinson *et al.*, 2012]). Further, progesterone is able to modulate immune responses, specifically at mucosal sites (reviewed in [Hall *et al.*, 2017]). Progesterone or progestin, belonging to the gestagens, are used in contraceptives and after administration change the thickness of vaginal epithelium, enhance expression of virus-entry co-receptors, and induce an anti-inflammatory cytokine milieu with reduced levels of the pro-inflammatory cytokines IFN-γ and TNF-α [Hall *et al.*, 2017].

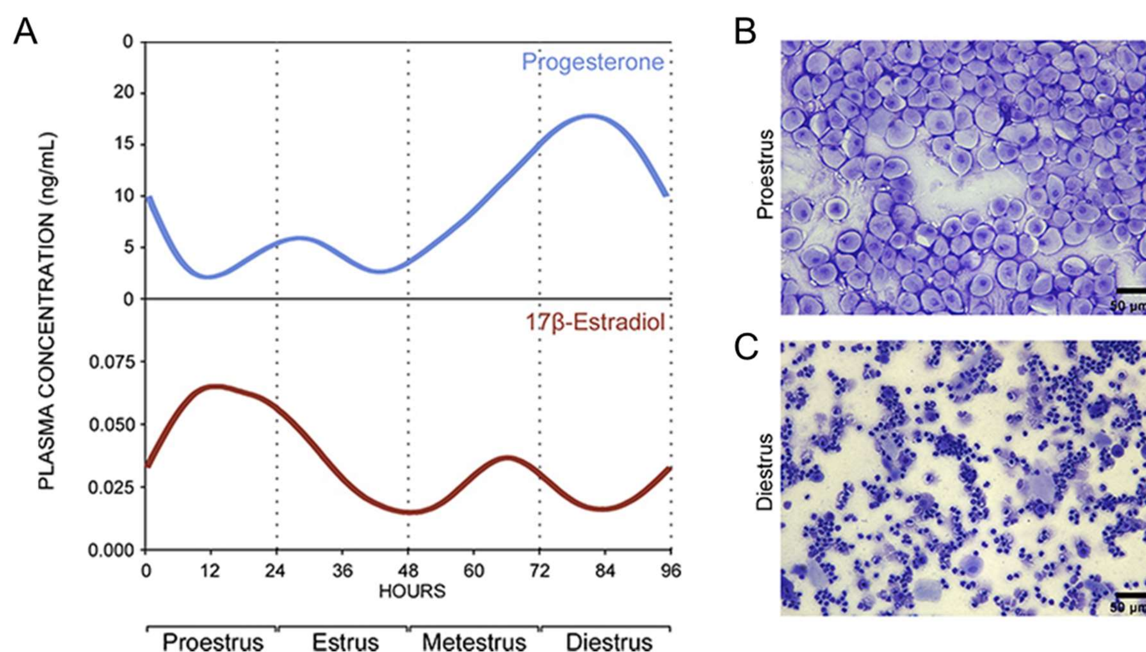

Figure 2: Hormone levels during estrous cycle and microscopy of vaginal smears. (A) Plasma concentration (ng/mL) of progesterone (blue) and 17β-estradiol (red). The progesterone level is highest in diestrous, whereas 17β-estradiol has its peak in proestrous and has a small additional increase in metestrous. **(B)** Crystal violet stain of vaginal smear in proestrous. Characteristic are the oval nucleated epithelial cells. **(C)** During diestrous leucocytes predominate and cornified epithelial cells are reduced. As such, vaginal smear cytology reflects underlying endocrine events. (modified from [McLean *et al.*, 2012])

The natural cycle of mice can be easily modified by administration of exogenous hormones, such as progesterone and estradiol. Furr *et al.* demonstrated that treatment of female mice with progesterone leads to a constant phase of diestrous 5 days post treatment and administration of estradiol leads to estrous stage within 24 h, both detectable for 27 days post treatment. The natural vaginal milieu of mice has a stable temperature of 33-35 °C and a pH of 7.2 to 7.4. Both parameters are not influenced by hormonal alterations [Furr *et al.*, 1991]. However, amounts of vaginal bacteria differ between the stages of menstrual cycle. During estrous, there is a natural increase of bacteria seen in vaginal smears and grown on agar plates, which further increases if estradiol was administered to the mice. In contrast, mice in diestrous have few vaginal bacteria, which is also true in mice treated with progesterone [Furr *et al.*, 1991]. Similar results have been shown that prove that the bacterial count is lowest in diestrous, compared to all other stages of the estrous cycle [Noguchi *et al.*, 2003]. The same is true for the relation of estrous cycle in rats, where the bacterial count is lower in diestrous compared to proestrous and estrous [Larsen *et al.*, 1976].

The estrous cycle, and its regularly fluctuating hormones, has a huge impact on the vaginal milieu as it affects the tissue, immune cells and supposedly composition of vaginal microbiota. Therefore, it is important to determine the influence of progesterone and estradiol in infection models. In particular the obligate usage of hormones to enhance infection should be reconsidered in regard of alterations within immune response and the first line of defense, the vaginal microbiota.

1.3.2 Sex hormones modulate various infections

Different viral and bacterial infections are dependent on the stage of the estrous cycle, during which the female genital tract undergoes structural changes. Therefore, several animal models exist, that make use of exogenous sex hormones to increase the susceptibility to infections, as well as to enhance the sequels. However, protective effects of hormones on the success and outcome of infections on various mucosal sites are also published.

HSV-2 has been shown to be dependent on progesterone levels in C57BL/6 mice if inoculated in the genital tract. Kaushic *et al.* show that the formulation of the progesterone is important, as Depo-Provera (dihydroxyprogesterone acetate) enhanced the HSV-2 infection by 10 times compared to a saline-progesterone compound with a comparable concentration. Further, Depo-Provera prolonged the duration of diestrous up to 4 weeks after administration [Kaushic *et al.*, 2003]. This is a key finding, as the choice of formulation is crucial for the outcome. Most studies utilize depo forms of progesterone, mainly diluted liquid hormonal contraceptives. Viral as well as bacterial infections were studied elucidating the effect of progesterone.

Genital infection of mice with *Mycoplasma pulmonis* is dependent on the stage of menstrual cycle, as in diestrous they are more susceptible to infection [Furr *et al.*, 1993]. However, persistence of the organism is of short duration, assumed to be caused by the rapidly fluctuating stages during menstrual cycle. If mice are treated with progesterone, *M. pulmonis* is able to colonize the genital tract longer than without treatment. In contrast, if treated with estradiol, mice become refractory to the infection. The authors attribute that effect not to a stimulation of the mucosal surface, but rather a higher number of progesterone-induced receptors on target cells of *M. pulmonis* [Furr *et al.*, 1993]. *N. gonorrhoeae* is a common sexually transmitted pathogen that is able to ascend the female genital tract and cause among others PID. Islam *et al*. showed that infection of female mice during diestrous, either natural staged or generated by exogenous progesterone, leads to clinical signs resembling discomfort, extensive gonococcal penetration to the submucosa, and severe inflammation, whereas only modest effects were achieved in estrous stage. Further, a gonococcal-specific humoral response is provoked only during diestrous [Islam *et al.*, 2016]. The most common sexually transmitted pathogen worldwide, *C. trachomatis*, is dependent on progesterone [Tuffrey *et al.*, 1981]. In rats, the influence of progesterone on chlamydial infections is well studied. Kaushic *et al.* proved inbred female Lewis rats as a valid model for infections with *C. trachomatis* MoPn. However, if infected during natural estrous or diestrous, no chlamydial inclusion could be found in the vagina or uterus of all tested rats, hinting at an acute, self-limiting infection. If progesterone

pretreated, chlamydial inclusions are found in epithelial cells 14 days post infection. All infected rats showed a specific immune response to *C. trachomatis* MoPn, which varies with endocrine balance at the time of infection [Kaushic *et al.*, 1998]. Further, in ovariectomized rats, it was shown that estradiol reduces the susceptibility of infection with *C. trachomatis* MoPn and accompanied inflammation. In contrast, progesterone enhances the susceptibility to chlamydial infection coincident with an inflammatory response. The combination of both hormones in one rat shows increased susceptibility while inflammation is reduced [Kaushic *et al.*, 2000].

Outside the urogenital tract, progesterone has protective effects on infections with lethal or sub-lethal concentrations of influenza A virus in female C57BL/6 mice [Hall *et al.*, 2016]. Within the lung tissue, progesterone promotes faster recovery and cellular proliferation, as well as improving pulmonary function, reducing protein leakage into the airway, and producing epidermal growth factor amphiregulin [Hall *et al.*, 2016]. A study showed a specific effect of depo medroxyprogesterone acetate on respiratory *Mycobacterium tuberculosis* infections [Kleynhans *et al.*, 2013]. C57BL/6 mice are resistant to *M. tuberculosis* infection, whereas BALB/c mice are susceptible, leading to contrary outcomes in the regulation of cytokines, such as TNF-α or IFN-γ. Furthermore, hormone treatment leads to an increased bacterial burden in C57BL/6 mice compared to untreated mice; it also affects the colony forming unit count in C57BL/6 mice but not in BALB/c mice [Kleynhans *et al.*, 2013].

Independent from the mucosal site of infection, progesterone influences the success and outcome of bacterial and viral infections. Mouse strain specific differences caused by diverse immune responses need to be considered in planning experiments, as well as the formulation of the hormone compounds, to achieve results closely related to the clinical setting.

2. Aim of the study

Sexually transmitted bacteria such as *C. trachomatis* can lead to severe sequels if either asymptomatic or insufficiently treated. To unravel both, initial situation and consequence, mice are often used in experimental models of chlamydial infection. In this respect, progesterone is administered to mice to synchronize them to the same stage of estrous cycle, thereby making them more susceptible to chlamydial infection. Sex hormones are well known modulators of immune responses; however the influence on urogenital microbiota is so far only suspected. The mode of action of progesterone in genital infection with *C. muridarum* remains unknown; its influence on the composition of vaginal microbiota, as well as proportions and infiltration of immune cells, needs to be elucidated.

Within this thesis the following aims were focused on:

I. Deciphering the composition of commensal vaginal microbiota in C57BL/6J mice.

II. Investigating which role progesterone and β-estradiol play with regard to abundance of vaginal microbiota and immune cell composition in the uterine tissue.

III. Defining the impact of progesterone during infection with *C. muridarum* in respect to pathology formation, vaginal microbiota changes, and immune cell invasion to the uterine tissue.

IV. Characterize the impact of antibiotics on the vaginal microbiota and the outcome of infections with *C. muridarum*.

3. Material and methods

3.1 Material

3.1.1 Devices

The subsequent table shows all used devices.

Table 1: Devices

Device	Version	Supplier
Centrifuge	4-15C	Sigma Laborzentrifugen GmbH, Osterode, Germany
	Biofuge 22R	Amplicor PCR Diagnostics, Roche, Basel, Switzerland
	Biofuge fresco	Heraeus Instruments GmbH, Hanau, 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
Clean bench	Safe 2020	Thermo Fischer Scientific, Waltham, MA, USA
	EN 12469	Clean Air Techniek B.V. Woerden, Netherlands
	PCR Workstation Pro	Peqlab, Darmstadt, Germany
Dispenser pipette	Ripette®	Ritter GmbH, Schwabmünchen, Germany
	Multipette	Eppendorf AG, Hamburg, Germany
Flow cytometer	BD LSR II	BD Biosciences, San Jose, USA
Fluorescence microscope	BZ-9000	Keyence, Osaka, Japan
Fluorometer	Qubit 2.0	Invitrogen, Carlsbad, California, USA
Freezer	Ultra-low -80°C	Sanyo, Leicestershire, UK
	Ultra-low -80°C	Thermo Fischer Scientific, Waltham, MA, USA
	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, UK
	Thermomixer comfort	Eppendorf AG, Hamburg, Germany
	Thermal Shake lite	VWR, Radnor, PA, USA
Horizontal gel chamber	EasyPhor Maxi	Biozym, Hessisch Oldendorf, Germany
Imagingsystem Fusion	FX7	Vilber Lourmat GmbH, Eberhardzell, Germany
Incubator (37°C, 5% CO_2)	CB Series	Binder GmbH, Tuftlingen, Germany
	Forma Series II 3131	Thermo Fisher Scientific, Waltham, MA, USA
Magnetic stirrer	RET basic	IKA®-Labortechnik, Staufen, Germany
Microscope	Axiovert 25	Carl Zeiss Microscopy GmbH, Jena, Germany
	Standard 20	Carl Zeiss Microscopy GmbH, Jena, Germany
	Stemi 508	Carl Zeiss Microscopy GmbH, Jena, Germany
Mouse cage	IVC green line II	Tecniplast, Bugugiatte, Italy
Multichannel pipette	Research plus (1000 µl)	Eppendorf AG, Hamburg, Germany
pH meter	MP 220	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 EV231	Biometra, Göttingen, Germany Peqlab, Darmstadt, Germany
Scale	KB 600-2	Kern & Sohn GmbH, Ballingen-Frommen, Germany
	AT261 delta range	Mettler-Toledo, OH, USA
Sequencer	MiSeq	Illumina®, San Diego, California, USA
Shaker	Polymax 1040	Heidolph Instruments GmbH, Schwalbach, Germany
	SM-30 Control	Edmund Bühler GmbH, Bodelshausen, Germany
Tissue homogenizer	Precellys 24	Bertin technologies, Montigny-le-Bretonneux, France
Thermocycler	C1000	Bio-Rad, Munich, Germany
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

3.1.2 Consumables

Table 2 contains all consumable supplies used during the course of this thesis.

Table 2: Consumables

Consumable supply	Supplier
$175cm^2$-tissue culture flask	Greiner Bio-One GmbH, Frickenhausen, Germany
24-well-tissue culture plate (1.9 cm^2 per well)	Greiner Bio-One GmbH, Frickenhausen, Germany
6-well-tissue culture plate (9.6 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)	Sarstedt AG&Co, Nürnbrecht, Germany
Cell Scraper 195 mm/14 mm	TPP Techno Plastic Products AG, Trasadingen, Switzerland
Cell strainer (100 µm, nylon)	Falcon, NY, USA
Costar® Thermowell® 96-well-PCR Plate	Corning Incorporated, Corning, New York, USA
FACS tube	Sarstedt AG&Co, Nürnbrecht, Germany
Falcon tubes (12, 15, 50 mL)	Sarstedt AG&Co, Nürnbrecht, Germany
Feeding tubes FTP-20-38 (20GAx38mm)	Instech Laboratories, Inc., PA, USA
Gene Catcher Tip	Gelcompany, San Francisco, California, USA
Glass beads	Karl Hechst GmbH & Co KG, Sondheim, Germany
Homogenizer Tubes Precellys® Ceramic-Kit 5.0/2.8 mm	Bertin technologies, Montigny-le-Bretonneux, France
Neubauer counting chamber	Hassa, Laborbedarf, Lübeck, Germany
Parafilm	Carl Roth GmbH, Karlsruhe, Germany
Pipette tips	Sarstedt AG&Co, Nürnbrecht, Germany
Reaction tubes (0.5, 1.5, 2 mL)	Sarstedt AG&Co, Nürnbrecht, Germany
Serologic pipette (5, 10, 25 mL)	Sarstedt AG&Co, Nürnbrecht, Germany
Syringe BD Discardit™ II (10 mL)	BD Bioscience, San Jose, USA
Syringe BD Plastipak (1 mL 25GAx5/8in)	BD Bioscience, San Jose, USA
Syringe Omnican® 40	B. Braun Melsungen AG, Melsungen, Germany
Tissue culture dish (60x15 mm)	BD Bioscience, San Jose, USA
Transsystem Amies w/o CH Aluminum Applicator Rayon Tipped	Copan Diagnostics Inc., Murrieta, USA

3.1.3 Chemicals

Below listed all utilized chemicals.

Table 3: Chemicals

Chemical	Supplier
Acetic acid (100%)	Merck KgaA, Darmstadt, Germany
Ammonium chloride (NH_4Cl)	Sigma-Aldrich Corporation, St. Louis, USA
Bovine serum albumin (BSA)	Sigma-Aldrich Corporation, St. Louis, USA
DMEM	Gibco®, Thermo Fisher Scientific, Waltham, MA, USA
DMSO	Sigma-Aldrich Corporation, St. Louis, USA
dNTP set	Thermo Fisher Scientific, Waltham, MA, USA
EDTA (Tritriplex III)	Merck KgaA, Darmstadt, Germany
Ethanol, absolute	Merck KgaA, Darmstadt, Germany
Fetal bovine serum	PAN-Biotech, Aidenbach, Germany
Formaldehyde solution (4%)	Merck KgaA, Darmstadt, Germany
GeneRuler 100 bp DNA Ladder (0.5 µg/µL)	Thermo Fischer Scientific, Waltham, MA, USA
HEPES	Invitrogen GmbH, Darmstadt, Germany
Isopropanol	Thermo Fisher Scientific, Waltham, MA, USA
L-Glutamic acid	Fluka Chemie GmbH, Buchs, Switzerland
Loading dye (6x)	Thermo Fisher Scientific, Waltham, MA, USA
Methanol	Merck KgaA, Darmstadt, Germany
PeqGold Universal Agarose	Peqlab, Darmstadt, Germany
Paraformaldehyde (1%)	Merck KgaA, Darmstadt, Germany
Potassium chloride (KCl)	Merck KgaA, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck KgaA, Darmstadt, Germany
Potassium hydrogen carbonate ($KHCO_3$)	Carl Roth GmbH, Karlsruhe, Germany
RedSafe	iNTRON Biotechnology, Korea
RPMI	Gibco®, Thermo Fisher Scientific, Waltham, MA, USA
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
Trypan blue (0.4%)	Sigma-Aldrich Corporation, St. Louis, USA
Trypsin/ EDTA	Invitrogen GmbH, Darmstadt, Germany

3.1.4 Medical products

All antibiotics and hormones used for experiments in this thesis.

Table 4: Medical products

Drug	Supplier
Amoxicillin	Sigma-Aldrich Corporation, St. Louis, USA
Amphotericin B	HyClone, Thermo Fisher Scientific, Schwerte, Germany
Ampicillin	Merck KgaA, Darmstadt, Germany
Azithromycin HEXAL® 500 mg	Hexal AG, Holzkirchen, Germany
β-estradiol	Sigma-Aldrich Corporation, St. Louis, USA
Cycloheximide	Sigma-Aldrich Corporation, St. Louis, USA
Doxycycline	Sigma-Aldrich Corporation, St. Louis, USA
Gentamycin	Sigma-Aldrich Corporation, St. Louis, USA
Medroxyprogesterone acetate (Depo-Clinovir®)	Pfizer Inc., New York, USA
Vancomycin	Sigma-Aldrich Corporation, St. Louis, USA

3.1.5 Buffers and solutions

Buffers and solutions, as well as their ingredients, used for work done during this thesis are listed below.

Table 5: Buffers and solutions

Buffer or solution	Ingredients
Antibiotic AVG cocktail	1.6 g Amphotericin B, 41.6 mg Vancomycin and 16.6 mg Gentamycin in 10 ml distilled water
Cycloheximide	1 g cycloheximide in 1 mL distilled water
Loading dye colorless	50 mM Tris (pH 8), 40 mM EDTA, 40% Sucrose, fill up to 100% with distilled water
PBS buffer	80 g NaCl, 2 g KCl, 11.5 g $NaH_2PO_4 x 12H_2O$ and 2 g KH_2PO_4 in 1 l distilled water (pH 7.2)
PBS-BSA buffer	BSA diluted in PBS to a final concentration of 0.5%
RBC lysis buffer	155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA fill up to 100% with distilled water (pH 7.2)
RPMI + 10% FBS	50 mL FBS in 450 mL RPMI medium
SPG buffer	75 g Saccharose, 2.47 g Na_2HPO_4, 0.36 g NaH_2PO_4 and 0.72 g L-Glutamic acid in 1 L distilled water (pH 7.3)
TAE buffer (50x)	242 g Tris/HCl, 18.6 g EDTA, 57.1 ml acetic acid fill up to 1L with distilled water (pH 8.0)

3.1.6 Cultivation media

Table 6 notes used cultivation media.

Table 6: Cultivation media

Cultivation medium	Ingredients	Supplier
HEp-2 standard cultivation medium	DMEM (4.5 g/L) with 10% FBS, 1 M HEPES, Gentamycin (10 mg/mL)	Invitrogen GmbH, Darmstadt, Germany
HEp-2 experimental medium	DMEM (4.5 g/L) with 10% FBS, 1 M HEPES	Invitrogen GmbH, Darmstadt, Germany

3.1.7 Antibodies

The following table lists antibodies used during this thesis.

Table 7: Antibodies

Antibody	Clone	Category	Dilution	Supplier
Anti-Chlamydia-LPS (mouse)		primary	1:50	Prof. H. Brade, FZ Borstel, Germany
Anti-mouse, FITC conjugated		secondary	1:250	Dako, Germany
CD3e, Anti-mouse	17A2	FACS	1:300	Biolegend, San Diego, CA, USA
CD4, Anti-mouse	L3T4 RM4-4	FACS	1:300	BD Bioscience, San Jose, USA
CD45, Anti-mouse	30-F11	FACS	1:300	Biolegend, San Diego, CA, USA
CD8a, Anti-mouse	53.6-7	FACS	1:300	eBioscience, Thermo Fisher Scientific, Waltham, MA, USA
Fc-Block (CD16/CD32)		FACS	1:100	eBioscience, Thermo Fisher Scientific, Waltham, MA, USA
F4/80, Anti-mouse	BM8	FACS	1:300	eBioscience, Thermo Fisher Scientific, Waltham, MA, USA
Ly6G, Anti-mouse	1A8	FACS	1:300	BD Bioscience, San Jose, USA
Zombie NIR™		FACS	1:500	Biolegend, San Diego, CA, USA

3.1.8 Enzymes

Enzymes used in this thesis for either DNA isolation or PCR.

Table 8: Enzymes

Enzyme	Supplier
Deoxyribonuclease I	Sigma-Aldrich Corporation, St. Louis, USA
Liberase TL	Roche, Basel, Switzerland
Phusion High Fidelity Polymerase	Thermo Fisher Scientific, Waltham, MA, USA
OB Proteinase	Peqlab, Darmstadt, Germany
Proteinase K	Peqlab, Darmstadt, Germany

3.1.9 Kits

The commercially available kits are listed in the table below.

Table 9: Kits

Kit	Supplier
BD™ CompBead Anti-Rat and Anti-Hamster Ig Ƙ/ Negative Control Compensation Particles Set	BD Bioscience, San Jose, USA
IMAGEN Chlamydia Kit	Oxoid, Cambridgeshire, UK
innuPREP DNA/RNA Mini Kit	Analytik Jena, Jena, Germany
Min Elute Gel Extraction Kit	Qiagen, Düsseldorf, Germany
MiSeq Reagent Kit v3 (600 cycles)	Illumina, San Diego, California, USA
QIAamp DNA Microbiome Kit	Qiagen, Düsseldorf, Germany
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA

3.1.10 Primer for PCR

Primers for PCR were obtained from Metabion (Planegg). The lyophilisates were dissolved to 100 µM in RT-PCR Grade Water (Thermo Fisher Scientific) according to the supplier's instructions. Primers were further diluted to 2 µM concentration for experiments. The primer sequences are listed in the appendix (Table 16).

3.1.11 Bacterial strains

Table 10 shows the bacterial strain used for *in vitro* and *in vivo* experiments.

Table 10: Bacterial strains

Strain	Supplier	Order No.
Chlamydia muridarum NiggII	ATCC, Manassas, USA	ATCC VR-123

3.1.12 Cell lines

The following table depicts the cell line used for all experiments in this thesis

Table 11: Cell lines

Cell line	Organism/tissue	Supplier	Order No.
HEp-2	Human/ epidermoid laryngeal carcinoma	ATCC, Manassas, USA	ATCC CCL-23

3.1.13 Mice

Mice (*mus musculus*) used for all *in vivo* experiments in this thesis were obtained from Janvier Labs, France. For all experiments, 7 to 8 week old female C57BL/6J mice were delivered directly from the Janvier breeding facility. Animals were kept in 500 cm^2 green line individually ventilated cages at the Gemeinsame Tierhaltung (Lübeck, Germany). Mice received standard chow and water *ad libitum*. All conducted experiments were approved by the Ministry of Energy, Agriculture, the Environment, Nature and Digitalization (Schleswig-Holstein, Germany) in file reference V311-7224.122(54-4/13).

3.2 Cell culture

HEp-2 cells were permanently cultivated in a suitable incubator with a concentration of 5% CO_2 and 21% O_2 at a temperature of 37 °C. Cells were kept in sterile cell culture flasks (175 cm^2) in 25 mL HEp-2 standard cultivation medium.

To passage cells, culture medium was removed, discarded, and cells were subsequently washed with 10 mL phosphate buffered saline (PBS). To detach the cells from the bottom of the flask, trypsin-EDTA solution was added for 5 min at 37 °C. Afterwards, the flask was gently shaken for final removal of the cells, which were then resuspended in fresh HEp-2 standard cultivation medium. Depending on the experiment, cells were split in a ratio of 1:2 or 1:4 in a new flask.
For experiments, cells were seeded in 24-well plates with either 0.5×10^5 cells per well for infection experiments or 2.5×10^6 cells for recovery assays, each in 1 mL experimental medium. Afterwards, cells were incubated for 24 h under standard cultivation conditions.

Cell counting was performed with 10 µl of the cell suspension, which was mixed with 80 µl PBS and 10 µl trypan blue (0.4%) for a final dilution of 1:10. From this final dilution, 10 µl were pipetted on a Neubauer counting chamber (depth 0.1 mm). Living cells were counted in four squares with a 10-x magnification. Cells which appeared blue were assigned as dead

as the trypan blue dye diffuses through disrupted cell membranes. Determination of the cell number of the suspension was performed as follows:

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

3.3 Infection biology

3.3.1 Cell infection with *Chlamydia muridarum*

To enhance the infectious success, 0.1 µg/mL cycloheximide was added to the medium to inhibit host cell protein synthesis. Cells were then infected with 15 IFUs *C. muridarum* NiggII per cell and centrifuged for 1 h with 700x *g* at 30 °C. Afterwards, plates were incubated with 5% CO_2 at 37 °C for 24 h.

3.3.2 Recovery assay

To assign the infectious progeny, cells were removed from the plate using a cell scraper. Cells were resuspended in medium (1 mL) and transferred into a 10 mL tube containing 1 mL glass beads. The tubes were shaken on a vibrating shaker for 5 min at a speed of 1500 rpm to break up the cells and release the *Chlamydia*. Prepared 24-well recovery plates were supplemented with 1 µg/mL cycloheximide. 50 µL of the suspension was added to every well with 200 µL of medium, for a total of 250 µL to each well. After mixing, 250 µL was transferred to the next well for infection. This was repeated for four more wells resulting in a dilution series of 1:5. The plate was then centrifuged for 1 h at 30 °C and 700x *g*, and afterwards incubated at 37 °C and 5% CO_2. After 24 h, the medium was removed and the cells were fixed using 1 mL methanol per well. Plates were placed at -20 °C for at least 30 min before chlamydial inclusions could be visualized with an indirect immunofluorescence test (IFT, see 3.4.1) and counted to assess the recovery rate.

3.3.3 Quantification of *Chlamydia*

Chlamydial inclusion from recovery assays were stained with an indirect IFT (see 3.4.1) for counting inclusions and calculating the amount in correlation to the dilution. Using the Keyence microscope and software, pictures of 24 visual fields were captured and merged. Inclusions were then manually counted using a 20x magnification. A formula for the determination of recovered *Chlamydia* was obtained including the following factors: initial volume of the *Chlamydia*-suspension; the dilution factor; and the size of the merged field (5.3 mm^2) to adjust it to the area of a well (200 mm^2).

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

3.3.4 Infection modulation with antibiotics

The infection model could be modulated by applying different antibiotics to the infected cells to determine their influence on *C. muridarum*. Therefore, antibiotics were applied at the moment of infection; their effect was evaluated 24 h post infection with direct IFT. The concentrations of the antibiotics were tested by using a serial dilution in comparison to an infected, but untreated, control.

3.4 Microscopy

3.4.1 Indirect immunofluorescence of *Chlamydia*

The indirect IFT was used to determine the number of chlamydial inclusions in the recovery assay. Cells were fixed with methanol at -20 °C for a minimum of 30 min. Hereafter, the methanol was discarded and the cells were washed with PBS to remove any remaining methanol. Afterwards, 250 µL of mouse anti-chlamydial lipopolysaccharide (LPS) was added to the cells, which were then incubated for 45 min at 37 °C. The antibody was removed and the cells washed two times with PBS. Subsequently, 250 µL FITC conjugated second antibody was added to the cells and incubated for 30 min at 37 °C. Cells were washed twice with PBS and IFUs were quantified with the Keyence microscope (see 3.3.3).

3.4.2 Direct immunofluorescence of *Chlamydia*

The direct IFT was used to determine the infection rate, as well as the success, of antibiotic treatment *in vitro*. Cells were therefore grown on coverslips in a 24-well plate and were fixed with methanol at -20 °C. After removing the methanol, coverslips were dried at room temperature. IMAGEN Chlamydia Kit was prepared by adding 1 mL PBS to the stock solution. To stain the inclusions and cells, 10 µL of the diluted antibody was applied and incubated for 30 min at 37 °C. Cells were washed and the coverslips fixed on microscope slides with mounting fluid. Through the process of immunofluorescence staining, chlamydial inclusion appeared green and host cells appeared red using the Keyence fluorescence microscope for quantification.

3.5 Animal experiments

Animal experiments were performed in the animal facility of the Gemeinsame Tierhaltung at the University of Lübeck. Figure 3 depicts the general experimental setup of all conducted mouse experiments. More detailed descriptions of the single steps are given in the following subchapters.

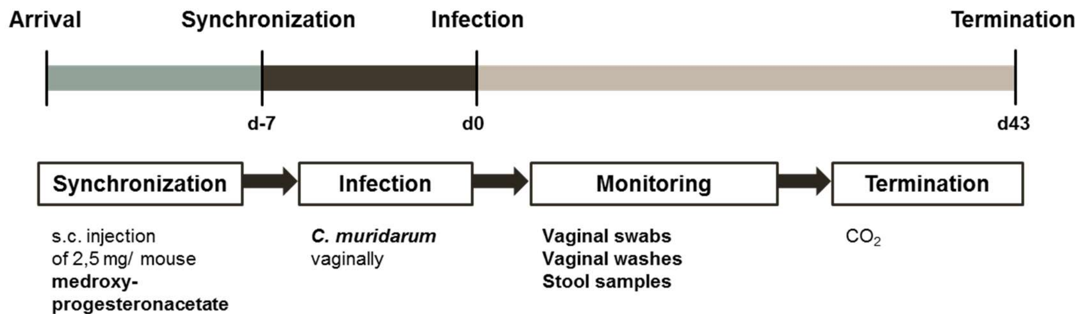

Figure 3: General setup of animal experiment. Female C57BL/6J mice, 7-8 weeks old upon arrival, were settled in the animal facility for one week before they were synchronized, and seven days later infected with *C. muridarum*. Over a course of 43 days mice were monitored and samples were collected. The experiment was terminated with the sacrifice of laboratory animals for evaluation.

3.5.1 Experimental procedures

Upon arrival, mice were 7-8 weeks old and settled in our facility for one week. Mice were synchronized to the same stage of estrous cycle. Therefore, 2.5 mg medroxyprogesterone acetate (Depo-Clinovir®) was injected subcutaneously in the nuchal fold per mouse seven days prior to infection [Gondek *et al.*, 2012]. Seven days after synchronization, mice were vaginally infected with *C. muridarum* NiggII. Via a pipette tip, 10^6 IFUs *C. muridarum* diluted in 4 µL SPG buffer was applied to the vagina of mice. To further enhance the infectious success and prevent discharge, mice were kept headlong for 1 min. During the course of the experiment, mice were monitored in a 6 or 7 day interval and samples were collected. Murine droppings, later referred to as stool samples, were gathered in 1.5 mL reaction tubes and stored at -80 °C until further processing. Additionally, vaginal swabs were taken. The cotton swabs were then placed in a sterile universal transport medium (UTM) until a recovery assay was performed to determine the bacterial load over time, and subsequent DNA isolation (see 3.6.1) of bacterial suspension. In other experiments, the vagina of mice was washed with sterile PBS using three times 50 µL and suspended each six times within the vagina. All three PBS-bacteria suspensions were transferred in one sterile 1.5 mL reaction tube and stored at -20 °C. One portion, 50 µL, of the suspension was used for recovery assay, while the remaining portion was used for DNA isolation. Every experiment ended 43 days post infection by exposition of the mice to a

gradually increasing concentration of CO_2 following the guidelines for euthanasia of rodents (American Veterinary Medical Association). The genital tracts of the mice were either macroscopically examined, or further prepared for flow-cytometric analysis or histological staining.

For recovery assays, 10 mL tubes containing 1 mL glass beads were filled with 1 mL sterile PBS. Vaginal swabs were placed into the tubes, which were then shaken on a vibrating shaker for 5 min at a speed of 1500 rpm to detach the bacteria from the cotton swab. Depending on the time point post infection, 50 or 250 µL of the bacterial suspension was added to a well of a 24-well plate containing a confluent monolayer of HEp-2 cells. Additionally, the plate contained 1 µg/mL cycloheximide and a 10 µL/mL AVG-cocktail to avert the growth of fungi or bacteria other than *Chlamydia*. According to the *in vitro* recovery assay (see 3.3.2), a serial dilution of 1:5 was done in six wells per sample and the plate afterwards centrifuged for 1 h at 700x *g* at 30 °C. After incubation for 24 h at 37 °C and 5% CO_2, the medium was removed and the cells, as well as bacteria, were fixed with methanol at -20 °C. For quantification of the bacterial load indirect IFT was performed (see 3.4.1). For vaginal washes, 200 µL PBS was transferred to a 10 mL tube containing 1 mL glass beads; 50 µL of the vaginal wash fluid was also added and following steps were performed as described before.

3.5.2 Pathology score

For evaluation of the severity of infection, pathologies of the urogenital tract were scored. The pathology score is derived from the macroscopically/ microscopically visible formation of the so-called hydrosalpinx, which is composed of clear fluid that accumulates in the fallopian tube due to distal occlusion. Pathologies were scored (Table 12) by two independent persons following criteria from [Yang *et al.*, 2014].

Table 12: Pathology score and evaluation criterion

Pathology Score	Evaluation criterion
0	no hydrosalpinx
1	hydrosalpinx microscopically visible
2	hydrosalpinx size smaller than ovary, but visible by eye
3	hydrosalpinx size similar to ovary
4	hydrosalpinx size bigger than ovary

3.6 Microbiota sequencing

Samples, either vaginal or gut-derived, were collected from female mice as described (see 3.5.1) and further processed for final barcoded-sequencing approach on an Illumina MiSeq sequencer.

3.6.1 Library preparation

Bacterial DNA from murine stool samples and vaginal swabs was isolated using the innuPREP DNA/RNA Mini Kit. Before beginning, the suspension from vaginal swabs was thawed, and 250 µL was used for subsequent DNA isolation. Depending on the size, one to two murine droppings were used for isolation. To support lysis of the sample, a homogenization step with the Precellys homogenizer was added (two 15 sec runs with 45 sec intermission). Afterwards, 1 h incubation with 0.4 units OB protease or protease K in a thermal shaker with 600 rpm and at 50 °C was included in the procedure. For the remaining isolation, the supplier's protocol was followed. Until further usage, the obtained DNA was stored at -20 °C.

For isolation of bacterial DNA from vaginal washes, the QIAamp DNA Microbiome Kit was used following the manufacturer's instructions. Depending on the sample, 100 to 150 µL of the vaginal wash solution was used. Host DNA was first demolished to enhance the yield in pure bacterial DNA, which was then isolated in a second isolation part. DNA was stored at -20 °C until it was further required.

Polymerase chain reaction (PCR) was used to amplify the V3/V4 region of the bacterial 16S rRNA gene. Therefore, specialized primers [Fadrosh *et al.*, 2014] were modified [Graspeuntner *et al.*, 2018a] and utilized. All primers contained the region 319F or 806R, followed by a heterogeneity spacer and an index sequence. A unique combination of indices was used to barcode every sample during the PCR to make it suitable for Miseq sequencing. For amplification of the 16S rRNA gene, a PCR mix was used (Table 13) and mixed with the respective DNA template. PCR was performed in a 96-well plate on a thermocycler (Table 14).

Table 13: PCR reagents and pipetting scheme per sample

Reagent	Volume [µL per reaction]
RT-PCR grade water	8.25
5 x HF buffer	5
10 µM dNTPs	0.5
2 mM Primer	5 each
Phusion polymerase	0.25
DNA	1 (stool) / 3 (vaginal sample)
Total	**25 / 28**

Table 14: PCR cycling conditions

Step	Temperature [°C]	Time [sec]	Cycles
Initial denaturation	98	300	
Denaturation	98	9	30x
Primer hybridization	55	60	
Elongation	72	90	
Cooling	4	∞	

DNA concentration and size of the amplicons was determined with gel electrophoresis. Therefore, agarose was mixed with 1x TAE to a 2% concentration and microwaved until it was completely dissolved. Before cooling, RedSafe was added at a ratio of 1:17,000 for later visualization of the bands. The PCR product (3.5 µL) was prepared with a dilution of 6x Loading Dye and water (1.2 µL and 2.3 µL per sample). A final amount of 6 µL was loaded on the gel, which ran for 75 min at 110 V before visualization. GeneRuler 100 bp DNA Ladder was used in a known amount to estimate the concentration of the DNA in each band by determining the band size. The marker contained a DNA amount of 0.5 µg and the intensities of the sample bands were compared to either the brighter 500 bp (115 ng), or the inferior 600 bp (45 ng) marker band. Pictures of the gels were taken using a Fusion FX7 imager, and analysis of the bands was done using Bio1D software. All barcoded samples were pooled into one complete library with a DNA concentration of 50 ng per sample. The library was mixed with colorless loading dye (1:7) for subsequent gel electrophoresis on a thick 2% agarose gel (run time of 90 min at 120 V). On an UV transilluminator, the bands were picked using Gene Catcher tips on a pipette. Three gel pieces were pooled for DNA elution from the gel.

The MinElute Gel Extraction Kit was used for elution of the DNA from agarose gels, following the manufacturer's protocol. Elution was performed twice with 10 µL from each column and pooled to a final volume of 20 µL. If more than one elution column was used, all elution volumes were pooled into one. The concentration of the eluted sample was fluorometrically quantified using the Qubit dsDNA BR Assay Kit following the manufacturer's instructions.

3.6.2 Next-generation sequencing

Sequencing of the library was performed at the Max Planck Institute of Evolutionary Biology (Plön). The library was sequenced using a sequencing-by-synthesis technique on an Illumina MiSeq desktop sequencer following an approach published by Kozich *et al.* [Kozich *et al.*, 2013] with modified primers as described above.

3.7 Flow cytometry

3.7.1 Tissue digestion and preparation of a single cell suspension

A 6-well plate was prepared by adding a 100 µm cell strainer to the well and 4 mL of pure RPMI. Both uterine horns per mouse were excised and pooled per well. For digestion, 1.25 mg Liberase TL, diluted in 50 µL pure RPMI, and 1 mg DNase, diluted in 20 µL PBS, were added per well and the plate incubated on a shaker for 90 min at 37 °C and 95 rpm.

After digestion, the cell strainer was placed onto a 50 mL tube and the uterine tissue was pressed through the strainer using the stamp of a 10 mL syringe until it was smooth. The medium of the well was pushed through the filter, and the well was washed twice with 5 mL RPMI + 10% FBS, which was then also pressed through the filter. The tubes were centrifuged for 10 min at 4 °C and 2019 rcf. The supernatant was discarded and the pellet broken by vortexing. To lyse remaining blood cells, 3 mL red blood cell (RBC) lysis buffer was used to resuspend the pellet. The lysis was stopped by filling the suspension up to 30 mL with 0.5% PBS-BSA and 1 mg DNase. The suspension was filtered again through a 100 µm cell strainer. Cells were counted in a Neubauer counting chamber, by mixing 20 µL trypan blue with 20 µL solution. Four fields were counted, and the total number of cells calculated by the following formula.

$$total\ number\ of\ cells = \frac{sum\ of\ four\ counted\ fields}{2} \times 30\ mL\ \times 10^4$$

The remaining cell suspension was centrifuged at 2019 rcf for 10 min at 4 °C. The supernatant was removed.

3.7.2 Staining

To prevent unspecific binding, cell epitopes were blocked using Fc-block in a 1:100 dilution and incubated on ice for 10 min. The suspension was transferred into a 1.5 mL tube, which was then centrifuged at 3.5x1000 rcf for 2 min. After discarding the supernatant, cells were resuspended in 1 mL PBS-BSA and split to a final cell number of 2 Mio. Cells were then centrifuged at 3.5x1000 rcf for 2 min and afterwards the supernatant discarded.
The life-dead dye Zombie NIR™ was diluted to 1:500 in PBS, mixed with the cells, and incubated at room temperature for 20 min in the dark. After incubation, 1 mL PBS-BSA was added and cells spun at 3.5x1000 rcf for 2 min. The supernatant was discarded and the staining master mix (all antibodies diluted 1:300 in PBS-BSA; 100 µL solution per 1 Mio cells) was applied and incubated on ice for 60 min in darkness. The cells were centrifuged, in the same manner as before, and the supernatant discarded and 500 µL PBS-BSA added for washing. Centrifugation and removal of supernatant were once more repeated.
Due to safety guidelines, cells were fixed using 1% paraformaldehyde (PFA) for 15 min on ice. Cells were again centrifuged, and in a second step washed with PBS-BSA. The cells were resuspended in 400 µL PBS-BSA and transferred into a FACS tube.

3.7.3 Measurements and gating strategy

All samples and controls were measured with the BD LSRII, which is located in the Cell Analysis Core (CAnaCore) at the Institute for Systemic Inflammation Research. In total 100,000 events of cells, which were alive, singlets and $CD45^+$ were measured for each sample.
To prevent an overlap between the different colors of the fluorochromes, all colors were compensated. A life-dead sample was prepared by incubating cells at 95 °C for 5 min in a thermal shaker and then cooled down on ice for a further 5 min. One million each of dead and alive cells were mixed and stained exclusively with the life-dead dye Zombie NIR™. All other colors were prepared on beads. A FACS tube was filled with 250 µL PBS, and one drop each of the negative control and the anti-rat and anti-hamster beads were added. Finally, 0.5 µL antibody was mixed with the beads and the specimen was measured with the LSRII. A negative control containing one drop of negative control beads in PBS was additionally measured. A compensation calculation was done using the BD FACSDiva™ software.
Fluorescence minus one (FMO) is used to accurately set the gates for the evaluation of flow cytometry data. Therefore, 50,000 cells were stained with a mix containing all dyes except for one. Results were used to determine the gating.
All data was measured on the LSRII using BD FACS Diva™ software. The gathered data was analyzed using FlowJo® software. To begin, cells were gated for their size using

Forward Scatter (FSC) and their granularity by Side Scatter (SSC). Single cells were then determined by gating for FSC-Height (FSC-H) by FSC-Area (FSC-A) via exclusion of duplets. Zombie NIR™ negative events were determined as living cells. To further specify immune cells, the start gate SSC by CD45 (leucocytes) was set. Afterwards, the population of $CD45^{+}$ cells was downstream analyzed for T cells with either CD3e/CD4 gate or CD3e/CD8a. Non-T cells ($CD3e^{-}$) were further distinguished in granulocytes and macrophages with a Ly6G by F4/80 gate (Figure 4).

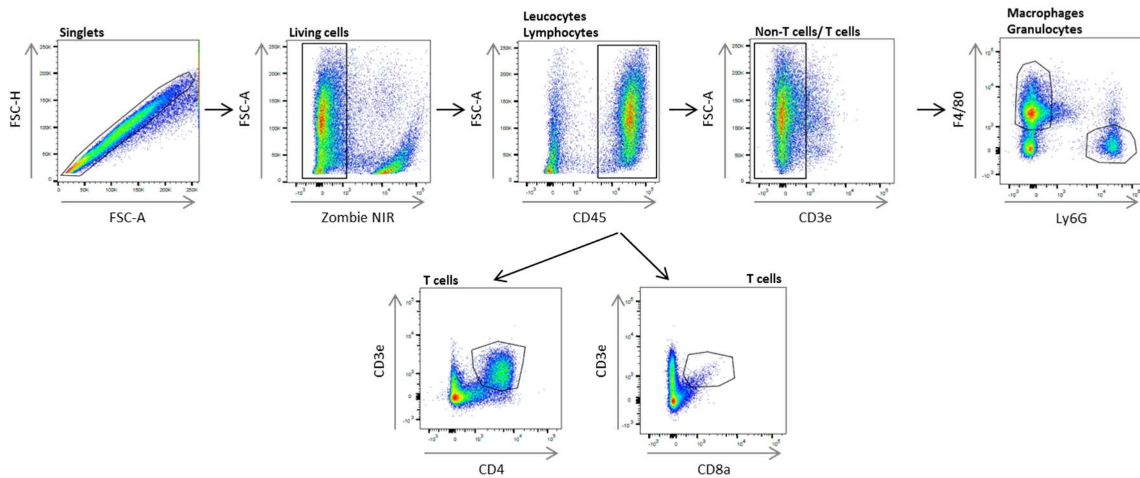

Figure 4: Flow cytometry gating strategy for analysis of immune cell subpopulations. For every sample measured on the LSR II, evaluation was done using FlowJo® software. Cells were determined as being singlets and life cells as a starting point and then further gated for CD45. Afterwards, T cell subpopulations, as well as granulocytes and macrophages, were determined.

3.8 Histological examination

Seven days post hormone treatment vaginal tissue was collected by cutting the vaginal tract from the uterus horns and fixed in formalin solution. Staff of the Pathology Department of the University Medical Center Schleswig-Holstein, Campus Lübeck embedded the vaginal specimens, cut them into 6-µm thick sections, and mounted them on slides for further hematoxylin and eosin staining. Together with an experienced pathologist, specimens were examined on a light microscope and pictures of regions of interest were taken using a Ventana iScan HT slide scanner (Roche) and its corresponding software.

3.9 Bioinformatics and statistics

3.9.1 Software

Table 15 shows all software required for this thesis.

Table 15: Software

Software	Distributor	License or freeware
Acrobat Reader	Adobe INC., Delaware, USA	License
BD FACSDiva	BD Bioscience, San Jose, USA	License
Bio1D	Vilber Lorumat, Eberhardzell, Germany	License
Excel	Microsoft Corporation, Redmond, WA, USA	License
BZII Analyzer	Keyence, Osaka, Japan	License
BZII Viewer	Keyence, Osaka, Japan	License
GraphPad Prism	GraphPad Software, La Jolla, CA, USA	License
FlowJo®	FlowJo, LLC, Ashland, OR, USA	License
Fusion	Vilber Lorumat, Eberhardzell, Germany	License
mothur	Mothur Project	Freeware
R	R-Project	Freeware
RStudio	R-Project	Freeware
Uchime	Drive5	Freeware
Word	Microsoft Corporation, Redmond, WA, USA	License
ZEN 2	Carl Zeiss Microscopy GmbH, Jena, Germany	License

3.9.2 Processing of raw NGS data

Fastq files received from sequencing were processed using mothur, version 1.38.0 [Schloss *et al.*, 2009], in which an algorithm was used to produce contigs combining forward and reverse sequences. Afterwards, all sequences with ambiguous bases, a homopolymer length > 12 or a length greater than the amplified fragment were removed. A SILVA reference data base [Pruesse *et al.*, 2007] was customized for subsequent alignment of the remaining sequences, and unaligned sequences were removed. Chimeras were detected and removed using the UCHIME algorithm [Edgar *et al.*, 2011], as implemented in mothur. Sequences were classified using mothur-formatted RDP [Wang *et al.*, 2007] trainset version

14 with a cutoff of 80. Furthermore, non-bacterial sequences were removed, as well as all reads belonging to the genera *Shewanella* and *Halomonas*, which were determined as contamination from further analysis [Wong *et al.*, 2017]. SourceTracker [Knights *et al.*, 2011] was used to further ascertain contamination within the samples in comparison to controls. Thereby, samples with a contamination greater than 15% were removed. A random subset of 1000 reads per vaginal sample and 5000 reads for stool samples was prepared for further analysis, either using operational taxonomic units (OTUs) or based on taxonomic assignment.

3.9.3 Graphical plotting

Data derived from recovery assay, pathology scoring, and flow cytometry were evaluated and visualized in GraphPad Prism version 7. Statistics and graphical visualizations for sequencing data were produced with R version 3.3.0 [RCoreTeam, 2015].

3.9.4 Statistical analysis

Recovery assay data was tested with two-way ANOVA and pairwise t tests with Holm-Sidak correction for multiple testing to assess differences in bacterial shedding between different treatments. Further, occurrence of pathology formation in changing conditions was evaluated with Chi square test. Flow cytometry results (day 0, n=3) were analyzed with pairwise t tests with false discovery rate correction following the method of Benjamini, Krieger and Yekutieli.

R package vegan [Oksanen *et al.*, 2015] was used to assess alpha diversity by calculating Simpson's and Shannon's diversity indices for every sample on the basis of OTUs. For calculation of beta diversity, R package labdsv [Roberts, 2016] was used. Differences over time in the diversity of microbiota within the stool samples were determined using Spearman's rank correlation coefficient and Spearman's rho. Changes within the abundance of microbiota in vaginal samples, as well as Shannon's Diversity Index, were analyzed using Wilcoxon Rank Sum test with Continuity Correction, and subsequently adjusting p-values for multiple comparisons using Holm correction. Beta diversity was analyzed using adonis test, which is a measure for permutational multivariate analysis of variances using distance matrices.

4. Results

4.1 Characterization of murine vaginal microbiota and tissue composition

The vaginal milieu is well characterized yet highly dynamic, caused by hormonal changes during menstrual cycle, pregnancy, and menopause in humans. The vagina is inhabited by microbes, such as *Lactobacillus* spp., which roughly maintain a typical pH of 4. In experimental setups, mice are often used as model organisms for many diseases, such as infections with sexually transmitted pathogens. Parameters, such as vaginal pH, temperature, and cellular composition are well known. Yet, the murine vaginal microbiota was not analyzed and considered as contributing factor in genital infections. Therefore, the impact of the microbiota in regard to its influence on the course of infection needs to be investigated in a more comprehensive manner in order to understand the interplay of commensal microbiota, tissue composition and pathogens.

4.1.1 Commensal vaginal microbiota

Mice arrive in our housing at 7-8 weeks of age and are given one week of rest in order to recover from the stress of the transportation. In addition, they adapt to the 12 h bright-dark-cycle, conditions and provided chow in our facility. Vaginal samples are collected in intervals of 6 to 7 days with swabs. Swab material is used to further isolate bacterial DNA and amplify the V3/V4 hypervariable region of the bacterial 16S rRNA gene for subsequent sequencing-by-synthesis approach on an Illumina Miseq sequencer. Vaginal microbiota was mainly composed of the phylum Firmicutes (Figure 5 A), which underwent minor variations over time. However, microbiota did not significantly change over the course of time on the phylum level. Likewise, on genus level the composition was slightly fluctuating, as enterococci were minorly decreased while lactobacilli were not significantly increased (Figure 5 B). These alterations had no significant impact on the alpha diversity of murine vaginal microbiota calculated as Shannon's Diversity Index (Figure 5 C).

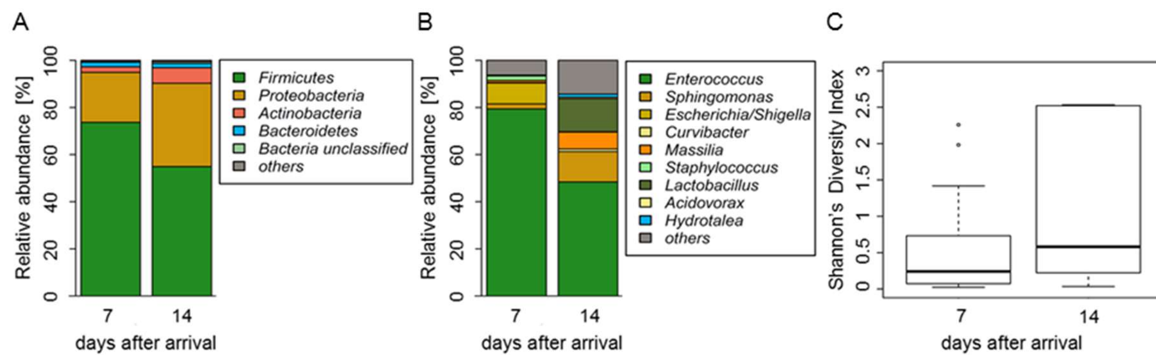

Figure 5: Vaginal microbiota of C57BL/6 mice. (A) Firmicutes had the highest abundance, which did not differ significantly over time. **(B)** On genus level, enterococci had the highest relative abundance. **(C)** The alpha diversity of the two groups did not significantly differ between the tested days. (n=3, Wilcoxon Rank Sum test with Continuity Correction: not significant (ns))

4.1.2 Vaginal tissue composition

The murine vagina is composed of the adventitia (loose connective tissue), a longitudinal and circular smooth muscle cell layer and a luminal mucosal layer. Generally, the mucosal layer comprises stratified squamous epithelium, which alters its morphology during the estrous cycle due to the abundance of circulating sex hormones [Scudamore, 2014]. The murine estrous cycle is composed of four stages: proestrous, estrous, metestrous and diestrous, which are each distinguishable by vaginal smears and appearance of the vagina.

Tissue samples for histological examination were fixed, embedded in paraffin, and stained with hematoxylin and eosin to identify estrous stage and to further look at the tissue composition. Naturally cycling mice, which remained untreated, had a thick mucosal, which was coated with a layer of keratinized cells (Figure 6 A, B). Immune cells were sparse. Only minor differences in tissue composition were observed between three tested mice, suggesting the mice were at corresponding stages of the estrous cycle. In contrast, if mice were treated with progesterone 7 days prior to examination, the vaginal tissue was displaying a layer of mucin-producing goblet cells (Figure 6 C, D). Immune cells, specifically granulocytes, were not present.

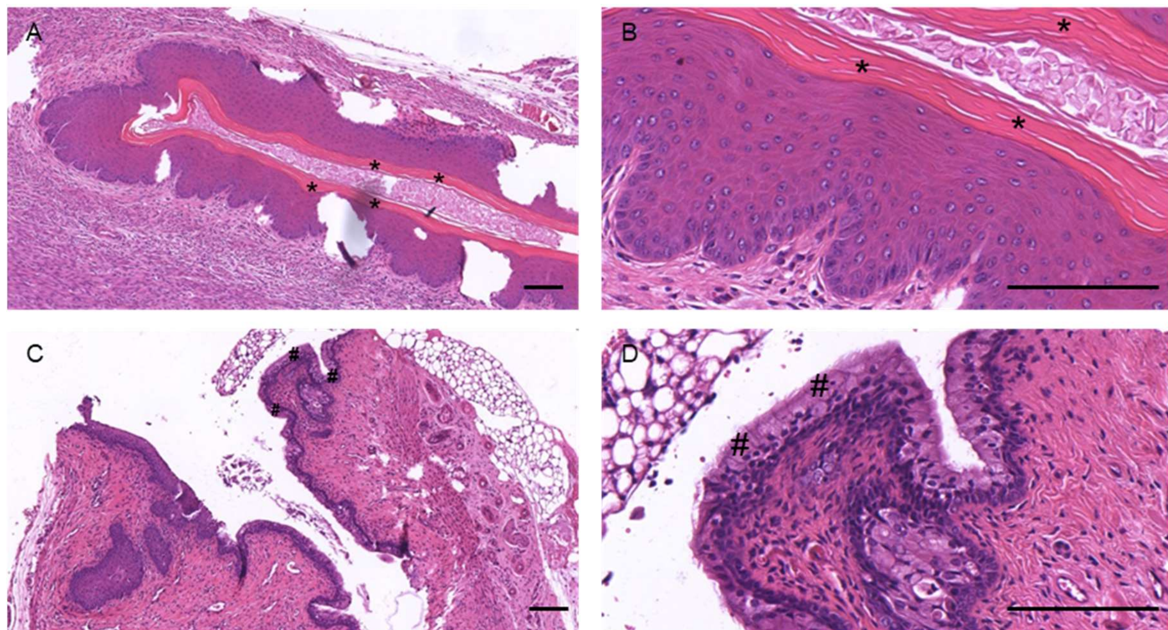

Figure 6: Vaginal tissue composition. Hematoxylin and eosin staining of vaginal tissue. **(A + B)** Longitudinal slides of vaginal tissue from naturally cycling mice with cornified epithelium (representative areas indicated with asterisk). **(C + D)** Vaginal tissue of progesterone-treated mice displayed characteristic mucin-producing goblet cells (representative areas indicated with hash). (magnification A + C 10x, B + D 40x, scale bar = 100 µm, n=3)

4.2 Modulation of the vaginal milieu by sex hormones

Sex hormones are major modulators of host immune response, thereby shaping the response towards pathogens. Therefore, the impact of the two sex hormones progesterone and β-estradiol on the composition of resident immune cells, as well as the abundance of vaginal microbiota, was investigated.

4.2.1 Reduced immune cells in uterine tissue after progesterone and β-estradiol treatment

Mice received 2.5 mg of medroxyprogesterone-acetate (referred to as progesterone), 0.5 mg of β-estradiol, or no treatment (referred to as untreated or naturally cycling) in order to compare the impact of the intervention on immune cell composition. Uterine tissue was harvested 7 days post-hormone treatment, and subsequently digested to obtain a single cell suspension. Flow cytometric analysis revealed a significant reduction in $CD45^+$ cells when comparing untreated mice to those that received either hormone treatment (Figure 7). However, the hormone-treated groups did not differ between each other. Within the reduced number of leucocytes, there were no significant differences in T cells ($CD3e^+/CD4^+$ and $CD3e^+/CD8a^+$), as well as granulocytes ($Ly6G^+$). However, there was a tendency that the $CD3e^+/CD4^+$ T cell subset was slightly increased in progesterone-treated animals.

Solely progesterone was capable of significantly reducing the amount of macrophages (F4/80$^+$), compared to both, untreated and β-estradiol-treated mice.

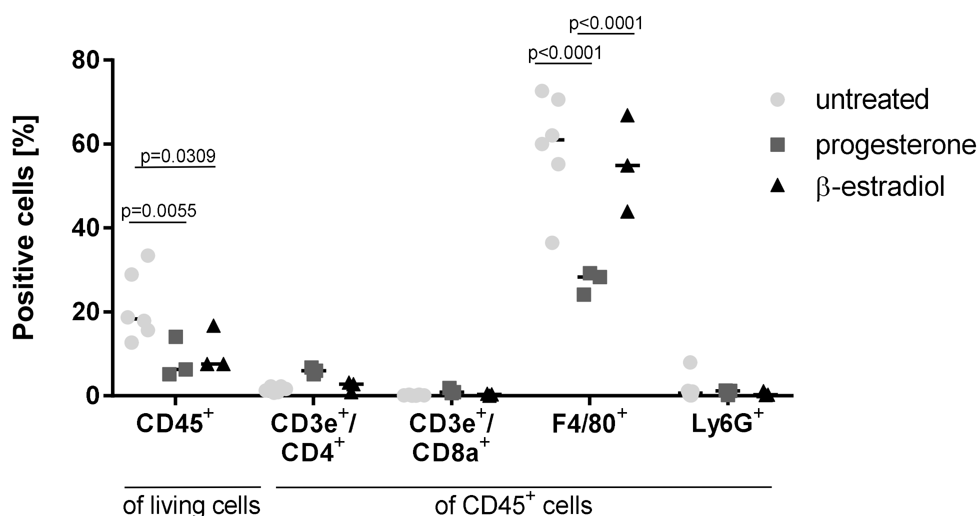

Figure 7: Hormones altered the abundance of immune cells in uterine tissue. The amount of CD45$^+$ cells was significantly decreased in progesterone- (dark grey square, p=0.0055) and β-estradiol- (black triangle, p=0.0309) treated mice compared to the untreated control (grey circle). Solely progesterone-treated mice had a significant decrease in F4/80$^+$ cells. Other subsets of CD3e$^+$/CD8a$^+$, CD3e$^+$/CD4$^+$, and Ly6G$^+$ were not hormonally regulated. (n=3, pairwise t tests with false discovery rate correction for multiple testing following the method of Benjamini, Krieger and Yekutieli)

4.2.2 Vaginal microbiota was altered in mice treated with progesterone, but not with β-estradiol

As described before, mice were treated with hormones at day -7 to determine their impact on vaginal microbiota. For sexually transmitted infections, the composition of vaginal microbiota, as well as the abundance of specific bacteria, may be of importance. This is especially critical in regards to the acquisition of pathogens, and as such the success of infection. Thus, the impact of different hormones on the first line of defense, namely the microbiota, is of interest.

Progesterone treatment led to a significant decrease in Firmicutes abundance 7 days after administration, whereas other phyla, such as Proteobacteria, Actinobacteria, Bacteroidetes, and unclassified Bacteria, were significantly enhanced (Figure 8 A). On genus level, the abundance of enterococci was significantly reduced and only a few other genera, *Curvibacter* and *Acidovorax*, were significantly enhanced (Figure 8 B). Shannon's diversity index reveals the genus diversity within one sample. Before treatment, alpha diversity was low; however, after progesterone treatment, the microbial diversity was significantly enhanced (Figure 8 C). Beta diversity is a measure for the extent of alterations in the composition of a community. Calculations showed that if mice were not treated with

progesterone, groups did not significantly differ between the two tested time points. However, if mice were treated with progesterone, there was a significant shift in beta diversity between pre-administration and 7 days post-administration (Figure 8 D).

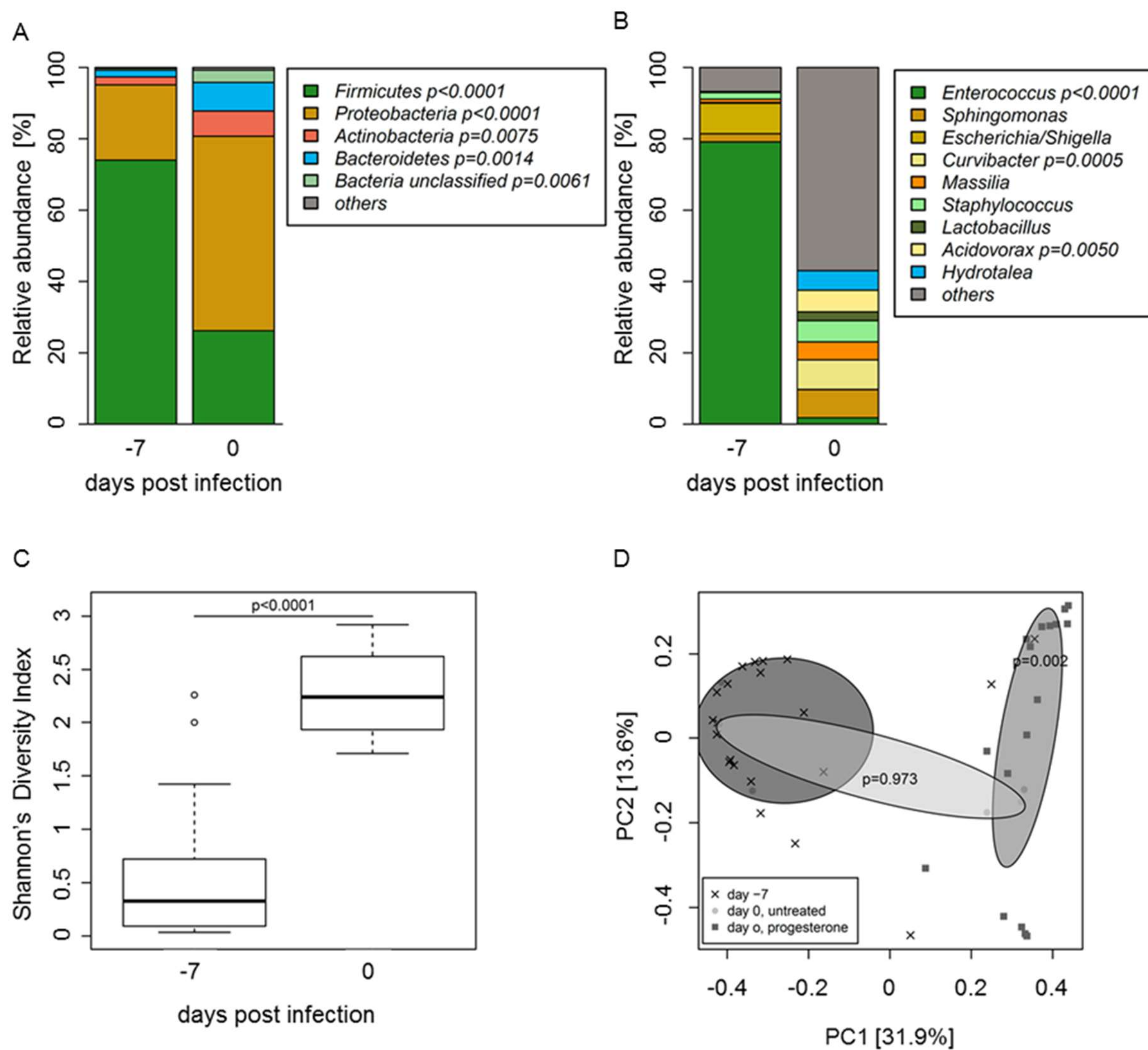

Figure 8: Progesterone significantly changed the abundance of vaginal microbiota and enhanced its diversity. (A) Progesterone administration 7 days prior to infection led to a significant reduction in Firmicutes abundance (p<0.0001), thereby significantly increasing Proteobacteria (p<0.0001), Actinobacteria (p=0.0075), Bacteroidetes (p=0.0014), and unclassified Bacteria (p=0.0061). **(B)** Enterococci were significantly decreased by progesterone treatment (p<0.0001). Other bacterial genera also increased significantly, such as *Curvibacter* (p=0.0005) and *Acidovorax* (p=0.0050). **(C)** Shannon's diversity index was significantly increased in progesterone treatment (p<0.0001). **(D)** Beta diversity analysis showed no difference between untreated mice at day 0 and day 7 (p=0.973), whereas microbiota of mice treated with progesterone significantly differed from the original composition (p=0.002). (n=4, subsampled to 1000 reads, relative abundance and alpha diversity: Wilcoxon Rank Sum test with Continuity Correction and correction for multiple testing, beta diversity: adonis)

The administration of β-estradiol to mice did not induce changes to the vaginal microbiota, contrary to progesterone, and thereby resembled the composition of vaginal microbiota of untreated mice. Firmicutes abundance remained high 7 days after hormone treatment (Figure 9 A), and neither composition, nor the proportions changed due β-estradiol administration. On genus level, β-estradiol did not significantly alter the proportions, even though there was a slight increase in *Staphylococci* abundance (Figure 9 B). These results are additionally supported through the observation of alpha (Figure 9 C) and beta diversity (Figure 9 D), which remained unchanged due to the β-estradiol treatment.

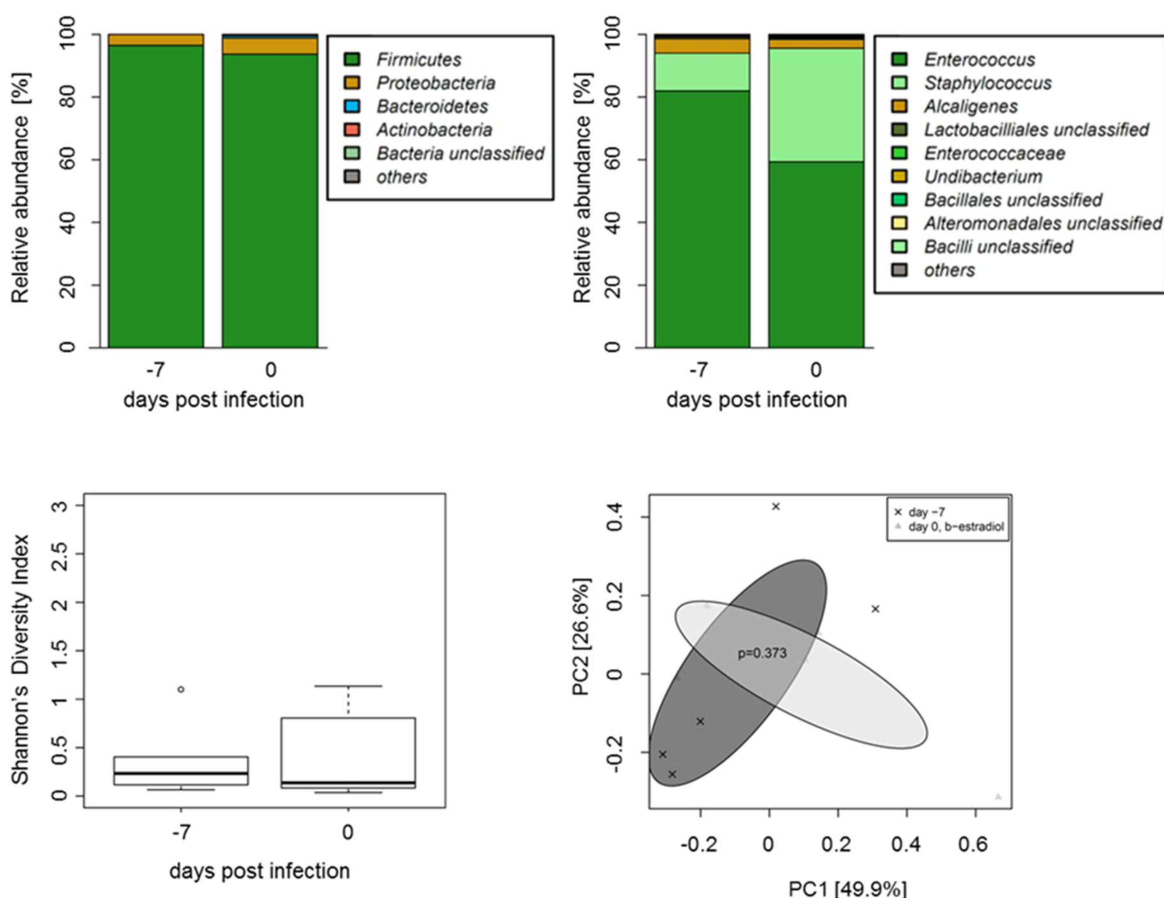

Figure 9: β-estradiol had no influence on vaginal microbiota. (A) The administration of β-estradiol 7 days before infection did not affect the composition and proportions of vaginal microbiota on phylum level, as Firmicutes abundance remained high. **(B)** On genus level, *Staphylococci* increase slightly; however, overall, the compositions remained alike. **(C)** Alpha diversity remained low despite β-estradiol treatment. **(D)** Over time, β-estradiol-treated group's beta diversity did not alter significantly (p=0.373). (n=4, subsampled to 1000 reads, relative abundance and alpha diversity: Wilcoxon Rank Sum test with Continuity Correction and correction for multiple testing, beta diversity: adonis)

4.2.3 Gut microbiota was unaffected by progesterone treatment

Administration of progesterone or β-estradiol led to a local reduction of leucocytes and Firmicutes/ enterococci abundance. As the intestinal microbiota plays a crucial role in the induction and function of host immune responses, the role of sex hormones on changes within the gut microbiota was examined. Therefore, murine droppings were collected in order to sequence the V3/V4 region of the bacterial 16S rRNA gene for further analysis, as already described.

If mice remain untreated, there were some slight, but not significant, fluctuations within the proportions of phyla visible; however, the overall composition remained stable (Figure 10 A). Gut microbiota was mainly composed of Bacteroidetes and Firmicutes. Neither progesterone treatment (Figure 10 B), nor β-estradiol administration (Figure 10 C) had an influence on the gut microbiota. Further expanding the tested time points and including alpha diversity analysis (Figure 20), there was no significant influence detectable.

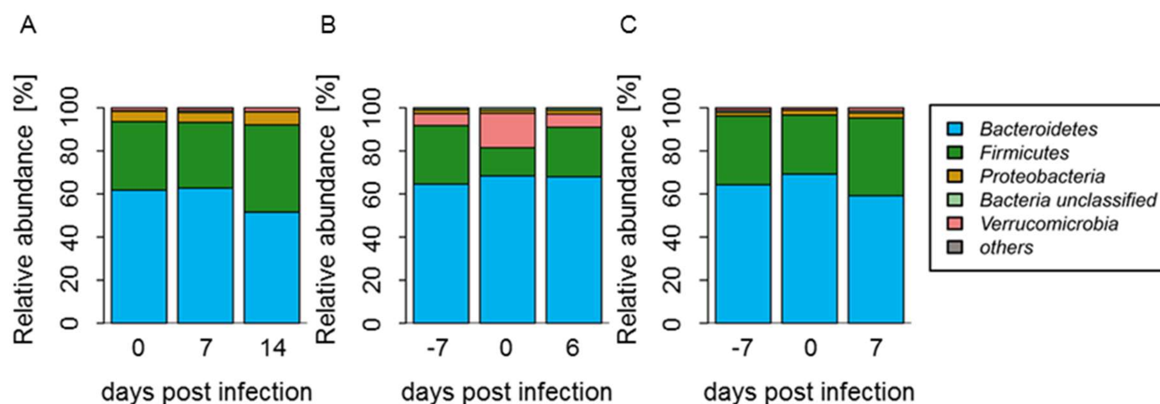

Figure 10: Gut microbiota was not affected by hormone treatment. (A) Untreated mice did not show any significant differences in relative abundance of bacterial phyla over time. **(B)** Progesterone administration at day -7 did not change the composition and proportions of gut microbiota. **(C)** No variations were detectable after β-estradiol treatment. (n=4, subsampled to 5000 reads, Spearman's rank correlation coefficient and Spearman's rho: untreated p=0.6845, rho =-0.1581; progesterone p=0.4032, rho= 0.2661; β-estradiol p=0.9273, rho=0.0296)

4.3 Progesterone enhanced susceptibility of mice to genital *C. muridarum* infection

Sex hormones, such as progesterone, have been shown to be crucial for infections at various mucosal sites. In animal models, genital infections, including *Chlamydia*, and their dependence on hormones was intensively studied. Rats, for example, require progesterone in order to develop severe chlamydial infection [Kaushic *et al.*, 2000]. In mice, progesterone has been described as being beneficial for *Chlamydia*; however, the mechanisms are not yet well studied. Sex hormones are described as modulators of immune responses [Beagley *et al.*, 2003], and further data indicates a role in shaping urogenital microbiota. Both factors are important for vaginal health, but the distinct mechanisms of sex hormones on vaginal milieu and STDs is not well understood. The established mouse model was therefore used to elucidate the effect of progesterone and partly β-estradiol on the course and outcome of a chlamydial infection, as well as involved immune cells and vaginal microbiota.

4.3.1 Enhanced bacterial shedding by progesterone-treatment

Over the course of infection with *C. muridarum*, vaginal swabs were regularly collected for subsequent recovery assay to determine the bacterial load and shedding by recovering infectious progeny. During the first days, the chlamydial load increased in progesterone-treated mice, compared to a reduced number in untreated and β-estradiol-treated mice. Initially, mice were infected with 10^6 IFUs *C. muridarum*, whose number was significantly higher within the first days post infection if mice were progesterone-treated compared to untreated mice (Figure 11). Over the course of time, the bacterial load decreased and *C. muridarum* was no longer recovered in untreated, β-estradiol-treated and progesterone-treated mice after 18, 21 and 28 days post infection, respectively. Remarkably, not all untreated or β-estradiol-treated mice were successfully infected.

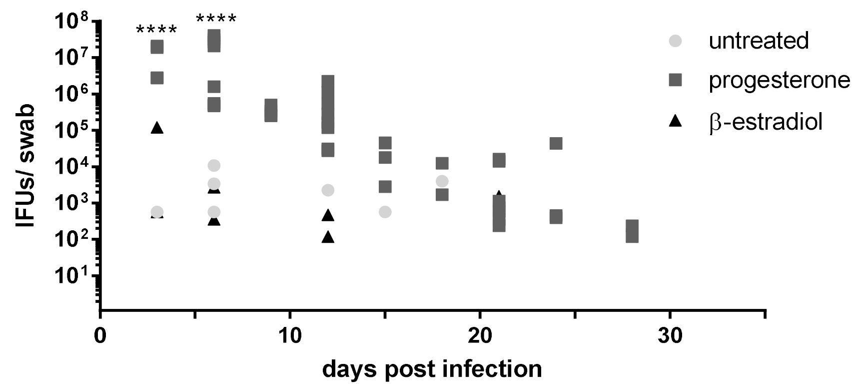

Figure 11: Progesterone-treated mice were more susceptible to chlamydial infection. Mice were infected with 10^6 IFUs *C. muridarum*. In progesterone-treated mice (dark grey square), the bacterial load increased within the first days post infection and decreased until day 29, when *Chlamydia* was not recoverable. At day 3, 6 and 7 post infection, the IFUs/swab was significantly higher than in untreated (grey circle) and β-estradiol- (black triangle) treated mice. The chlamydial load in untreated and β-estradiol-treated mice was comparably low but steady over 18 and 21 days, respectively. (n=4, two-way ANOVA and pairwise t tests with Holm-Sidak correction for multiple testing ****$p<0.0001$)

4.3.2 Progesterone-treated mice developed pathology

Usage of the murine pathogenic strain *C. muridarum* consistently leads to the formation of pathology. Hydrosalpinx is an accumulation of clear fluid caused by distal occlusion in the fallopian tubes, which is comparable to tissue occlusion and generalized inflammation as a result of chronic infection with *C. trachomatis* [Brunham *et al.*, 2015]. Whether sex hormones play a direct role in the development of pathology, or are a key factor for *Chlamydia* is so far unknown.

Pathology development was macroscopically evaluated 43 days post infection by comparing the size of hydrosalpinx to the ovary and scoring it with values from 0-4 (Table 12). Mice which were not treated with hormones did not develop any hydrosalpinx (Figure 12 A), and the urogenital tract appeared macroscopically (Figure 12 B) comparable to uninfected tissue. Similar, β-estradiol-treated mice scarcely developed pathology, and macroscopic evaluation revealed the appearance of the urogenital tract (Figure 12 C) resembled untreated. If mice were progesterone-treated, the formation of hydrosalpinx was significantly higher compared to untreated and β-estradiol-treated mice, as more than 60% of all evaluated urogenital tracts carried pathology. Pathologies occurred either bi- (Figure 12 D) or uni-laterally (Figure 12 E), and were mostly scored with a value of 4, indicating the most severe case.

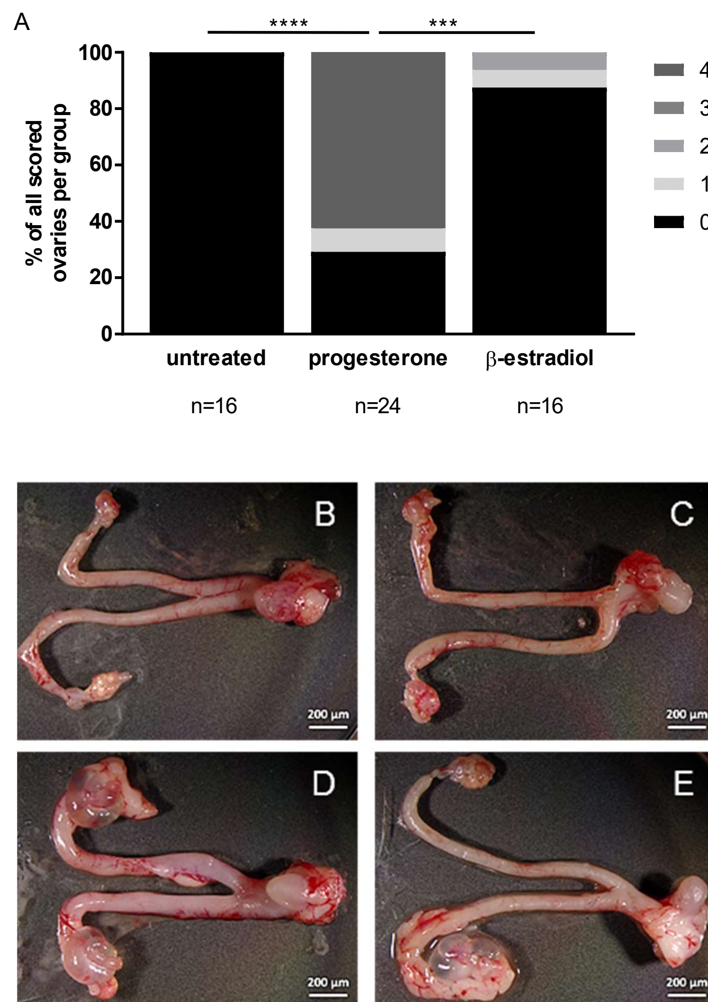

Figure 12: Hydrosalpinx formation was characteristic for progesterone-treated mice. (A) Untreated mice did not develop any hydrosalpinx after *C. muridarum* infection (n=16). Similarly, β-estradiol-treated mice (n=16) did develop small hydrosalpinx, though only rarely. Progesterone-treated mice developed pathologies on more than 60% of all examined uterus horns (n=24). **(B)** Urogenital tract of an untreated mouse with no signs of inflammation or pathology. **(C)** Picture of β-estradiol-treated urogenital tract with no hydrosalpinx. **(D)** Urogenital tract with bilateral hydrosalpinx with a score of 4 from a progesterone-treated mouse. **(E)** Unilateral hydrosalpinx with a score of 4 from a progesterone-treated mouse. Pathologies were scored, comparing the proportion of hydrosalpinx to the size of the ovary, following the scheme: 0=no pathology; 1=microscopically visible; 2=smaller than ovary; 3=same size as ovary; 4=bigger than ovary. (scale bar 200µm, Chi square test, ***p=0.001, ****p<0.0001)

4.3.3 Vaginal microbiota during chlamydial infection

Additional to the bacterial shedding, vaginal microbiota analysis gives insight into the abundance of taxa other than *Chlamydia*. Over the course of the experiment, every 6th or 7th day, vaginal swabs or washes were collected and the V3/V4 region was subsequently sequenced. An abundance of bacterial phyla and genera were determined and alpha diversity calculated based on Illumina MiSeq results.

Vaginal microbiota of C57BL/6J mice housed in our facility was mainly composed of Firmicutes (Figure 13 A), which were stable if mice remained untreated. On genus level, enterococci and lactobacilli were most abundant (Figure 21 A). Infection with *C. muridarum* (d0) led to an increase in alpha diversity (Figure 13 B). Chlamydial reads, however, were barely detectable, corresponding to bacterial shedding (see 4.3.1). Over time, mice were able to restore their vaginal microbiota to approximately the initial composition. By contrast, progesterone administration decreased the abundance of Firmicutes (Figure 13 C), specifically enterococci and lactobacilli (Figure 21 B), 7 days after treatment when mice were infected with *C. muridarum*. Infection led to a high abundance of *Chlamydia* among sequenced reads, which was stable until 24 days post infection. Following resolved infection, vaginal microbiota was restored in spite of high Firmicutes/ enterococci abundance. Alpha diversity was enhanced after progesterone treatment and decreased post infection (Figure 13 D). After chlamydial shedding, alpha diversity once again increased. In β-estradiol-treated mice, the abundance of Firmicutes (Figure 13 E)/ enterococci (Figure 21 C) remained high even after hormonal treatment. The low alpha diversity was only slightly enhanced after infection with *C. muridarum* (Figure 13 F). Chlamydial reads were barely observable 7 days post infection and increased 14 days post infection; however, their level remained lower than in progesterone-treated mice.

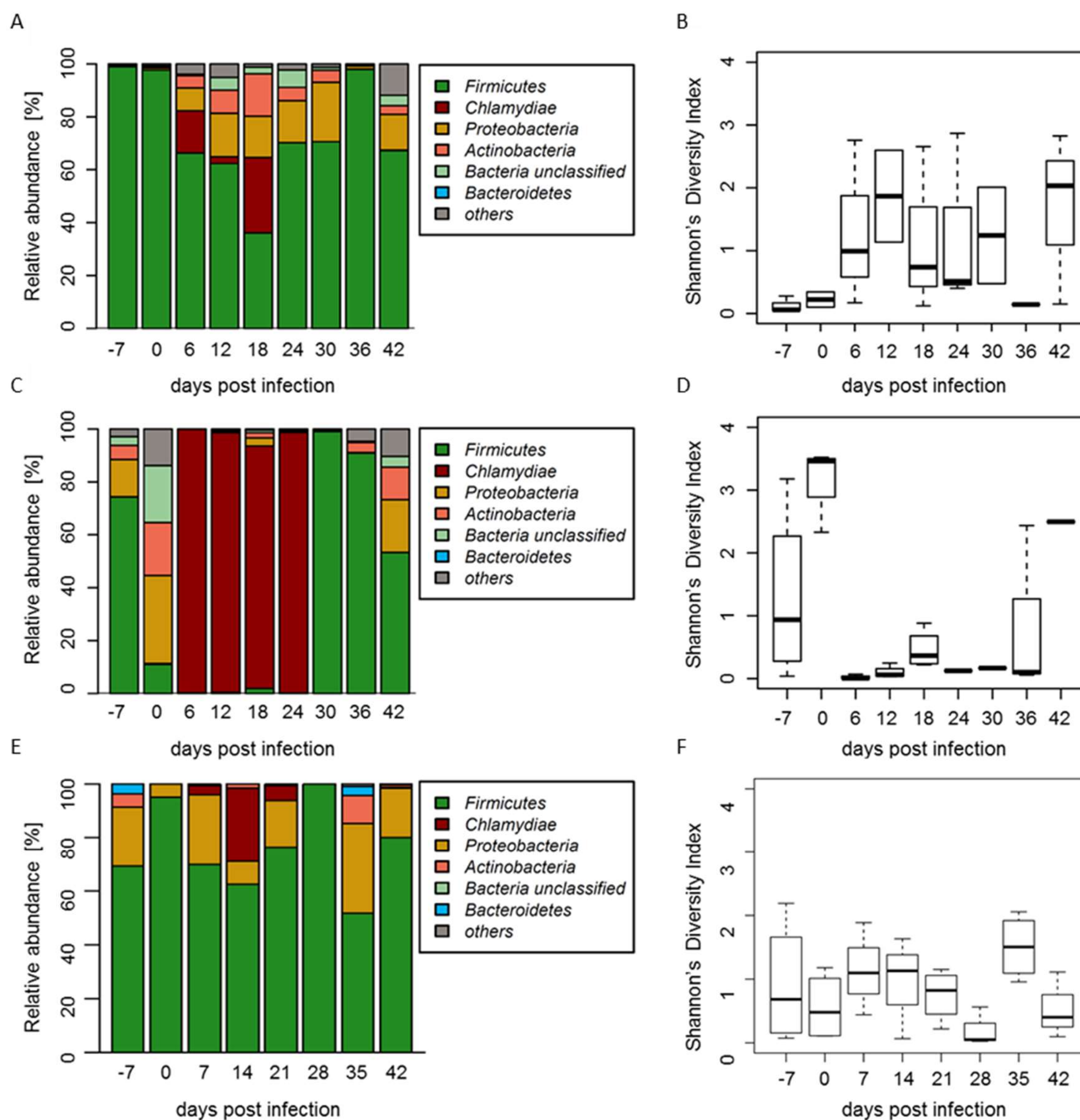

Figure 13: Vaginal microbiota during *C. muridarum* infection. (A, C, E) Vaginal microbiota on phylum level. **(B, D, F)** Alpha diversity of vaginal microbiota. **(A)** Vaginal microbiota of untreated mice remained highly abundant with Firmicutes before infection. Infection with *C. muridarum* led to a non-significant change within the composition of microbiota, which was restored over time. **(B)** Diversity was low when mice were untreated. Infection led to an increase in diversity. **(C)** Firmicutes abundance decreased after progesterone treatment, and chlamydial reads were highly abundant 6 to 24 days post infection. **(D)** Progesterone treatment led to an increase in diversity that was reverted by infection with *C. muridarum* 6 days post infection. **(E)** β-estradiol administration did not alter the composition of vaginal microbiota. Inoculation of *Chlamydia* led to a change in proportions of relative abundances. **(F)** Diversity of vaginal microbiota of β-estradiol-treated mice was affected neither by hormone treatment, nor by infection. (n=1-4, subsampled to 1000 reads, Wilcoxon Rank Sum test with Continuity Correction and correction for multiple testing)

4.3.4 Infection-induced host immune response was elevated in progesterone-treated mice

Sex hormones are modulators of immune responses, thereby altering the success of infections. Therefore, the involved immune cells are important, especially for mechanisms concerning bacterial shedding and induction of pathology formation. As a first idea of subsets involved in immune response in this study, leucocytes, T cells, macrophages, and granulocytes were studied.

Progesterone significantly reduced $CD45^+$ cells prior to infection (Figure 14 A). Naturally cycling mice had a slight decline in leucocytes 3 days post infection, but returned to the initial amount of approximately 30% in uterine tissue. Progesterone treatment led to a strong increase of leucocytes 7 days post infection, which further decreased until day 14 and slightly increased again. T cells were discriminated in $CD3e^+/CD4^+$ T helper cells (Figure 14 B) and also $CD3e^+/CD8a^+$ T killer cells (Figure 14 C), which were both elevated in progesterone-treated animals compared to untreated animals. T helper cells migrated to the uterine tissue 14 days post infection and declined afterwards in progesterone-treated mice, whereas in untreated mice no $CD4^+$ T cells were recruited to the infected site. T killer cells peaked twice at 3 and 14 days post chlamydial infection. Untreated mice exhibited a minor increase of $CD8a^+$ cells 3 days after infection, which declined afterwards to a nearly undetectable level. Tissue macrophages ($F4/80^+$) were significantly reduced by progesterone (Figure 14 D). During infection, macrophages remained lower in progesterone-treated animals, while continued to be more abundant in untreated animals. Granulocytes ($Ly6G^+$) were comparable in progesterone-treated and untreated mice (Figure 14 E). However, infection with *C. muridarum* led to an increase in granulocytes 7 days post infection and the number remained higher than before infection. In untreated mice, at 7 days post infection the amount of granulocytes was increased, which remained stable until day 21 post infection, when it decreased to initial levels.

Progesterone treatment led to a significant reduction in total leucocytes and specifically macrophages. During the infection, there was a massive influx of immune cells to the uterine tissue 7 days post infection. Smaller immune cell subpopulations occurred at later time points, as $CD4^+$ cells and granulocytes.

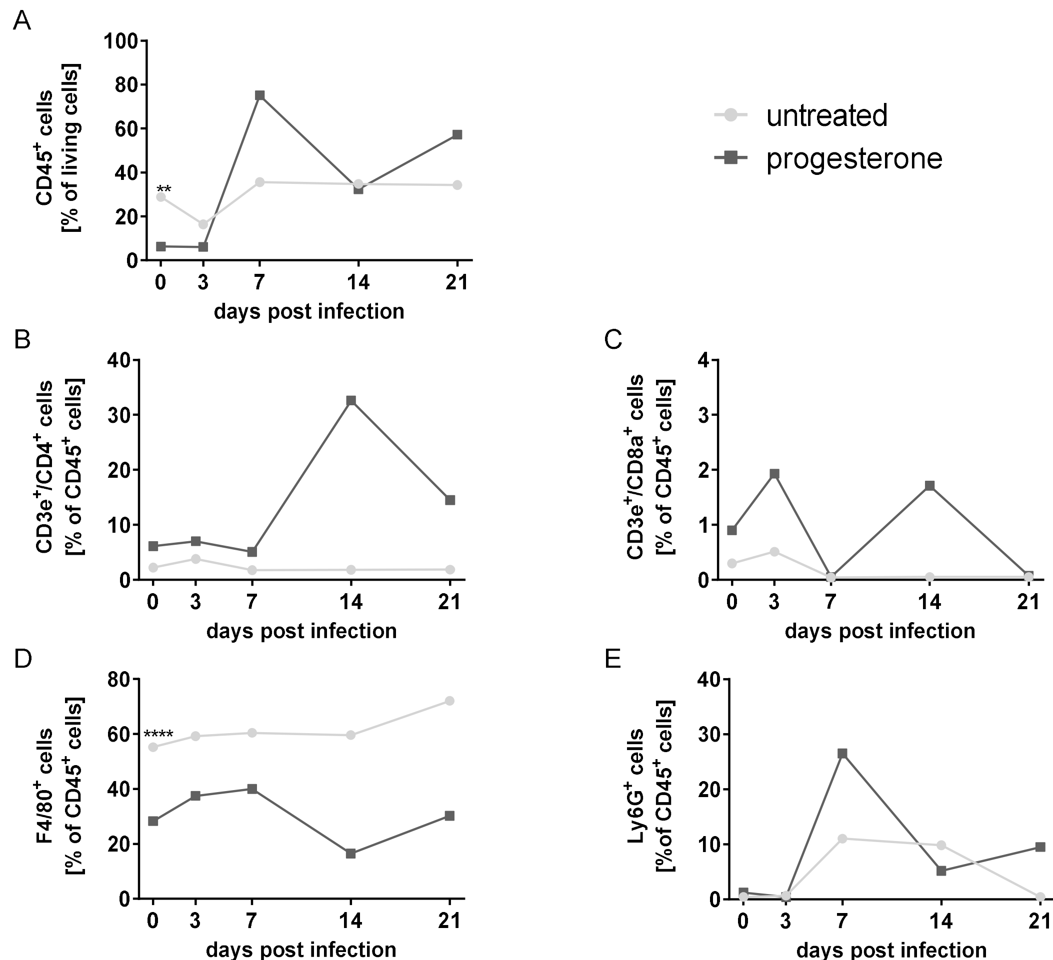

Figure 14: Immune cell response towards chlamydial infection differed in progesterone-treated mice. **(A)** $CD45^+$ leucocytes were significantly reduced in progesterone-treated mice (dark grey squares) compared to untreated mice (grey circles). 7 days post infection macrophages were highly abundant in uterine tissue in progesterone-treated mice, but not in untreated. **(B)** Influx of $CD3e^+/CD4^+$ T cells 14 days post infection in progesterone-treated mice. Abundance of T helper cells in untreated tissue did not change. **(C)** T killer cells, $CD3e^+/CD8a^+$, were slightly abundant, but in progesterone-treated tissue there was an influx to the uterine tissue 3 and 14 days post infection. **(D)** $F4/80^+$ cells were significantly reduced in progesterone-treated mice which remained even over the course of infection. **(E)** Progesterone-treated mice had an influx of granulocytes ($Ly6G^+$) to the uterine tissue 7 days post infection. (d0 progesterone n=3, d0 untreated n=6: pairwise t tests with false discovery rate correction following the method of Benjamini, Krieger and Yekutieli, **$p<0.01$, ****$p<0.0001$; d3-21: n=1)

4.4 Application of antibiotics – effects on *Chlamydiae* and microbiota

Recommended treatments in the clinics for infections with *C. trachomatis* are either doxycycline or azithromycin, whereas for pregnant women amoxicillin is the antimicrobial of choice. Antibiotics do not specifically target the pathogen; however, some have an additional effect on commensal microbiota. Antibiotic-induced changes within the commensal composition might have an impact on the acquisition and course of chlamydial infections. Therefore, antibiotic pretreatment and its influence on chlamydial infection were tested.

4.4.1 Antibiotic efficacy *in vitro*

Cells can be infected with the intracellular pathogen *C. muridarum*. Therefore, cells were seeded in a 24-well plate and 24 h later infected with *C. muridarum*. Antibiotics were added in a serial dilution to find a working concentration. Cells were fixed and stained 24 h post infection.

Chlamydial inclusions are intracellular vacuoles containing chlamydial EBs and RBs, which were stained with fluorescent dye and appear green, while host cells appear red. In the untreated control, approximately 95% of all cells were infected (Figure 15 A). 1 µg/mL Doxycycline (Figure 15 B) and 10 µg/mL azithromycin (Figure 15 C) were effective as no chlamydial inclusions could be detected. However, 10-50 µg/mL amoxicillin (Figure 15 D) did not avert chlamydial infection. Thus, inclusions appear to be different from the control and appear morphologically like aberrant bodies. Aberrant bodies are a sign for induction of persistence, which is feasible *in vitro* by administration of β-lactam antibiotics.

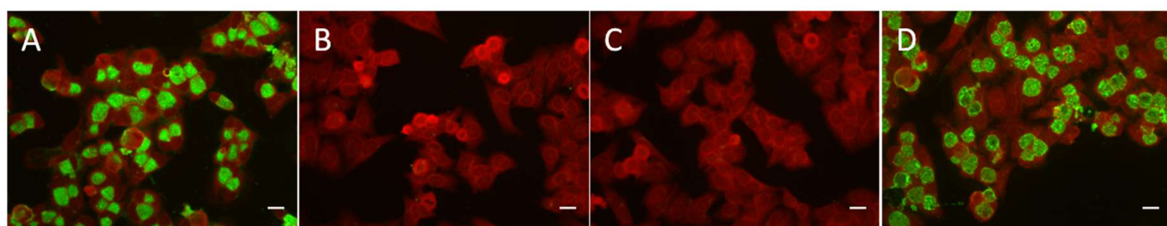

Figure 15: Antibiotic efficacy against *C. muridarum in vitro*. (A) Cells were infected with 15 IFUs *C. muridarum* per seeded cell and not treated with any antibiotic. Approximately 95% of all cells were infected. **(B)** In cells treated with 1 µg/ mL doxycycline there were no inclusions of *C. muridarum* visible. **(C)** There were no chlamydial inclusions found in cells treated with 10 µg/ mL azithromycin. **(D)** Amoxicillin was not able to eradicate *Chlamydia* at a concentration of 50 µg/ mL. (magnification 40x, scale bar = 20 µm)

4.4.2 Efficacies of antibiotics varied in eradicating chlamydial infection

Antibiotics are validated in *in vivo* models before utilization in the clinics. The test against *C. muridarum* was conducted utilizing concentrations that are comparable to physiologic serum concentrations reached in humans. All antibiotics were administered perioral, meaning direct application to the stomach via feeding needles. This method assures compliant dosage of the antibiotics. Doxycycline (10 mg/ kg) and amoxicillin (20 mg/ kg) were administered in a 12 h rhythm for 7 days, azithromycin in one high dose (40 mg/ kg) and three subsequent low doses (20 mg/ kg) once daily following regular treatment schemes.

Control animals showed a regular shedding curve with an increase of bacterial load in the first days and a decline until day 28 post infection. Afterwards, *Chlamydia* was no longer recoverable (Figure 16). Doxycycline treatment significantly reduced the chlamydial IFUs. No *Chlamydia* was detectable at any time point. Until 14 days post infection, no chlamydial inclusions were recoverable after azithromycin treatment; however, two mice tested positive 14 days post infection and one 21 days post infection. Amoxicillin significantly reduced chlamydial IFUs 3 and 7 days post infection compared to the control, but was not as efficient as doxycycline and azithromycin. Shedding duration was comparable to the control.

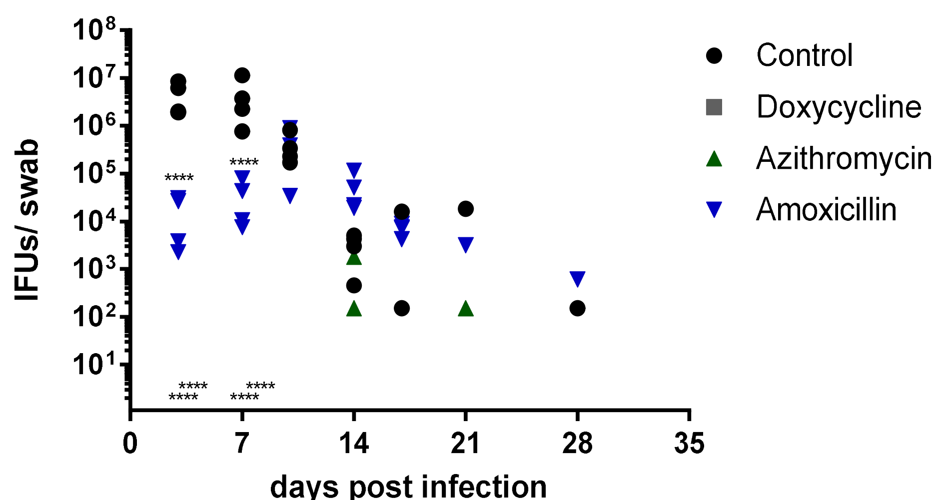

Figure 16: Doxycycline effectively eradicated *C. muridarum*, whereas azithromycin and amoxicillin fail. Infected but untreated mice (black circle) displayed a regular shedding curve. 3 and 7 days post infection, doxycycline (10 mg/ kg, grey square) and azithromycin (40 mg/ kg and 20 mg/ kg, green triangle) significantly reduced the bacterial load. However, chlamydial inclusions were visible 14 and 21 days post infection after azithromycin treatment. Amoxicillin (20 mg/ kg, blue inverted triangle) significantly reduced the number of IFUs 3 and 7 days post infection. (n=4, two-way ANOVA and pairwise t tests with Holm-Sidak correction for multiple testing ****$p<0.0001$)

4.4.3 Ampicillin affected vaginal microbiota but not chlamydial infection

Antibiotics are prescribed for several infectious diseases. As they do not function locally but generalized, the idea of a randomly altered commensal microbiota led to the question of a potential influence on the acquisition and course of chlamydial infection. Doxycycline and ampicillin were tested for their ability to alter the vaginal microbiota, and thereby vaginal chlamydial infection independent from the hormone status. Mice were treated with antibiotics 3 consecutive days and had a 3 day washout-phase before infection to be certain antibiotics would not directly interfere with the pathogen.

Both antibiotics significantly reduced the initial bacterial load in progesterone-treated mice; however, during the further course infection, there were no differences in shedding. In the control animals, as well as the antibiotic pretreated ones, the shedding continued 24 days before *Chlamydia* was no longer recoverable (Figure 17 A). If mice were not treated with progesterone, *Chlamydia* had difficulty propagating in the vaginal tract and was hard to recover from swabs. There were no differences between the control and antibiotic pretreatment (Figure 17 B).

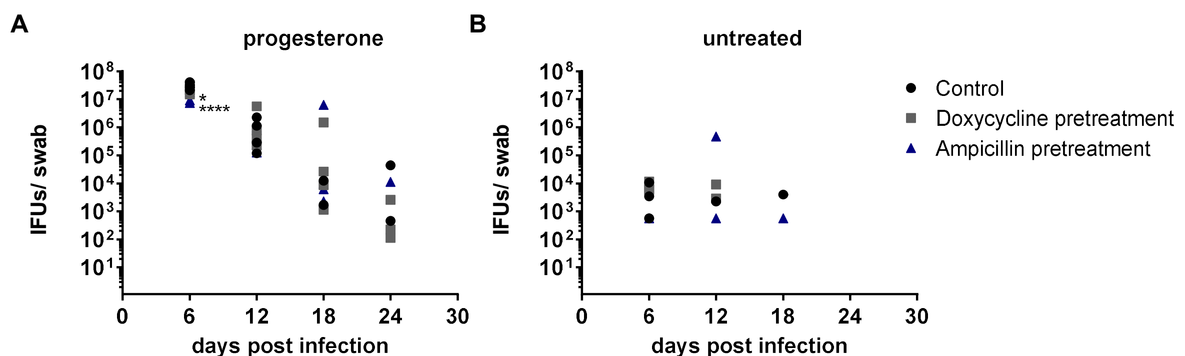

Figure 17: Antibiotic pretreatment did not prevent infection with *C. muridarum*. (A) Doxycycline (grey square) and ampicillin (blue triangle) treatment prior to infection significantly reduced bacterial load 6 days post infection. No more changes in bacterial shedding compared to the control (black circle) were detected. Shedding ended in all three groups 24 days post infection. **(B)** Hormonally untreated mice yielded less IFUs/ swab after infection. Shedding ended at day 18 with no differences. (n=4, two-way ANOVA and pairwise t tests with Holm-Sidak correction for multiple testing *p<0.05, ****p<0.0001)

Doxycycline is one of the first-choice antimicrobials for treatment of *C. trachomatis* infections due to its high effectivity in acute infection. The tetracycline has a broad spectrum efficacy against Gram-positive and Gram-negative bacteria. Pretreatment with doxycycline did not alter the composition of vaginal microbiota in naturally cycling mice. In hormone-untreated mice, administration of doxycycline did not affect the composition of microbiota on phylum level, as Firmicutes were still present in high abundance (Figure 18 A). Correspondingly, enterococci remained high in abundance and the proportions did not change (Figure 22 A). Over time, low numbers of chlamydial reads were found 6 and 12 days post infection and not later. Vaginal microbiota was restored to their initial composition and proportions. Alpha diversity was fluctuating over time; however, it was apparently neither influenced by doxycycline treatment, nor by infection (Figure 18 B). Vaginal microbiota of mice pretreated with progesterone and doxycycline had a reduction in Firmicutes (Figure 18 C), specifically enterococci (Figure 22 B), prior to infection. High numbers of chlamydial reads were detected 6 to 18 days post infection. Afterwards, the microbiota retrieved to the incipient state. Pretreatment with hormone and antibiotics led to an increase in alpha diversity due to reduction of Firmicutes/ enterococci (Figure 18 D). Following infection, diversity was reduced and remained low over time.

Vaginal microbiota was unaffected by 3-day doxycycline treatment; additionally, no changes to the chlamydial infection were observed. If additionally pretreated with progesterone, Firmicutes/ enterococci were decreased and high abundance of *Chlamydia* was observed.

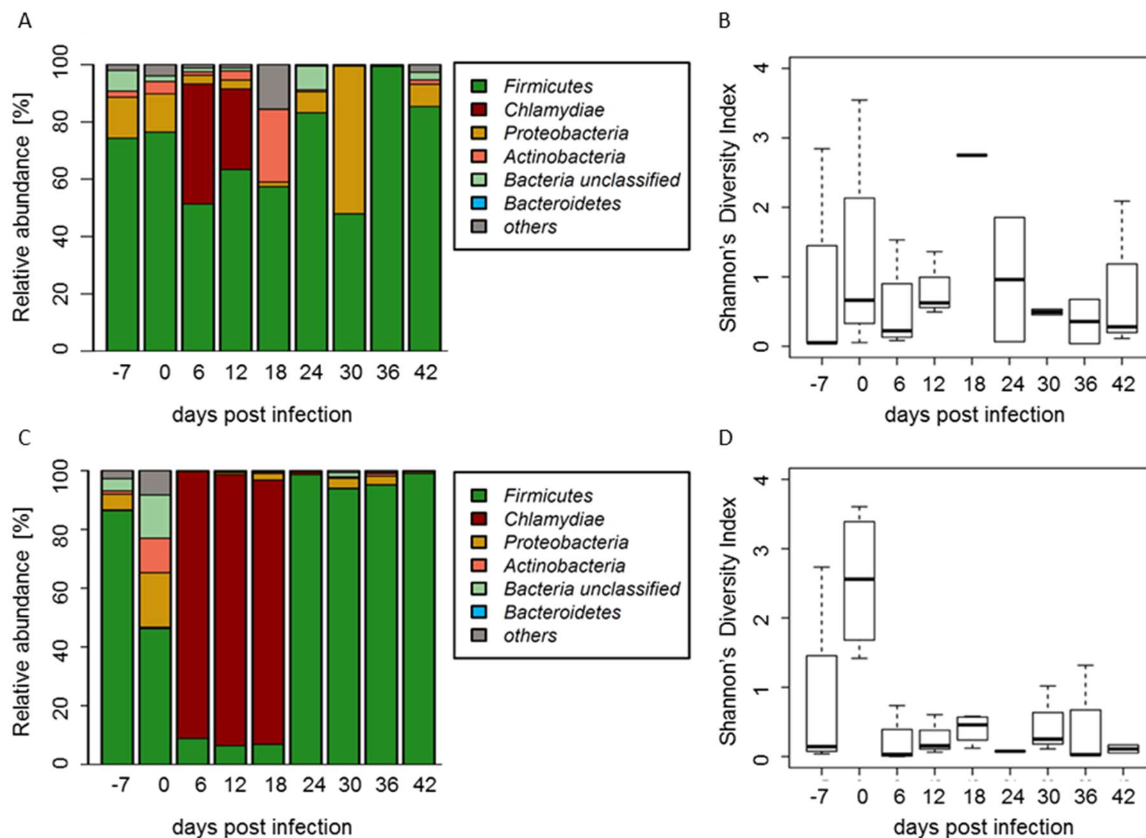

Figure 18: Doxycycline pretreatment did not alter vaginal microbiota and the course of infection. (A) Mice pretreated with doxycycline showed no differences in the composition and proportions of vaginal microbiota at day 0. Chlamydial reads were only detected 6 and 12 days post infection in low abundance. **(B)** Alpha diversity varied not significantly over the time. **(C)** Progesterone and doxycycline pretreatment led to a decrease in Firmicutes. Chlamydial abundance was high 6 to 18 days post infection, whereupon the initial state restored. **(D)** Due to pretreatment, alpha diversity was enhanced at day 0, which decreased post infection. Over time, diversity remained low. (n=1-4, subsampled to 1000 reads, Wilcoxon Rank Sum test with Continuity Correction and correction for multiple testing)

Ampicillin, belonging to the β-lactam antibiotics, has a broad antibacterial spectrum. It is effective against streptococci, enterococci, some *Clostridia* and *Neisseria* species. Pretreatment with ampicillin in hormone-untreated mice led to a reduction in Firmicutes abundance (Figure 19 A) and, accordingly, enterococci decreased while other genera increased (Figure 23 A). Over time, *Chlamydiae* were highly abundant. Ampicillin treatment further led to an increase in alpha diversity at day 0, which was decreased by infection with *C. muridarum* and fluctuated over time (Figure 19 B). Combining progesterone administration with ampicillin pretreatment also led to decreased Firmicutes (Figure 19 C) and enterococci (Figure 23 B) abundance at day 0. Thereby, diversity index was enhanced (Figure 19 D). Over time, *C. muridarum* propagated and chlamydial reads were highly abundant until day 24, whereupon the vaginal microbiota reversed to the initial condition. Alpha diversity was lowered by infection and fluctuated during the course of experiment.

Ampicillin pretreatment was effective in eradicating Firmicutes/ enterococci but did not enhance the infection as dynamically as progesterone administration prior to infection.

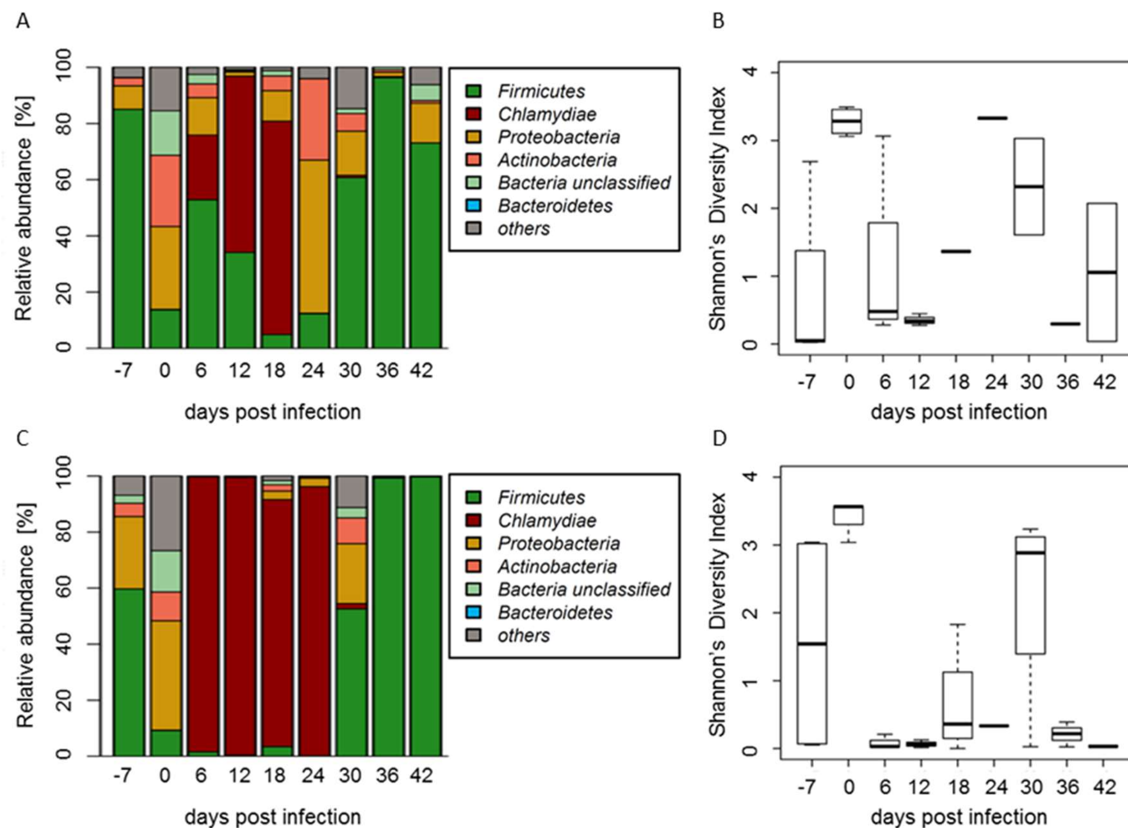

Figure 19: Ampicillin shifted vaginal microbiota to a progesterone-like composition. (A) Firmicutes were reduced by ampicillin pretreatment. Chlamydial reads were found 6-18 days post infection in high abundance. **(B)** Alpha diversity increased due to ampicillin pretreatment until day 0 and was reduced by infection with *C. muridarum*. Over time, the diversity fluctuates. **(C)** Mice treated with progesterone and pretreated with ampicillin had a reduced amount of Firmicutes compared to their initial state. *Chlamydiae* were highly abundant over the course of infection until day 24 post infection. **(D)** Alpha diversity was increased by double treatment and directly dropped after infection and thereafter remained low. (n=1-4, subsampled to 1000 reads, Wilcoxon Rank Sum test with Continuity Correction and correction for multiple testing)

5. Discussion

5.1 Sex hormones alter site specific microbiota

Sex hormones, such as β-estradiol and progesterone, are known regulators of immune cells in females, and thereby shape the host immune response. As of yet, it is uncertain to what extent sex hormones influence urogenital microbiota in humans; however, studies hint at the role of the menstrual cycle [Chaban *et al.*, 2014] and contraceptives in altering urogenital microbiota [Achilles *et al.*, 2018]. In contrast, the murine vaginal microbiota is rarely studied. Therefore, to begin, this study analyzed commensal vaginal microbiota and next the influence of the two commonly used sex hormones progesterone and β-estradiol on vaginal microbiota composition.

5.1.1 Commensal vaginal microbiota shape a stable vaginal milieu

The vaginal milieu is shaped by factors such as vaginal temperature and pH. In humans, the temperature is approximately 37 °C [Rodrigues *et al.*, 2009], which differs from the one found in mice, which is 33-35° C [Furr *et al.*, 1991]. Furthermore, vaginal pH fluctuates around 3.8 to 4.5 in humans [Ravel *et al.*, 2011, O'Hanlon *et al.*, 2013], and varies in a range of 7.2 to 7.4 in mice [Furr *et al.*, 1991]. These differences certainly shape unlike surroundings.

In healthy humans, the urogenital tract is colonized mainly by *Lactobacillus spp*. [Ravel *et al.*, 2011, Nunn *et al.*, 2015]. Besides a CST dominated by one single species, a diverse CST is described that is characterized by lacking a dominant species [Ravel *et al.*, 2011]. C57BL/6J mice, obtained from Janvier, housed and adjusted to our animal facility, were vaginally swabbed. Subsequently, DNA was isolated from the swabs, the V3/V4 region of the 16S rRNA gene amplified, and the prepared library sequenced on a MiSeq sequencer. The choice of hypervariable region to be sequenced is essential, as it has been recently shown that the V1/V2 region, which is commonly used in microbiota studies, misses bacteria which are crucial for the female urogenital tract, such as *Chlamydia* and *Gardnerella* [Graspeuntner *et al.*, 2018b]. Therefore, the V3/V4 region was utilized as it is more suitable to vaginal samples. Both in humans and in mice, the vaginal microbiota is mainly composed of Firmicutes (Figure 5 A); however, on genus level, microbiota differs. In humans, lactobacilli are highly abundant; whereas in mice, enterococci have the highest abundance and lactobacilli are only found in a minor fraction (Figure 5 B). Enterococci belong to the order of *Lactobacillales* and share their anaerobic and non-spore forming characteristics with lactobacilli. However, the production of lactic acid, a key feature of *Lactobacilli spp*., is not a characteristic of *Enterococci spp.* Therefore, the origin of the

neutral pH in mice is likely relying on lacking lactic acid production in the vagina. The proportions of bacterial genera fluctuate slightly over time, which may be dependent on the stage of estrous cycle, which was not determined in this corresponding study. Over time, alpha diversity is not stable, but varies around a comparable median if mice remain untreated (Figure 5 C). However, the mechanical intervention of swab sampling needs to be carefully considered. Narrow sampling intervals might lead to profound and detectable changes in microbial composition, as swabbing disrupts the commensal microbiota. Therefore, the long intervals of 6 to 7 days between the collections were chosen for our studies for microbiota to restore in a natural manner. In vaginal flushes, a high abundance of Firmicutes and Proteobacteria was also found by Barfod *et al.* using the V3/V4 hypervariable region [Barfod *et al.*, 2013]. In this thesis, *Halomonas* and *Shewanella* reads were accounted as contamination and removed from further analysis (see 3.9.2 [Wong *et al.*, 2017]). Thereby, the abundance of Proteobacteria is decreased in the samples, leading to difficulties for the direct comparison of our obtained and already published data [Barfod *et al.*, 2013]. Furthermore, differences between individual mice were observed, indicating variations caused by as of yet undetermined host characteristics. The estrous cycle or genetic factors are most likely responsible for variations.

The vaginal milieu, specifically temperature and pH, are independent of the estrous cycle [Furr *et al.*, 1991], and thereby offer a stable milieu for commensal bacteria. In C57BL/6 mice, the vaginal microbiota is mainly composed of Firmicutes/ enterococci, whose abundance is steady. The impact of sex hormones progesterone and β-estradiol on vaginal microbiota is so far undescribed.

5.1.2 Progesterone shifts murine vaginal epithelial cells to diestrous stage and induces dysbiosis within vaginal microbiota

The administration of progesterone to the animals is supposed to synchronize them to the same stage of estrous cycle, namely diestrous. Thereby, profound changes were induced to the epithelial cells [Furr *et al.*, 1991], as well as to the vaginal microbiota.

Vaginal epithelial cells of mice differ throughout the estrous cycle. During estrous, the vaginal mucosal is comparably thick and covered by a layer of cornified, flattened, highly eosinophilic and usually anuclear cells. Further, there is no infiltration of neutrophils to the epithelium or lamina propria [Scudamore, 2014]. This stage was found in all three tested mice on different days (Figure 6 A, B), which were naturally cycling and did not receive any treatment. In contrast, diestrous is characterized by a thinning of the epithelium. Neutrophils, which infiltrated the tissue during metestrous, gradually reduce and stay in the lamina propria. Epithelial cells begin to proliferate and the surface layer becomes mucified

at the end of diestrous [Scudamore, 2014]. Progesterone was administered subcutaneously to mice to induce diestrous. Characteristics described in literature could be found in histological examination of hematoxylin and eosin staining (Figure 6 C, D). A layer of mucus-producing goblet cells was found in all specimens. However, the occurrence of high numbers of neutrophils in the lamina propria was not visible. Differences between regular progesterone and the medroxyprogesterone acetate, which is a depot form administered for hormonal contraception, might be true for the influence on vaginal epithelial cells and thereby on estrous stage specific features.

Vaginal microbiota plays a crucial role in the defense against sexually transmitted pathogens, such as infections with *N. gonorrhoeae* and *C. trachomatis*. In humans, a high abundance of *Lactobacillus spp*. is described as protective via the production of lactic acid, lowering the pH [Ravel *et al.*, 2011], and bactericidal substances are in discussion. Besides sexual and socioeconomic behavior (e.g. [Brotman *et al.*, 2010, Fashemi *et al.*, 2013, Cools *et al.*, 2016]), the menstrual cycle effects microbiota as well [Chaban *et al.*, 2014], while it remains unknown whether directly or indirectly. Dysbiosis of microbiota is related to a higher probability of acquiring STDs in humans [Foschi *et al.*, 2017, Di Pietro *et al.*, 2018]. In mice, progesterone significantly reduces the relative abundance of Firmicutes 7 days post administration. Thereby, other phyla such as Proteobacteria, Actinobacteria, Bacteroidetes and, unclassified Bacteria were significantly enhanced (Figure 8 A). On genus level, the abundance of only enterococci is decreased, while other bacterial genera increase (Figure 8 B). Shifts in the composition are leading to a more diverse composition, confirmed by significantly increased alpha diversity calculated via Shannon's diversity index (Figure 8 C). Associating this state to the characteristics of a diverse CST, progesterone presumably induced a dysbiosis within the vaginal microbiota. As calculations are on a basis of relative abundance, there is no assertion of the absolute numbers of bacteria within the vagina. It was proven that the total number of bacteria is lowest in mice during diestrous [Noguchi *et al.*, 2003]. Therefore, the dysbiosed state 7 days post progesterone treatment may be at the basis of a reduction in total amount of bacteria, mostly enterococci, leading to an artificial increase in other genera in relative abundance.

Progesterone treatment leads to changes in epithelial cells, shifting them to a diestrous-like state. Additionally, progesterone reduces Firmicutes/ enterococci, thereby increasing alpha diversity of vaginal microbiota, which is creating a dysbiosis-like state. Together, these alterations could have an impact on the success on genital infections in mice.

5.1.3 β-estradiol stabilizes composition of vaginal microbiota

Administration of β-estradiol did not cause significant changes in the composition of vaginal microbiota on phylum level. Firmicutes were permanently on a high level within the microbiota, resembling the untreated state. Also on genus level, no significant changes were induced by β-estradiol; however, the abundance of enterococci slightly decreased whereas staphylococci increased (Figure 9 A, Figure 21 C). These shifts might be reasoned by the mechanical intervention of vaginal washes, which to an unknown extent could alter vaginal microbiota and epithelial cells. Neither alpha, nor beta diversity revealed changes of examined vaginal microbiota or between the two groups, respectively (Figure 9 C, D). Low alpha diversity hints at a homogenous sample composition with one genus being mainly abundant. Estradiol treatment leads to an onset of estrous stage 24 h post treatment and is observable up to 27 days [Furr *et al.*, 1991]. During estrous, which is initiated by high estrogen levels, the absolute number of bacteria in cultivation-based experiments is highest compared to other stages [Furr *et al.*, 1991, Noguchi *et al.*, 2003]. Correspondingly, samples from mice treated with β-estradiol had a low amount of contamination after first analysis. In a pilot study, we demonstrated that the amount of contamination is dependent on the biomass that was initially applied (data not shown). Vaginal samples from β-estradiol-treated mice were presumably of higher biomass than those from progesterone-treated mice. This was supported by Furr *et al.* who show that during estrous, the bacterial proliferation is enhanced in the vaginal tract. The authors find a possible explanation for this difference in the deposition of glycogen in the vagina, which occurs combined with cornification of the epithelium [Furr *et al.*, 1991]. In humans, the amount of glycogen correlates with a high abundance of lactobacilli and a low pH in high estrogen state [Miller *et al.*, 2016, Amabebe *et al.*, 2018]. During estrous and high estradiol levels, high glycogen levels may be advantageous for other bacteria than lactobacilli, not lowering the pH but still forming a protective barrier against invading pathogens.

β-estradiol administration stabilizes the microbiota composition, which is detectable in an untreated state. Characteristics are a constantly high abundance of Firmicutes and a low alpha diversity.

5.1.4 Gut microbiota is unaffected by hormonal state

Progesterone, but not β-estradiol, altered significantly the abundance of phyla/ genera and alpha as well as beta diversity of vaginal microbiota. As both hormones were administered with subcutaneous injection, they could have generalized effects on microbiota, and not only site specific effects. Therefore, gut microbiota was sequenced to determine effects of sex hormones on the composition and diversity.

Murine droppings were collected in parallel to vaginal swabs/ washes and similarly processed to obtain sequencing data of the V3/V4 region of the bacterial 16S rRNA gene, as previously described. Analysis of the gut microbiota revealed stable relative abundance of Bacteroidetes of around 60% over time, regardless of the hormonal treatment (Figure 10). In addition, when looking at longer sampling periods, there were no significant changes over time in the composition of gut microbiota on phylum and genus level, as well as alpha diversity (Figure 20). Administration of neither progesterone, nor β-estradiol altered the composition and diversity within gut microbiota of female mice. According to our results, it was recently demonstrated that the estrous cycle of female C57BL/6 mice does not significantly alter the intestinal microbial community [Wallace *et al.*, 2018]. Therefore, neither natural hormonal variations, nor exogenously supplied hormones alter the composition of gut microbiota. Even though hormones are not inducing changes in the composition, microbiota has been shown to be dependent on specific aspects of mouse husbandry. For example, alpha diversity was reduced if the chow used was irradiated, and the facility plays a major role for intestinal microbiota [Rausch *et al.*, 2016]. Interestingly, intrinsic factors such as hormones have no impact. At another site, the oropharynx, progesterone, and estradiol also did not induce changes to the amount of bacteria [Furr *et al.*, 1991]. It was shown that during estrous, the bacterial proliferation was enhanced in the vaginal tract, which was not found in the oropharynx [Furr *et al.*, 1991].

Progesterone is a potent modulator of vaginal microbiota composition, whereas β-estradiol appears to influence the biomass, but not the composition. However, both hormones are unable to induce changes in the gut and oropharyngeal microbiota in mice.

5.2 Progesterone enhances susceptibility to *C. muridarum* infection

Infections with *C. trachomatis* are a major health concern as it is the most common sexually transmitted infection worldwide. In mice, infections are apparently asymptomatic if they are infected vaginally with 10^6 IFUs *C. muridarum*. However, these infections lead to severe sequel such as formation of pathology and infertility [Swenson *et al.*, 1983, de la Maza *et al.*, 1994]. Microenvironmental and influencing factors are so far unknown, and the elucidation of either supportive or impeding factors is of interest to modulate the infection.

5.2.1 Immune response towards *C. muridarum* infection is elevated by progesterone, while macrophages are constantly diminished

Progesterone is a known modulator of immune responses at diverse mucosal sites [Hall *et al.*, 2017]. Therefore, the local immune response within the digested uterine tissue was analyzed. Since there are no differences between the abundance of resident and infiltrating immune cells in ovaries and uterine tissue, they can be considered equal [Lijek *et al.*, 2018]. Hence, in this study, solely uterine tissue was digested for single cell suspension and subsequent flow cytometric analysis of immune cell abundance and infiltration.

Progesterone significantly reduced the local abundance of $CD45^+$ cells in the uterine tissue of mice compared to untreated mice (Figure 7). CD45, called leucocyte common antigen (LCA), is expressed on all nucleated hematopoietic cells, including leucocytes and lymphocytes. A first assumption on the presence of immune cells can be rendered by CD45 staining. Over the course of infection, $CD45^+$ cells remained low in untreated mice, whereas progesterone-treated mice had an increase in $CD45^+$ cells 7 days post infection (Figure 14). This immune response towards genital infection with *C. muridarum* was further analyzed by more detailed analysis of immune cell subsets. To distinguish immune cell populations, T cells were determined by CD3e antibody. Neither $CD3e^+$/ $CD4^+$, nor $CD3e^+$/ $CD8a^+$ cells were present in high numbers after progesterone treatment, and did not differ in comparison to naturally cycling mice. It is known that $CD4^+$ and $CD8^+$ T cells are present during and after infection but not resident in the naïve uterine tissue [Johnson *et al.*, 2016]. Post infection, the amount of $CD4^+$ T cells increased drastically, whereas untreated mice remain low in T cell abundance. $CD4^+$ T cells are a major component in the clearance of genital infections with *Chlamydiae*, but the exact mechanism remains unknown [Gondek *et al.*, 2012]. However, authors suggest that the IFN-γ production is a major component [Mosolygo *et al.*, 2014]. To a lesser extent, $CD8a^+$ cells were upregulated 3 and 14 days post infection in progesterone-treated mice. It is known that $CD8^+$ T cells are not involved in chlamydial clearance, but rather oviduct pathology via TNF-α production [Murthy *et al.*, 2011, Vlcek *et al.*, 2016]. A higher abundance of $CD3e^+$/$CD8a^+$ T cells in the uterine horns might be

indicative for ensuing pathology formation, which is true for solely progesterone-treated mice compared to a low level of $CD3e^+/CD8a^+$ cells in untreated mice.

The abundance of macrophages ($F4/80^+$) was significantly decreased by progesterone treatment compared to untreated mice (Figure 7). Untreated mice had a higher abundance of macrophages in the uterine tissue, which was stable over the course of infection. In progesterone-treated mice, the macrophage abundance remained lower in comparison to untreated mice and dropped slightly 14 days post infection (Figure 14). Within the mouse uterus, macrophages are found in the endometrium and myometrium [De *et al.*, 1990]. Immunohistochemical analysis of the murine uterus revealed no quantitative differences between the stages of the estrous cycle. Ovariectomy of naturally cycling mice resulted in a reduction of uterine macrophages, which could be restored with administration of estrogen and progesterone [De *et al.*, 1990]. Hormones are distinct regulators of uterine macrophages. Progesterone is thereby a negative regulator of macrophages by reducing the migration into the uterus and impairing effector molecule production [Hunt *et al.*, 1998]. Corresponding to our findings, progesterone significantly decreases macrophages after administration and keeps the abundance low over the course of infection. Furthermore, the high number of macrophages in naturally cycling mice is supporting their key role in the rapid defense against *C. muridarum* infection, as no immune response developed. Granulocytes, specifically neutrophils (Ly6G+, Lymphocyte antigen 6 complex locus G6D), were low in abundance and remained unchanged by hormone treatment (Figure 7); they are also known to infiltrate infected tissue, but not as being resident in uterine tissue [Johnson *et al.*, 2016]. We could also show that granulocytes infiltrated the uterine tissue 7 days post infection in progesterone-treated animals (Figure 14). Macrophages and neutrophils are main producers of IL-1β, which is a factor in shortening the chlamydial clearance, but on the other hand mediating oviduct pathology [Prantner *et al.*, 2009]. Similarly, it was shown that neutrophils are upregulated following challenge with *C. trachomatis* serovar D, and are of importance for the development of pathology but negligible in clearance of the pathogen [Lijek *et al.*, 2018]. The early upregulation of granulocytes in our study might be an indication for the participation in chlamydial clearance. However, as it was not tested at later time points, there is no data on the presence of granulocytes regarding pathology formation.

Progesterone significantly decreases $CD45^+$ cells and macrophages prior to infection. Naturally cycling mice have a weak immune response towards genital challenge with *C. muridarum*; whereas in progesterone-treated mice, the infection provokes a strong immune response. The significant difference of macrophages between the two groups is of interest, as uterine macrophages might play a role in early clearance of the pathogen.

5.2.2. Progesterone-induced dysbiosis is advantageous for *C. muridarum*

Prior to infection, progesterone reduced the relative abundance of Firmicutes/ enterococci. If *C. muridarum* is inoculated into the murine vagina, *Chlamydia* propagate and 6 days post infection there are more than 95% of all reads belonging to Chlamydiae/ *Chlamydia*. This high abundance remains stable for approximately 24 days. Afterwards, chlamydial reads were no longer detected in the samples, fitting the bacterial shedding curve. Following ended shedding; vaginal microbiota recovers and returns to the initial state with high abundance of Firmicutes. The increase in diversity caused by progesterone fits to the diverse CST, which occurs in women in a dysbiosed state [Ravel *et al.*, 2011]. Vaginal dysbiosis is connected with bacterial vaginosis, which is a vaginal syndrome in women. Furthermore, BV and the acquisition of STDs has been studied on *N. gonorrhoeae* and *C. trachomatis* [Wiesenfeld *et al.*, 2003], and others proved women with BV being more likely to become infected with sexually transmitted pathogens [Allsworth *et al.*, 2011]. Diverse microbiota is composed of an increased amount of anaerobic bacteria, of which some are known producers of indole. Some chlamydial species are able to convert indole to tryptophan; however, in a surrounding of non-indole producers, *Chlamydia* starve [Ziklo *et al.*, 2016a]. Further, the activation of IFN-γ plays an important role in immune response against *Chlamydia* and intracellular tryptophan metabolism. *C. trachomatis* is highly susceptible to IFN-γ response in mice, as they express p47 GTPases that are responsible for clearance. Due to co-evolution, *C. muridarum* has evolved an escape mechanism to p47 GTPases [Nelson *et al.*, 2005]. Further to be considered is the absolute amount of bacteria in progesterone-treated animals. They are artificially set in diestrous by progesterone administration. During diestrous, there is less bacteria in the vaginal tract of mice than during estrous [Noguchi *et al.*, 2003], which might be another explanation for the enhanced infectious success during progesterone-induced diestrous.

Progesterone enhances diversity prior to infection, comparable to a vaginal dysbiosis, which is beneficial for *C. muridarum*. The amount and genus of bacteria are presumably important factors to form a physical barrier to infections.

5.2.3 Progesterone induces severe pathology formation following chlamydial infection

The profound changes in immune response and vaginal microbiota caused by progesterone were further regarded concerning the bacterial shedding and pathology formation. These readouts give an insight into the success and outcome of genital chlamydial infection.

Recovery assays are conducted from the material of vaginal swabs/ washes to determine bacterial shedding. In untreated mice, the shedding was low and ended early (Figure 11).

Further, these mice did not develop any pathology (Figure 12). These results resemble those of humans, whose general course of chlamydial infection is asymptomatic and self-limiting [Geisler, 2010]. In contrast, the chlamydial load in progesterone-treated mice increased within the first days post infection, and *C. muridarum* was shed within 28 days. Other research groups commonly publish this course of infection as well (e.g. [O'Connell *et al.*, 2007, Ramsey *et al.*, 2009]). However, shedding also depends on the inoculated chlamydial strain; as mice infected with human isolates of *C. trachomatis* have a low and fast bacterial shedding [Gondek *et al.*, 2012, Yang *et al.*, 2017]. There is likely a relation of high and long shedding and pathology formation. Thereby, the characteristic formation of hydrosalpinx, an accumulation of clear fluid caused by distal occlusion in the fallopian tubes, was scored by size comparison to the ovary (3.5.6, [Yang *et al.*, 2014]). If treated with progesterone, mice developed hydrosalpinx in more than 60% of all cases, mostly with the highest score of 4. Occurrence of hydrosalpinx was either uni- or bilateral, but as of yet there is no explanation for this phenomenon. Shah *et al.* showed that oviduct occlusion appears after infection with *C. muridarum* and correlates with hydrosalpinx development and infertility [Shah *et al.*, 2005]. Furthermore, it was shown that *C. muridarum* leads to inflammation and dilatation of the glandular ducts as it can further infect glandular epithelial cells [Sun *et al.*, 2015]. C57BL/6J mice are highly susceptible to pathology formation and direct infection of the uterus horn enhanced glandular ducts, dilatation and hydrosalpinx occurrence [Sun *et al.*, 2015]. Macroscopic evaluation is a final readout but does not give insights into inflammation of the tissue, as swelling and redness are hard to evaluate due to individual differences in size of the urogenital tract. Therefore, experienced pathologists are needed for further microscopic evaluation and for pathology scoring of stained tissue sections.

Progesterone-induced alterations in vaginal microbiota and immune response make mice more susceptible to infection with *C. muridarum*. The impact of vaginal epithelial cells, Firmicutes/ enterococci and macrophages is thereby not clearly determinable. β-estradiol and antibiotic pretreatment were used to specifically alter single involved components to assess their role in chlamydial infection.

5.3 Alteration of either immune cells or vaginal microbiota is not sufficient for modulation of chlamydial infection

5.3.1 β-estradiol reduces leucocytes prior to infection but does not change course and outcome of *C. muridarum* infection

Mice were treated with β-estradiol, and 7 days later, uteri were examined with flow cytometry to determine immune cell subsets. Local abundance of $CD45^+$ cells was

significantly reduced when compared to untreated mice (Figure 7). This downregulation is comparable to progesterone-dependent reduction of $CD45^+$ cells. However, the abundance of macrophages ($F4/80^+$) in β-estradiol-treated mice was comparable to untreated mice and significantly higher than in progesterone-treated. Accordingly, it has been shown that estrogen is a positive regulator of uterine macrophages, creating an active macrophage profile [Hunt *et al.*, 1998]. T cells and granulocytes are low in uterine tissue [Johnson *et al.*, 2016], which fits the observed data, as $CD3e^+/CD4^+$, $CD3e^+/CD8a^+$, and $Ly6G^+$ cells were all not regulated by β-estradiol treatment. However, Henderson *et al.* describe uterine natural killer (NK) cells, which are a unique subset in humans and mice. Their function is mainly controlled by estradiol and glucocorticoids, supporting their highest abundance in the mid-late secretory phase of the menstrual cycle [Henderson *et al.*, 2003]. Surface markers to detect these uterine NK cells need to be further included in upcoming analysis.

Vaginal microbiota was mainly composed of Firmicutes/ enterococci, which remained stable, even after β-estradiol administration. *C. muridarum* propagated poorly, and only a few reads were detected by sequencing 6 to 18 days post infection (Figure 13). The microbiota, however, changed due to inoculation of the pathogen in their abundance and composition on genus level, which was reversible over time. Corresponding, Neuendorf *et al*. showed that the inoculation of *C. caviae* to the vaginal tract of guinea pigs leads to alterations of the abundance of bacterial phylotypes but not the composition of vaginal microbiota [Neuendorf *et al.*, 2015].

Bacterial shedding post infection was low and had an average duration of 21 days, which was comparable to normally cycling mice. Due to the poor infection success, mice did not develop any pathology if untreated. Few β-estradiol-treated mice developed small pathologies. Besides these few occurrences, urogenital tracts were macroscopically indistinguishable from uninfected control or hormone-untreated infected ones and did not display any sign of inflammation, such as swelling or redness.

Untreated and β-estradiol-treated mice model the general course of chlamydial infection in humans, such as being asymptomatic and self-limiting [Geisler, 2010]. The administration of β-estradiol does not affect microbiota, but rather the number of leucocytes. A high abundance of Firmicutes/ enterococci might have other protective features than solely lowering the pH like lactobacilli. Further, estradiol has been shown to have generalized anti-inflammatory properties, as the formulation of 17-β-estradiol was protective for mice with warm ischemia/reperfusion injury, most likely because of an increased serum nitric oxide and decreased TNF-α, which was additionally measured [Eckhoff *et al.*, 2002]. These factors might favor the protective vaginal milieu and less severe course of chlamydial infections.

5.3.2 Ampicillin diminishes Firmicutes which has no impact on chlamydial infection

Chlamydial infections are commonly treated with doxycycline or azithromycin in the clinics. In pregnancy, amoxicillin is recommended for treatment. However, up to 8% treatment failures are reported [Horner, 2006]. *In vitro* studies on *C. trachomatis* infection revealed that the efficacy of doxycycline and azithromycin are reduced in low oxygen, so-called hypoxic, condition [Shima *et al.*, 2011], which occur physiologically in the female urogenital tract [Juul *et al.*, 2007]. The efficacy of doxycycline, azithromycin, and amoxicillin was tested against *C. muridarum in vitro* (Figure 15). Doxycycline and azithromycin both were efficient against *C. muridarum* with a concentration of 1 µg and 10 µg, respectively. Amoxicillin (10-50 µg), however, could not eradicate *Chlamydia*. These inclusions were shaped differently when compared to the control and visually resembled the state of persistence. Other β-lactam antibiotics have been shown to induce persistence *in vitro* in occulargenital *Chlamydia* in clinically relevant concentrations [Kintner *et al.*, 2014]. In this state, *Chlamydia* is viable but non-infectious; yet they can be recovered after stressor-removal.

All antibiotics were tested in 8-week old C57BL/6J mice against *C. muridarum* infection following the regular protocol of progesterone administration 7 days prior to infection. Treatment started 24 h post infection (Figure 16). Doxycycline was efficient, as no *Chlamydia* were recovered, which has also been shown by others, regardless if administration began immediately or 3 days post infection [Su *et al.*, 1999]. Azithromycin treatment led to a few recoverable IFUs 14 and 21 days post infection from two and one mice, respectively. Treatment of mice with azithromycin was already shown to carry a treatment failure rate of 9% if mice were treated after productive infection with *C. muridarum* Weiss [Phillips-Campbell *et al.*, 2014]. Amoxicillin reduced the bacterial burden significantly within the first week post infection, but further the course is comparable to the untreated infection control. BALB/c mice, which were administered amoxicillin orally, have a high shedding compared to untreated animals, but the antibiotic-induced persistence in *C. muridarum*. This was confirmed by transmission electron microscopy of uterine tissue, which revealed the appearance of aberrant bodies that are not visible in the controls [Phillips Campbell *et al.*, 2012]. The authors further validated the efficacy of azithromycin against amoxicillin-induced persistence in *C. muridarum* Weiss, which leads to an increase in treatment failure to 22% [Phillips-Campbell *et al.*, 2014]. The newly obtained data supports doxycycline as the most efficient antibiotic against genital chlamydial infections *in vivo*. However, the usage of other antibiotics, such as azithromycin and especially amoxicillin, needs to be reconsidered in spite of their restricted efficacy in murine genital infection with *C. muridarum*.

Doxycycline and ampicillin are commonly used in the clinics against several bacterial infections at many sites. Thereby, generalized changes of microbiota are induced, potentially also in the vaginal microbiota. Whether or not this might have an effect on the acquisition of sexually transmitted infections is not yet well studied. Further, the influence of sex hormones on antibiotic efficacy and the infectious success was tested. Therefore, antibiotics were administered intraperitoneally once daily for 3 consecutive days and a following 3 days washout phase to prevent direct interaction of the antibiotic and the pathogen. Doxycycline has a broad spectrum of efficacy, eradicating Gram-positive and Gram-negative bacteria. However, doxycycline did not induce changes to the vaginal microbiota prior to infection, either in naturally cycling, or in progesterone-treated mice (Figure 18). Previously, doxycycline has been shown to decrease diversity and richness in fecal microbiota of female mice, causing dysbiosis by mainly reducing the abundance of Firmicutes and thereby increasing Bacteroidetes [Becker *et al.*, 2017, Boynton *et al.*, 2017]. Progesterone decreased the relative abundance of Firmicutes/ enterococci as shown before, and *Chlamydia* was able to propagate in the dysbiosed microenvironment. The infection was comparable to regular infection without antibiotic treatment. In contrast, administration of ampicillin increased the alpha diversity post administration by reduction of Firmicutes/ enterococci, and the resulting composition on day 0 was similar to progesterone-treated animals (Figure 19). Ampicillin also effectively alters the murine gut microbiota composition [Ceylani *et al.*, 2018], as it significantly and immediately diminished bacterial species richness and diversity [Le Bastard *et al.*, 2018]. Castro-Mejía *et al.* suggest a suppression of the majority and elimination of only a few gut microbiota members with temporary ampicillin treatment in mice [Castro-Mejia *et al.*, 2018]. However, there was no additive effect of progesterone and ampicillin treatment on the reduction of Firmicutes/ enterococci in the vaginal microbiota. Even though chlamydial IFUs were inoculated into a comparable microbial environment, the course of infection differed. In mice pretreated with ampicillin, *C. muridarum* could hardly propagate and a few chlamydial reads were found, corresponding to a low shedding, which was comparable to shedding of doxycycline pretreated and antibiotic untreated animals. Nevertheless, changes within the murine vaginal microbiota were not sufficient enough to enhance chlamydial shedding and prolong it. However, treatment strategy might be shifted to prolonged and oral administration, possibly inducing enhanced changes within the microbiota. So far, it has been shown that doxycycline prevents protective immunity in successfully treated mice [Su *et al.*, 1999]. Further, it would be of interest whether antibiotic administration has an impact on primary immune response and if so, to what extent.

5.4 Conclusion

Progesterone administration to female mice leads to changes in the vaginal tissue composition, reduction in Firmicutes/ enterococci and significant decrease of leucocytes, specifically macrophages. Thereby, the susceptibility to vaginal infections with *C. muridarum* is enhanced, leading to severe pathology.

This study provides insights into the mode of action of progesterone in murine vaginal microbiota and immune cells before and during chlamydial infection, which was previously incoherently studied. We show a site-specific effect of progesterone on the composition of vaginal microbiota, whereas gut-derived remain unaffected by systemic progesterone treatment. The progesterone-initiated decrease of the genus *Enterococcus* leads to enhanced alpha diversity, thereby creating a state of dysbiosis. In contrast, mere reduction of enterococci via antibiotic treatment is not sufficient for a significant increase in course and outcome of chlamydial infection. This dysbiosis of the vaginal microbiota is beneficial to infections with *C. muridarum*; however, it appears to express its impact only in coherence with alterations of immune response. Sex hormones are well known to shape host immune response towards several infections at various mucosal sites. The reduction in total leucocytes by β-estradiol appears insufficient for an effect on chlamydial infection. An additional significant decrease of macrophages by progesterone is needed to enhance chlamydial infection. Macrophages have so far been rarely studied during *C. muridarum* infection; thus, their role in clearance of the pathogen could be more important than suggested. The interplay of commensal microbiota and resident immune cells is pivotal for the host's susceptibility to avert infections, especially genital infections with *C. muridarum*.

Taken together, this work emphasizes the role of sex hormones as major regulators of immune response towards infection, as well as vaginal epithelial cells and vaginal microbiota. The combination of alterations induced by progesterone is necessary to avert chlamydial infections *in vivo*.

5.5 Outlook

The following studies will contribute to progress in the (i) investigation of the role of immune cells in chlamydial infection; (ii) exploring the impact of anaerobic cultivation of murine vaginal samples to characterize bacterial species and produced metabolites; (iii) establishment of the clinical application using microbiota data.

The established mouse model can be used for studies of infections with *C. muridarum*, and to a lesser degree, *C. trachomatis*. Progesterone is a potent modulator of immune responses; however, the underlying mechanisms of the involved immune cells protecting

from chlamydial infections are still not completely understood. Cell populations of interest are uterus specific T cell subsets, such as uNK cells, as well as resident macrophages. Their role can be examined using different approaches, including depletion of target populations or specific knock-out mice strains. Furthermore, host-signaling modulation within immune cells could give an insight into the function of distinct factors contributing to propagation or aversion of infections. *In vitro* studies revealed hypoxia-inducible factor-1α (HIF-1α) plays a pivotal role in chlamydial infection. As the vaginal tract is hypoxic, it would be of interest to examine a knock-out of HIF-1α in myeloid cells to better gain insights into the role of oxygen during infection. To better characterize and treat infections it is important to elucidate underlying mechanisms in the host-pathogen interplay.

NGS opened possibilities to detect new and more bacteria in all colonized compartments of the body. However, NGS reveals limited information about the functionality and behavior of those bacteria. Anaerobic cultivation of so far uncultivated bacteria, especially from the urogenital tract, is important to gain new insights to antagonizing and propagating effects in a bacterial community. Further, possible advantageous or disadvantageous bacteria could be used to specifically modify the vaginal microbiota to elaborate their role in chlamydial co-infection models. Specific bacterial strains can be transferred back to mice, which were either hormonally or antibiotically treated, to determine the interplay of bacteria and pathogen. If bacteria were cultivated, metabolic analysis could be performed to further characterize important metabolites, such as tryptophan, which commensal bacteria, as well as pathogens, rely on. Furthermore, cultivation of bacteria would enhance missing taxonomic knowledge of murine commensal bacteria. Thereby, databases could be extended to species level, as to date there are only a few available. With the help of such databases, the specific assessment of information about the composition of microbiota on species level would facilitate the elucidation of the role of single bacteria within the community.

As of yet, data on the role of progesterone in humans remains sparse. Therefore, a cohort of women diagnosed clinically infertile with a wish for a child could be recruited, because they are commonly treated with progesterone to enhance their chance of conception. Prior and post treatment, cervical swabs can be collected and via NGS the microbiota determined. These results can be used for suggestions of how to use microbiota as a diagnostic tool and further the impact of the hormone state to protect from possible sexually transmitted infections.

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7. Appendix

7.1 Supplementary material

7.1.1 Sequences of V3/V4 primers

All primers used for the amplification of the V3/V4 region of the bacterial 16S rRNA gene for paired-end-sequencing on a MiSeq sequencer.

Table 16: List of V3/V4 sequencing primers

Primer	Nucleotide sequence (5'-3')
V3F_Seq	TATGGTAATT GG CCTACGGGAGGCAGCAG
V4R_Seq	AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
Index_V3V4	ATTAGAWACCCBDGTAGTCC GG CTGACTGACT
hV3F_MID_1	AATGATACGGCGACCACCGAGATCTACAC ATCGTACG ACACTCTTTC CCTACACGACGCTCTTCCGATCT CCTACGGGAGGCAGCAG
hV3F_MID_2	AATGATACGGCGACCACCGAGATCTACAC ACTATCTG ACACTCTTTCC CTACACGACGCTCTTCCGATCT T CCTACGGGAGGCAGCAG
hV3F_MID_3	AATGATACGGCGACCACCGAGATCTACAC TAGCGAGT ACACTCTTTC CCTACACGACGCTCTTCCGATCT GT CCTACGGGAGGCAGCAG
hV3F_MID_4	AATGATACGGCGACCACCGAGATCTACAC CTGCGTGT ACACTCTTTC CCTACACGACGCTCTTCCGATCT CGA CCTACGGGAGGCAGCAG
hV3F_MID_5	AATGATACGGCGACCACCGAGATCTACAC TCATCGAG ACACTCTTTC CCTACACGACGCTCTTCCGATCT ATGA CCTACGGGAGGCAGCAG
hV3F_MID_6	AATGATACGGCGACCACCGAGATCTACAC CGTGAGTG ACACTCTTTC CCTACACGACGCTCTTCCGATCT TGCGA CCTACGGGAGGCAGCAG
hV3F_MID_7	AATGATACGGCGACCACCGAGATCTACAC GGATATCT ACACTCTTTC CCTACACGACGCTCTTCCGATCT GAGTGG CCTACGGGAGGCAGCAG
hV3F_MID_8	AATGATACGGCGACCACCGAGATCTACAC GACACCGT ACACTCTTTC CCTACACGACGCTCTTCCGATCT CCTACGGGAGGCAGCAG
hV3F_MID_9	AATGATACGGCGACCACCGAGATCTACAC CTACTATA ACACTCTTTCC CTACACGACGCTCTTCCGATCT T CCTACGGGAGGCAGCAG
hV3F_MID_10	AATGATACGGCGACCACCGAGATCTACAC CGTTACTA ACACTCTTTCC CTACACGACGCTCTTCCGATCT GT CCTACGGGAGGCAGCAG
hV3F_MID_11	AATGATACGGCGACCACCGAGATCTACAC AGAGTCAC ACACTCTTTC CCTACACGACGCTCTTCCGATCT CGA CCTACGGGAGGCAGCAG
hV3F_MID_12	AATGATACGGCGACCACCGAGATCTACAC TACGAGAC ACACTCTTTC CCTACACGACGCTCTTCCGATCT ATGA CCTACGGGAGGCAGCAG
hV3F_MID_13	AATGATACGGCGACCACCGAGATCTACAC ACGTCTCG ACACTCTTTC CCTACACGACGCTCTTCCGATCT TGCGA CCTACGGGAGGCAGCAG
hV3F_MID_14	AATGATACGGCGACCACCGAGATCTACAC TCGACGAG ACACTCTTTC CCTACACGACGCTCTTCCGATCT GAGTGG CCTACGGGAGGCAGCAG

hV3F_MID_15	AATGATACGGCGACCACCGAGATCTACAC GATCGTGT ACACTCTTTC CCTACACGACGCTCTTCCGATCT CCTACGGGAGGCAGCAG
hV3F_MID_16	AATGATACGGCGACCACCGAGATCTACAC GTCAGATA ACACTCTTTC CCTACACGACGCTCTTCCGATCT T CCTACGGGAGGCAGCAG
hV3F_MID_17	AATGATACGGCGACCACCGAGATCTACAC ACGACGTG ACACTCTTTC CCTACACGACGCTCTTCCGATCT GT CCTACGGGAGGCAGCAG
hV3F_MID_18	AATGATACGGCGACCACCGAGATCTACAC CGTCGCTA ACACTCTTTC CCTACACGACGCTCTTCCGATCT CGA CCTACGGGAGGCAGCAG
hV3F_MID_19	AATGATACGGCGACCACCGAGATCTACAC GCTCTAGT ACACTCTTTC CCTACACGACGCTCTTCCGATCT ATGA CCTACGGGAGGCAGCAG
hV3F_MID_20	AATGATACGGCGACCACCGAGATCTACAC TGCGTACG ACACTCTTTC CCTACACGACGCTCTTCCGATCT TGCGA CCTACGGGAGGCAGCAG
hV4R_MID_A	CAAGCAGAAGACGGCATACGAGAT AACTCTCG GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT GGACTACHVGGGTWTCTAAT
hV4R_MID_B	CAAGCAGAAGACGGCATACGAGAT ACTATGTC GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT A GGACTACHVGGGTWTCTAAT
hV4R_MID_C	CAAGCAGAAGACGGCATACGAGAT AGTAGCGT GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT TC GGACTACHVGGGTWTCTAAT
hV4R_MID_D	CAAGCAGAAGACGGCATACGAGAT CAGTGAGT GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT CTA GGACTACHVGGGTWTCTAAT
hV4R_MID_E	CAAGCAGAAGACGGCATACGAGAT CGTACTCA GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT GATA GGACTACHVGGGTWTCTAAT
hV4R_MID_F	CAAGCAGAAGACGGCATACGAGAT CTACGCAG GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT ACTCA GGACTACHVGGGTWTCTAAT
hV4R_MID_G	CAAGCAGAAGACGGCATACGAGAT GGAGACTA GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT TTCTCT GGACTACHVGGGTWTCTAAT
hV4R_MID_H	CAAGCAGAAGACGGCATACGAGAT GTCGCTCG GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT GGACTACHVGGGTWTCTAAT
hV4R_MID_I	CAAGCAGAAGACGGCATACGAGAT GTCGTAGT GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT A GGACTACHVGGGTWTCTAAT
hV4R_MID_J	CAAGCAGAAGACGGCATACGAGAT TAGCAGAC GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT TC GGACTACHVGGGTWTCTAAT
hV4R_MID_K	CAAGCAGAAGACGGCATACGAGAT TCATAGAC GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT CTA GGACTACHVGGGTWTCTAAT
hV4R_MID_L	CAAGCAGAAGACGGCATACGAGAT TCGCTATA GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT GATA GGACTACHVGGGTWTCTAAT
hV4R_MID_M	CAAGCAGAAGACGGCATACGAGAT AAGTCGAG GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT ACTCA GGACTACHVGGGTWTCTAAT
hV4R_MID_N	CAAGCAGAAGACGGCATACGAGAT ATACTTCG GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT TTCTCT GGACTACHVGGGTWTCTAAT
hV4R_MID_O	CAAGCAGAAGACGGCATACGAGAT CATAGAGA GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT GGACTACHVGGGTWTCTAAT
hV4R_MID_P	CAAGCAGAAGACGGCATACGAGAT CGTAGATC GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT A GGACTACHVGGGTWTCTAAT

hV4R_MID_Q	CAAGCAGAAGACGGCATACGAGAT GCGCACGT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT TC GGACTACHVGGGTWTCTAAT
hV4R_MID_R	CAAGCAGAAGACGGCATACGAGAT GGTACTAT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTA GGACTACHVGGGTWTCTAAT
hV4R_MID_S	CAAGCAGAAGACGGCATACGAGAT TACGAGCA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT GATA GGACTACHVGGGTWTCTAAT
hV4R_MID_T	CAAGCAGAAGACGGCATACGAGAT TCAGCGTT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT ACTCA GGACTACHVGGGTWTCTAAT
hV4R_MID_U	CAAGCAGAAGACGGCATACGAGAT AGCTGCTA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT TTCTCT GGACTACHVGGGTWTCTAAT
hV4R_MID_V	CAAGCAGAAGACGGCATACGAGAT CTCGTTAC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT GGACTACHVGGGTWTCTAAT
hV4R_MID_W	CAAGCAGAAGACGGCATACGAGAT GTATACGC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT A GGACTACHVGGGTWTCTAAT
hV4R_MID_X	CAAGCAGAAGACGGCATACGAGAT TCGCTACG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT TC GGACTACHVGGGTWTCTAAT

7.2 Supplementary data

7.2.1 Representative list of all genus level classifications from stool samples using the V3/V4 region

Table 17: OTUs assessed by V3/V4 region sequencing in stool samples

OTU	Read Number	Taxonomy
Otu001	1769763	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);
Otu002	2457786	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);
Otu003	134810	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiales_unclassified(100);
Otu004	96298	Bacteria_unclassified(100);Bacteria_unclassified(100); Bacteria_unclassified(100);Bacteria_unclassified(100);
Otu005	119699	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Bacteroidales_unclassified(100);
Otu006	239969	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);Barnesiella(100);
Otu007	260318	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Rikenellaceae(100);Alistipes(100);
Otu008	92522	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);
Otu009	153516	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Clostridium_XlVa(100);
Otu010	36670	Bacteroidetes(100);Bacteroidetes_unclassified(100); Bacteroidetes_unclassified(100);Bacteroidetes_unclassified(100);
Otu011	36159	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Prevotellaceae(100);
Otu012	25930	Firmicutes(100);Firmicutes_unclassified(100); Firmicutes_unclassified(100);Firmicutes_unclassified(100);
Otu013	74112	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Bacteroidaceae(100);Bacteroides(100);
Otu014	85267	Verrucomicrobia(100);Verrucomicrobiae(100); Verrucomicrobiales(100);Verrucomicrobiaceae(100); Akkermansia(100);
Otu015	82733	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);Odoribacter(100);
Otu016	10436	Proteobacteria(100);Proteobacteria_unclassified(100); Proteobacteria_unclassified(100);Proteobacteria_unclassified(100);
Otu017	20235	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Oscillibacter(100);

Otu018	5919	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Rikenellaceae(100);
Otu019	69853	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Prevotellaceae(100);Alloprevotella(100);
Otu020	186353	Proteobacteria(100);Deltaproteobacteria(100); Desulfovibrionales(100);Desulfovibrionaceae(100);
Otu021	3925	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Prevotellaceae(100);Hallella(100);
Otu022	32424	Firmicutes(100);Bacilli(100);Lactobacillales(100); Lactobacillaceae(100);Lactobacillus(100);
Otu023	7983	Proteobacteria(100);Deltaproteobacteria(100); Deltaproteobacteria_unclassified(100); Deltaproteobacteria_unclassified(100);
Otu024	12414	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Butyricicoccus(100);
Otu025	9682	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Flavonifractor(100);
Otu026	12358	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Allobaculum(100);
Otu027	9943	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Turicibacter(100);
Otu028	7253	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Pseudoflavonifractor(100);
Otu029	7305	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Clostridium_XlVb(100);
Otu030	6387	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Ruminococcus(100);
Otu031	105738	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Prevotellaceae(100);Prevotella(100);
Otu032	1550	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiaceae_1(100);
Otu033	2834	Firmicutes(100);Clostridia(100);Clostridia_unclassified(100); Clostridia_unclassified(100);
Otu034	6829	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Roseburia(100);
Otu035	3891	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);Parabacteroides(100);
Otu036	1571	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Marvinbryantia(100);
Otu037	5064	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Anaerotruncus(100);
Otu038	3565	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Clostridium_IV(100);

Otu039	7812	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Sutterellaceae(100);Parasutterella(100);
Otu040	5301	Proteobacteria(100);Deltaproteobacteria(100);Desulfovibrionales(100);Desulfovibrionales_unclassified(100);
Otu041	25276	Proteobacteria(100);Betaproteobacteria(100);Rhodocyclales(100);Rhodocyclaceae(100);Methyloversatilis(100);
Otu042	1189	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100);Erysipelotrichaceae(100);
Otu043	13977	Firmicutes(100);Clostridia(100);Clostridiales(100);Clostridiaceae_1(100);Clostridium_sensu_stricto(100);
Otu044	1550	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Dorea(100);
Otu045	475	Proteobacteria(100);Betaproteobacteria(100);Betaproteobacteria_unclassified(100);Betaproteobacteria_unclassified(100);
Otu046	1391	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Ruminococcus2(100);
Otu047	414	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Robinsoniella(100);
Otu048	400	Firmicutes(100);Bacilli(100);Lactobacillales(100);Lactobacillales_unclassified(100);
Otu049	4595	Proteobacteria(100);Alphaproteobacteria(100);Alphaproteobacteria_unclassified(100);Alphaproteobacteria_unclassified(100);
Otu050	10655	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Oxalobacteraceae(100);Herbaspirillum(100);
Otu051	4186	Tenericutes(100);Mollicutes(100);Anaeroplasmatales(100);Anaeroplasmataceae(100);Anaeroplasma(100);
Otu052	10929	Proteobacteria(100);Gammaproteobacteria(100);Oceanospirillales(100);Halomonadaceae(100);Halomonas(100);
Otu053	2881	Firmicutes(100);Clostridia(100);Clostridiales(100);Peptostreptococcaceae(100);Clostridium_XI(100);
Otu054	298	Firmicutes(100);Clostridia(100);Clostridiales(100);Peptostreptococcaceae(100);
Otu055	755	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Burkholderiales_unclassified(100);
Otu056	442	Actinobacteria(100);Actinobacteria(100);Coriobacteriales(100);Coriobacteriaceae(100);
Otu057	1437	Actinobacteria(100);Actinobacteria(100);Bifidobacteriales(100);Bifidobacteriaceae(100);Bifidobacterium(100);
Otu058	215	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnoanaerobaculum(100);
Otu059	527	Firmicutes(100);Clostridia(100);Clostridiales(100);Eubacteriaceae(100);Eubacterium(100);

Otu060	159	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiaceae_1(100);Anaerobacter(100);
Otu061	164	Firmicutes(100);Bacilli(100);Bacilli_unclassified(100); Bacilli_unclassified(100);
Otu062	7150	Proteobacteria(100);Gammaproteobacteria(100); Alteromonadales(100);Shewanellaceae(100);Shewanella(100);
Otu063	154	Actinobacteria(100);Actinobacteria(100);Bifidobacteriales(100); Bifidobacteriaceae(100);
Otu064	239	Candidatus_Saccharibacteria(100); Candidatus_Saccharibacteria_unclassified(100); Candidatus_Saccharibacteria_unclassified(100); Candidatus_Saccharibacteria_unclassified(100);
Otu065	149	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Acetitomaculum(100);
Otu066	139	Firmicutes(100);Bacilli(100);Lactobacillales(100); Lactobacillaceae(100);
Otu067	3154	Proteobacteria(100);Deltaproteobacteria(100); Bdellovibrionales(100);Bdellovibrionaceae(100);Vampirovibrio(100);
Otu068	132	Actinobacteria(100);Actinobacteria(100);Coriobacteriales(100); Coriobacteriaceae(100);Enterorhabdus(100);
Otu069	590	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Sutterellaceae(100);
Otu070	85	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Coprococcus(100);
Otu071	80	Proteobacteria(100);Gammaproteobacteria(100); Alteromonadales(100);Alteromonadales_unclassified(100);
Otu072	629	Proteobacteria(100);Alphaproteobacteria(100);Rhodospirillales(100); Rhodospirillales_unclassified(100);
Otu073	91	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100); Johnsonella(100);
Otu074	65	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiaceae_1(100);Anaerosporobacter(100);
Otu075	84	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Acetanaerobacterium(100);
Otu076	61	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);Tannerella(100);
Otu077	2532	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);Sediminibacterium(100);
Otu078	61	Proteobacteria(100);Gammaproteobacteria(100); Oceanospirillales(100);Halomonadaceae(100);

Otu079	65	Firmicutes(100);Clostridia(100);Clostridiales(100); Eubacteriaceae(100);Anaerofustis(100);
Otu080	47	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Lactonifactor(100);
Otu081	50	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Anaerostipes(100);
Otu082	32	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Shuttleworthia(100);
Otu083	1066	Proteobacteria(100);Alphaproteobacteria(100);Rhodospirillales(100); Rhodospirillaceae(100);
Otu084	778	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);Hydrotalea(100);
Otu085	30	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Hydrogenoanaerobacterium(100);
Otu086	24	Firmicutes(100);Clostridia(100);Clostridiales(100); Peptostreptococcaceae(100);Sporacetigenium(100);
Otu087	35	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Clostridium_XVIII(100);
Otu088	22	Proteobacteria(100);Deltaproteobacteria(100); Desulfovibrionales(100);Desulfovibrionaceae(100);Bilophila(100);
Otu089	27	Actinobacteria(100);Actinobacteria(100);Coriobacteriales(100); Coriobacteriaceae(100);Olsenella(100);
Otu090	20	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);
Otu091	275	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Acidovorax(100);
Otu092	17	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Moryella(100);
Otu093	38	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Gemmiger(100);
Otu094	303	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Lachnospira(100);
Otu095	460	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadaceae(100); Sphingomonas(100);
Otu096	12	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Syntrophococcus(100);
Otu097	38	Firmicutes(100);Bacilli(100);Bacillales(100); Staphylococcaceae(100);Staphylococcus(100);
Otu098	10	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Hespellia(100);

Otu099	10	Actinobacteria(100);Actinobacteria(100); Actinobacteria_unclassified(100);Actinobacteria_unclassified(100);
Otu100	382	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiaceae(100);Cupriavidus(100);
Otu101	198	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Bradyrhizobiaceae(100);Bradyrhizobium(100);
Otu102	13	Firmicutes(100);Bacilli(100);Lactobacillales(100); Streptococcaceae(100);Streptococcus(100);
Otu103	158	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Schlegelella(100);
Otu104	7	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Blautia(100);
Otu105	146	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Bradyrhizobiaceae(100);Bosea(100);
Otu106	7	Proteobacteria(100);Gammaproteobacteria(100); Gammaproteobacteria_unclassified(100); Gammaproteobacteria_unclassified(100);
Otu107	77	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Moraxellaceae(100);Enhydrobacter(100);
Otu108	165	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Microbacteriaceae(100);Microbacterium(100);
Otu109	7	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Oribacterium(100);
Otu110	59	Proteobacteria(100);Gammaproteobacteria(100); Pasteurellales(100);Pasteurellaceae(100);Haemophilus(100);
Otu111	76	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);Undibacterium(100);
Otu112	6	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100); Escherichia/Shigella(100);
Otu113	53	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Actinomycetaceae(100);Actinomyces(100);
Otu114	4	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiales_Incertae_Sedis_XIII(100);Anaerovorax(100);
Otu115	4	Verrucomicrobia(100);Verrucomicrobiae(100); Verrucomicrobiales(100);Verrucomicrobiaceae(100);
Otu116	4	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Sporobacter(100);
Otu117	96	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Curvibacter(100);
Otu118	69	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Moraxellaceae(100);Acinetobacter(100);
Otu119	33	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Methylobacteriaceae(100);Methylobacterium(100);

Otu120	8	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Microbacteriaceae(100);
Otu121	96	Proteobacteria(100);Alphaproteobacteria(100);Rhodospirillales(100); Acetobacteraceae(100);Roseomonas(100);
Otu122	3	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiaceae(100);
Otu123	3	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Butyrivibrio(100);
Otu124	87	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Rhizobiaceae(100);Rhizobium(100);
Otu125	5	Firmicutes(100);Bacilli(100);Bacillales(100); Staphylococcaceae(100);Jeotgalicoccus(100);
Otu126	30	Proteobacteria(100);Betaproteobacteria(100); Burkholderiales(100);Comamonadaceae(100);Variovorax(100);
Otu127	6	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);Pantoea(100);
Otu128	2	Firmicutes(100);Negativicutes(100);Selenomonadales(100); Veillonellaceae(100);Veillonella(100);
Otu129	6	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Corynebacteriaceae(100);Corynebacterium(100);
Otu130	4	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);
Otu131	29	Bacteroidetes(100);Sphingobacteriia(100); Sphingobacteriales(100);Sphingobacteriaceae(100); Mucilaginibacter(100);
Otu132	2	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Catonella(100);
Otu133	2	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Parasporobacterium(100);
Otu134	2	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Bradyrhizobiaceae(100);
Otu135	2	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadaceae(100);
Otu136	2	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Rikenellaceae(100);Rikenella(100);
Otu137	2	Firmicutes(100);Bacilli(100);Lactobacillales(100); Carnobacteriaceae(100);Atopostipes(100);
Otu138	2	Firmicutes(100);Bacilli(100);Lactobacillales(100); Enterococcaceae(100);Enterococcus(100);
Otu139	2	Proteobacteria(100);Alphaproteobacteria(100); Caulobacterales(100);Caulobacteraceae(100); Phenylobacterium(100);
Otu140	31	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Brevibacteriaceae(100);Brevibacterium(100);

Otu141	1	Proteobacteria(100);Betaproteobacteria(100); Burkholderiales(100);Comamonadaceae(100);
Otu142	1	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Prevotellaceae(100) ;Paraprevotella(100);
Otu143	1	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiaceae_1(100);Sarcina(100);
Otu144	1	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Papillibacter(100);
Otu145	1	Proteobacteria(100);Betaproteobacteria(100);Neisseriales(100); Neisseriaceae(100);Neisseria(100);
Otu146	1	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100); Enterobacter(100);
Otu147	1	Bacteroidetes(100);Sphingobacteriia(100); Sphingobacteriales(100);Chitinophagaceae(100);
Otu148	21	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Micrococcaceae(100);Micrococcus(100);
Otu149	1	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Actinomycetaceae(100);Mobiluncus(100);
Otu150	1	Proteobacteria(100);Alphaproteobacteria(100); Rhodospirillales(100);Rhodospirillaceae(100);Insolitispirillum(100);
Otu151	10	Proteobacteria(100);Betaproteobacteria(100); Methylophilales(100);Methylophilaceae(100);
Otu152	1	Firmicutes(100);Clostridia(100);Clostridiales(100); Defluviitaleaceae(100);Defluviitalea(100);
Otu153	1	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Coprobacillus(100);
Otu154	26	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Micrococcaceae(100);
Otu155	1	Firmicutes(100);Bacilli(100);Bacillales(100); Staphylococcaceae(100);
Otu156	1	Firmicutes(100);Clostridia(100);Clostridiales(100); Eubacteriaceae(100);
Otu157	1	Proteobacteria(100);Deltaproteobacteria(100); Bdellovibrionales(100);Bdellovibrionales_unclassified(100);
Otu158	1	Proteobacteria(100);Deltaproteobacteria(100); Desulfovibrionales(100);Desulfovibrionaceae(100);Lawsonia(100);
Otu159	1	Fusobacteria(100);Fusobacteriia(100);Fusobacteriales(100); Leptotrichiaceae(100);Leptotrichia(100);
Otu160	46	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Propionibacteriaceae(100);Propionibacterium(100);

Otu161	1	Proteobacteria(100);Gammaproteobacteria(100); Oceanospirillales(100);Oceanospirillales_unclassified(100);
Otu162	1	Firmicutes(100);Bacilli(100);Bacillales(100);Bacillales_unclassified (100);

7.2.2 Representative list of all genus level classification from vaginal samples using the V3/V4 region

Table 18: OTUs assessed by sequencing of V3/V4 region of vaginal samples

OTU	Read number	Taxonomy
Otu001	1824407	Firmicutes(100);Bacilli(100);Lactobacillales(100); Enterococcaceae(100);Enterococcus(100);
Otu002	533281	Firmicutes(100);Bacilli(100);Bacillales(100);Staphylococcaceae(100); Staphylococcus(100);
Otu003	335643	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadaceae(100); Sphingomonas(100);
Otu004	6964	Firmicutes(100);Bacilli(100);Lactobacillales(100); Enterococcaceae(100);
Otu005	5677	Firmicutes(100);Bacilli(100);Lactobacillales(100); Lactobacillales_unclassified(100);
Otu006	218795	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100); Escherichia/Shigella(100);
Otu007	4384	Proteobacteria(100);Gammaproteobacteria(100); Oceanospirillales(100);Halomonadaceae(100);
Otu008	180359	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);Massilia(100);
Otu009	44249	Bacteria_unclassified(100);Bacteria_unclassified(100); Bacteria_unclassified(100);Bacteria_unclassified(100);
Otu010	13332	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);
Otu011	3280	Proteobacteria(100);Gammaproteobacteria(100); Alteromonadales(100);Alteromonadales_unclassified(100);
Otu012	43100	Firmicutes(100);Bacilli(100);Bacillales(100); Bacillales_unclassified(100);
Otu013	34847	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Pseudomonadaceae(100); Pseudomonas(100);
Otu014	59450	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);Hydrotalea(100);

Otu015	156573	Firmicutes(100);Bacilli(100);Lactobacillales(100); Lactobacillaceae(100);Lactobacillus(100);
Otu016	27505	Proteobacteria(100);Betaproteobacteria(100);Rhodocyclales(100); Rhodocyclaceae(100);Methyloversatilis(100);
Otu017	5817	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadaceae(100);
Otu018	14255	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Schlegelella(100);
Otu019	34584	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Phyllobacteriaceae(100);Phyllobacterium(100);
Otu020	2604	Proteobacteria(100);Gammaproteobacteria(100); Gammaproteobacteria_unclassified(100); Gammaproteobacteria_unclassified(100);
Otu021	19139	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Acidovorax(100);
Otu022	8837	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);
Otu023	20155	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Curvibacter(100);
Otu024	1843	Proteobacteria(100);Alphaproteobacteria(100); Alphaproteobacteria_unclassified(100); Alphaproteobacteria_unclassified(100);
Otu025	15707	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);Sediminibacterium(100);
Otu026	12451	Proteobacteria(100);Alphaproteobacteria(100);Caulobacterales(100); Caulobacteraceae(100);Phenylobacterium(100);
Otu027	583	Firmicutes(100);Bacilli(100);Lactobacillales(100); Lactobacillaceae(100);
Otu028	9773	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);
Otu029	16526	Actinobacteria(100);Actinobacteria(100);Coriobacteriales(100); Coriobacteriaceae(100);Olsenella(100);
Otu030	12865	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Propionibacteriaceae(100);Propionibacterium(100);
Otu031	774	Firmicutes(100);Bacilli(100);Bacilli_unclassified(100); Bacilli_unclassified(100);
Otu032	620	Firmicutes(100);Bacilli(100);Bacillales(100);Staphylococcaceae(100);
Otu033	10269	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Moraxellaceae(100);Enhydrobacter(100);
Otu034	7373	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Moraxellaceae(100);Acinetobacter(100);

Otu035	9310	Firmicutes(100);Bacilli(100);Lactobacillales(100); Streptococcaceae(100);Streptococcus(100);
Otu036	7906	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);Undibacterium(100);
Otu037	682	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Pseudomonadaceae(100);
Otu038	2718	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);
Otu039	17390	Firmicutes(100);Bacilli(100);Lactobacillales(100); Carnobacteriaceae(100);Atopostipes(100);
Otu040	8722	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Alcaligenaceae(100);Alcaligenes(100);
Otu041	8111	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Brevibacteriaceae(100);Brevibacterium(100);
Otu042	15301	Firmicutes(100);Bacilli(100);Bacillales(100);Staphylococcaceae(100); Jeotgalicoccus(100);
Otu043	1599	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);
Otu044	2614	Parcubacteria(100);Parcubacteria_unclassified(100); Parcubacteria_unclassified(100);Parcubacteria_unclassified(100);
Otu045	4575	Candidatus_Saccharibacteria_unclassified(100); Candidatus_Saccharibacteria_unclassified(100);
Otu046	3366	Bacteroidetes(100);Bacteroidetes_unclassified(100); Bacteroidetes_unclassified(100);Bacteroidetes_unclassified(100);
Otu047	3748	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Xanthomonadaceae(100);
Otu048	5373	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Variovorax(100);
Otu049	5245	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiaceae(100);Burkholderia(100);
Otu050	4802	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiales_incertae_sedis(100);Aquabacterium(100);
Otu051	1124	Chlamydiae(100);Chlamydiia(100);Chlamydiales(100); Chlamydiaceae(100);Chlamydia(100);
Otu052	6510	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Methylobacteriaceae(100);Methylobacterium(100);
Otu053	4063	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Corynebacteriaceae(100);Corynebacterium(100);
Otu054	3039	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);Barnesiella(100);
Otu055	3540	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Bradyrhizobiaceae(100);
Otu056	8247	Firmicutes(100);Bacilli(100);Bacillales(100);Bacillaceae_2(100);

Otu057	1752	Proteobacteria(100);Proteobacteria_unclassified(100); Proteobacteria_unclassified(100);Proteobacteria_unclassified(100);
Otu058	5817	Actinobacteria(100);Actinobacteria(100);Bifidobacteriales(100); Bifidobacteriaceae(100);Bifidobacterium(100);
Otu059	2544	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Bacteroidaceae(100);Bacteroides(100);
Otu060	5066	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Bradyrhizobiaceae(100);Bosea(100);
Otu061	2254	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100); Flavobacteriaceae(100);Flavobacterium(100);
Otu062	1632	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);
Otu063	3915	Firmicutes(100);Bacilli(100);Bacillales(100);Planococcaceae(100);
Otu064	1701	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);
Otu065	3258	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Xanthomonadaceae(100); Stenotrophomonas(100);
Otu066	3965	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Blautia(100);
Otu067	4893	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Bradyrhizobiaceae(100);Bradyrhizobium(100);
Otu068	4699	Firmicutes(100);Clostridia(100);Clostridiales(100); Peptostreptococcaceae(100);Clostridium_XI(100);
Otu069	1978	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiales_unclassified(100);
Otu070	1682	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Actinomycetales_unclassified(100);
Otu071	1396	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Microbacteriaceae(100);
Otu072	1018	Proteobacteria(100);Betaproteobacteria(100); Betaproteobacteria_unclassified(100); Betaproteobacteria_unclassified(100);
Otu073	2326	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Allobaculum(100);
Otu074	282	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadales_unclassified(100);
Otu075	640	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Rhizobiales_unclassified(100);
Otu076	304	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiales_unclassified(100);

Otu077	1333	Proteobacteria(100);Gammaproteobacteria(100);Pasteurellales(100); Pasteurellaceae(100);Haemophilus(100);
Otu078	1457	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadaceae(100); Sphingobium(100);
Otu079	93	Proteobacteria(100);Gammaproteobacteria(100); Oceanospirillales(100);Oceanospirillales_unclassified(100);
Otu080	151	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);Naxibacter(100);
Otu081	384	Firmicutes(100);Firmicutes_unclassified(100); Firmicutes_unclassified(100);Firmicutes_unclassified(100);
Otu082	1029	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Sinobacteraceae(100);
Otu083	1701	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Prevotellaceae(100);Prevotella(100);
Otu084	439	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Bacteroidales_unclassified(100);
Otu085	2030	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Roseburia(100);
Otu086	1234	Proteobacteria(100);Gammaproteobacteria(100);Pasteurellales(100); Pasteurellaceae(100);
Otu087	757	Microgenomates(100);Microgenomates_unclassified(100); Microgenomates_unclassified(100); Microgenomates_unclassified(100);
Otu088	1187	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Actinomycetaceae(100);Actinomyces(100);
Otu089	1917	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Micrococcaceae(100);Micrococcus(100);
Otu090	2224	Proteobacteria(100);Alphaproteobacteria(100);Rhodobacterales(100); Rhodobacteraceae(100);Paracoccus(100);
Otu091	1649	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Microbacteriaceae(100);Microbacterium(100);
Otu092	1101	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100); Flavobacteriaceae(100);Chryseobacterium(100);
Otu093	1057	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Micrococcaceae(100);
Otu094	1622	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Micrococcaceae(100);Kocuria(100);
Otu095	1601	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiaceae_1(100);Clostridium_sensu_stricto(100);
Otu096	488	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Clostridium_XIVa(100);
Otu097	1356	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Pelomonas(100);

Otu098	445	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);Parabacteroides(100);
Otu099	598	Firmicutes(100);Negativicutes(100);Selenomonadales(100); Veillonellaceae(100);Veillonella(100);
Otu100	410	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Prevotellaceae(100);
Otu101	4580	Firmicutes(100);Bacilli(100);Bacillales(100);Planococcaceae(100); Sporosarcina(100);
Otu102	975	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Polaromonas(100);
Otu103	491	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Xanthomonadaceae(100); Pseudoxanthomonas(100);
Otu104	682	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Sphingobacteriaceae(100);Mucilaginibacter(100);
Otu105	1454	Firmicutes(100);Bacilli(100);Bacillales(100);Bacillaceae_1(100); Bacillus(100);
Otu106	517	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiales_incertae_sedis(100);Sphaerotilus(100);
Otu107	1020	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Micrococcaceae(100);Rothia(100);
Otu108	1202	Verrucomicrobia(100);Verrucomicrobiae(100); Verrucomicrobiales(100);Verrucomicrobiaceae(100); Akkermansia(100);
Otu109	440	Actinobacteria(100);Actinobacteria(100);Acidimicrobiales(100); Acidimicrobiales_unclassified(100);
Otu110	453	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Moraxellaceae(100);
Otu111	436	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Sphingobacteriales_unclassified(100);
Otu112	580	Actinobacteria(100);Actinobacteria(100);Acidimicrobiales(100); Acidimicrobiaceae(100);Ilumatobacter(100);
Otu113	566	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadaceae(100); Sphingopyxis(100);
Otu114	501	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Rikenellaceae(100);Alistipes(100);
Otu115	514	Actinobacteria(100);Actinobacteria(100);Gaiellales(100); Gaiellaceae(100);Gaiella(100);
Otu116	502	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Coprococcus(100);

Otu117	361	Proteobacteria(100);Betaproteobacteria(100);Hydrogenophilales(100); Hydrogenophilaceae(100);Petrobacter(100);
Otu118	430	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100); Ruminococcus(100);
Otu119	707	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Nocardioidaceae(100);Nocardioides(100);
Otu120	736	Proteobacteria(100);Alphaproteobacteria(100);Caulobacterales(100); Caulobacteraceae(100);Brevundimonas(100);
Otu121	240	Bacteroidetes(100);Cytophagia(100);Cytophagales(100); Cytophagales_unclassified(100);
Otu122	540	Actinobacteria(100);Actinobacteria(100);Solirubrobacterales(100); Solirubrobacterales_unclassified(100);
Otu123	120	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Micrococcaceae(100);Renibacterium(100);
Otu124	776	Firmicutes(100);Bacilli(100);Bacillales(100); Bacillales_Incertae_Sedis_XI(100);Gemella(100);
Otu125	697	Proteobacteria(100);Deltaproteobacteria(100);Bdellovibrionales(100); Bdellovibrionaceae(100);Bdellovibrio(100);
Otu126	348	Proteobacteria(100);Deltaproteobacteria(100);Myxococcales(100); Myxococcales_unclassified(100);
Otu127	434	Proteobacteria(100);Gammaproteobacteria(100); Aeromonadales(100);Aeromonadaceae(100);Aeromonas(100);
Otu128	383	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Dermabacteraceae(100);Brachybacterium(100);
Otu129	420	Proteobacteria(100);Alphaproteobacteria(100);Rhodobacterales(100); Rhodobacteraceae(100);
Otu130	143	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Oscillibacter(100);
Otu131	38	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiaceae_1(100);
Otu132	273	Acidobacteria(100);Acidobacteria_Gp6(100);Gp6(100); Gp6_unclassified(100);
Otu133	506	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100); Flavobacteriaceae(100);Cloacibacterium(100);
Otu134	373	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Butyricicoccus(100);
Otu135	237	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);Enterobacter(100);
Otu136	684	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);Duganella(100);

Otu137	32	Firmicutes(100);Clostridia(100);Clostridiales(100); Peptostreptococcaceae(100);
Otu138	456	Proteobacteria(100);Alphaproteobacteria(100);Caulobacterales(100); Caulobacteraceae(100);
Otu139	707	Proteobacteria(100);Betaproteobacteria(100);Hydrogenophilales(100); Hydrogenophilaceae(100);Thiobacillus(100);
Otu140	1443	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Nocardiaceae(100);Gordonia(100);
Otu141	578	Firmicutes(100);Bacilli(100);Lactobacillales(100); Carnobacteriaceae(100);Granulicatella(100);
Otu142	410	Bacteroidetes(100);Cytophagia(100);Cytophagales(100); Cytophagaceae(100);Hymenobacter(100);
Otu143	386	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100); Cryomorphaceae(100);
Otu144	376	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Clostridium_IV(100);
Otu145	550	Proteobacteria(100);Betaproteobacteria(100);Methylophilales(100); Methylophilaceae(100);
Otu146	303	Proteobacteria(100);Betaproteobacteria(100);Rhodocyclales(100); Rhodocyclaceae(100);
Otu147	373	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Prevotellaceae(100);Alloprevotella(100);
Otu148	493	Firmicutes(100);Bacilli(100);Bacillales(100);Bacillaceae_1(100); Geobacillus(100);
Otu149	624	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadaceae(100); Novosphingobium(100);
Otu150	260	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Alcaligenaceae(100);
Otu151	299	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Comamonas(100);
Otu152	726	Acidobacteria(100);Acidobacteria_Gp3(100);Gp3(100); Gp3_unclassified(100);
Otu153	567	SR1(100);SR1_unclassified(100);SR1_unclassified(100); SR1_unclassified(100);
Otu154	422	Proteobacteria(100);Deltaproteobacteria(100);Bdellovibrionales(100); Bdellovibrionaceae(100);Vampirovibrio(100);
Otu155	350	Bacteroidetes(100);Cytophagia(100);Cytophagales(100); Cytophagaceae(100);
Otu156	367	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Sutterellaceae(100);Parasutterella(100);

Otu157	617	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Micrococcaceae(100);Arthrobacter(100);
Otu158	246	Verrucomicrobia(100);Subdivision3(100); Subdivision3_unclassified(100);Subdivision3_unclassified(100);
Otu159	411	Acidobacteria(100);Acidobacteria_Gp4(100);Gp4(100); Gp4_unclassified(100);
Otu160	356	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Anaerostipes(100);
Otu161	1229	Proteobacteria(100);Alphaproteobacteria(100);Rhodobacterales(100); Rhodobacteraceae(100);Amaricoccus(100);
Otu162	263	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);Ferruginibacter(100);
Otu163	479	Firmicutes(100);Bacilli(100);Lactobacillales(100); Leuconostocaceae(100);Weissella(100);
Otu164	208	Proteobacteria(100);Deltaproteobacteria(100); Deltaproteobacteria_unclassified(100); Deltaproteobacteria_unclassified(100);
Otu165	149	Actinobacteria(100);Actinobacteria(100); Actinobacteria_unclassified(100);Actinobacteria_unclassified(100);
Otu166	23	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiaceae(100);
Otu167	420	Bacteroidetes(100);Cytophagia(100);Cytophagales(100); Cytophagaceae(100);Arcicella(100);
Otu168	116	Proteobacteria(100);Deltaproteobacteria(100);Myxococcales(100); Kofleriaceae(100);Kofleria(100);
Otu169	269	Bacteroidetes(100);Cytophagia(100);Cytophagales(100); Cytophagaceae(100);Emticicia(100);
Otu170	349	Proteobacteria(100);Alphaproteobacteria(100);Rhodospirillales(100); Acetobacteraceae(100);Roseomonas(100);
Otu171	472	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);Herbaspirillum(100);
Otu172	80	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Flavonifractor(100);
Otu173	167	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100); Flavobacteriaceae(100);
Otu174	179	Tenericutes(100);Mollicutes(100);Acholeplasmatales(100); Acholeplasmataceae(100);Acholeplasma(100);
Otu175	343	Proteobacteria(100);Alphaproteobacteria(100);Rhodobacterales(100); Rhodobacteraceae(100);Rubellimicrobium(100);
Otu176	171	Firmicutes(100);Bacilli(100);Bacillales(100);Listeriaceae(100); Brochothrix(100);

Otu177	324	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Sphingobacteriaceae(100);Pedobacter(100);
Otu178	296	Proteobacteria(100);Deltaproteobacteria(100); Desulfovibrionales(100);Desulfovibrionaceae(100);
Otu179	278	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Turicibacter(100);
Otu180	205	Gemmatimonadetes(100);Gemmatimonadetes(100); Gemmatimonadales(100);Gemmatimonadaceae(100); Gemmatimonas(100);
Otu181	213	Proteobacteria(100);Gammaproteobacteria(100);Legionellales(100); Coxiellaceae(100);Aquicella(100);
Otu182	502	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiales_Incertae_Sedis_XI(100);Anaerococcus(100);
Otu183	210	Latescibacteria(100);Latescibacteria_unclassified(100); Latescibacteria_unclassified(100);Latescibacteria_unclassified(100);
Otu184	198	Acidobacteria(100);Acidobacteria_Gp16(100);Gp16(100); Gp16_unclassified(100);
Otu185	210	Firmicutes(100);Bacilli(100);Lactobacillales(100); Aerococcaceae(100);Aerococcus(100);
Otu186	343	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Intrasporangiaceae(100);
Otu187	255	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Pseudomonadaceae(100);Cellvibrio(100);
Otu188	204	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiales_incertae_sedis(100);Inhella(100);
Otu189	293	Firmicutes(100);Bacilli(100);Lactobacillales(100); Carnobacteriaceae(100);Trichococcus(100);
Otu190	155	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);Klebsiella(100);
Otu191	335	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiales_Incertae_Sedis_XI(100);Peptoniphilus(100);
Otu192	47	Actinobacteria(100);Actinobacteria(100);Coriobacteriales(100); Coriobacteriaceae(100);
Otu193	294	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);Serratia(100);
Otu194	608	Firmicutes(100);Bacilli(100);Lactobacillales(100); Streptococcaceae(100);Lactococcus(100);
Otu195	196	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Faecalibacterium(100);
Otu196	487	Firmicutes(100);Bacilli(100);Bacillales(100);Planococcaceae(100); Ureibacillus(100);

Otu197	604	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);Citrobacter(100);
Otu198	260	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);Janthinobacterium(100);
Otu199	165	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Nakamurellaceae(100);Nakamurella(100);
Otu200	577	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Rhizobiaceae(100);Rhizobium(100);
Otu201	16	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Pseudomonadales_unclassified(100);
Otu202	412	Fusobacteria(100);Fusobacteriia(100);Fusobacteriales(100); Fusobacteriaceae(100);Fusobacterium(100);
Otu203	948	Firmicutes(100);Bacilli(100);Lactobacillales(100); Aerococcaceae(100);Facklamia(100);
Otu204	164	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Nocardiaceae(100);Rhodococcus(100);
Otu205	366	Proteobacteria(100);Epsilonproteobacteria(100); Campylobacterales(100);Campylobacteraceae(100);Arcobacter(100);
Otu206	196	Proteobacteria(100);Gammaproteobacteria(100);Legionellales(100); Coxiellaceae(100);Diplorickettsia(100);
Otu207	111	Chlamydiae(100);Chlamydiia(100);Chlamydiales(100); Chlamydiales_unclassified(100);
Otu208	158	Proteobacteria(100);Deltaproteobacteria(100);Bdellovibrionales(100); Bacteriovoracaceae(100);
Otu209	31	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Propionibacteriaceae(100);
Otu210	206	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Pseudonocardiaceae(100);Actinomycetospora(100);
Otu211	141	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Albidiferax(100);
Otu212	102	Chlamydiae(100);Chlamydiia(100);Chlamydiales(100); Parachlamydiaceae(100);
Otu213	75	Chloroflexi(100);Chloroflexi_unclassified(100); Chloroflexi_unclassified(100);Chloroflexi_unclassified(100);
Otu214	32	Acidobacteria(100);Acidobacteria_Gp3(100); Acidobacteria_Gp3_unclassified(100); Acidobacteria_Gp3_unclassified(100);
Otu215	337	Proteobacteria(100);Alphaproteobacteria(100);Rhodospirillales(100); Acetobacteraceae(100);
Otu216	260	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);Pantoea(100);

Otu217	**152**	Proteobacteria(100);Gammaproteobacteria(100);Legionellales(100); Legionellaceae(100);Legionella(100);
Otu218	95	Proteobacteria(100);Gammaproteobacteria(100); Oceanospirillales(100);Oceanospirillaceae(100);Marinospirillum(100);
Otu219	116	Bacteroidetes(100);Cytophagia(100);Cytophagales(100); Cytophagaceae(100);Dyadobacter(100);
Otu220	15	Firmicutes(100);Bacilli(100);Lactobacillales(100); Carnobacteriaceae(100);
Otu221	14	Proteobacteria(100);Gammaproteobacteria(100); Alteromonadales(100);Pseudoalteromonadaceae(100);Algicola(100);
Otu222	292	Firmicutes(100);Negativicutes(100);Selenomonadales(100); Veillonellaceae(100);Dialister(100);
Otu223	156	Verrucomicrobia(100);Verrucomicrobiae(100); Verrucomicrobiales(100);Verrucomicrobiaceae(100); Prosthecobacter(100);
Otu224	206	Bacteroidetes(100);Bacteroidetes_incertae_sedis(100); Ohtaekwangia(100);Ohtaekwangia_unclassified(100);
Otu225	117	Acidobacteria(100);Acidobacteria_Gp2(100);Gp2(100); Gp2_unclassified(100);
Otu226	112	Chloroflexi(100);Anaerolineae(100);Anaerolineales(100); Anaerolineaceae(100);
Otu227	281	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Limnohabitans(100);
Otu228	32	Firmicutes(100);Bacilli(100);Bacillales(100);Bacillaceae_1(100);
Otu229	186	Firmicutes(100);Bacilli(100);Bacillales(100);Staphylococcaceae(100); Nosocomiicoccus(100);
Otu230	142	Acidobacteria(100);Holophagae(100);Holophagales(100); Holophagaceae(100);Geothrix(100);
Otu231	77	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Alcaligenaceae(100);Paenalcaligenes(100);
Otu232	132	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Moraxellaceae(100);Psychrobacter(100);
Otu233	134	Proteobacteria(100);Betaproteobacteria(100);Methylophilales(100); Methylophilaceae(100);Methylophilus(100);
Otu234	134	Firmicutes(100);Bacilli(100);Lactobacillales(100); Carnobacteriaceae(100);Carnobacterium(100);
Otu235	170	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Delftia(100);
Otu236	250	Proteobacteria(100);Alphaproteobacteria(100); Alphaproteobacteria_incertae_sedis(100);Rhizomicrobium(100);

Otu237	176	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadaceae(100); Sphingorhabdus(100);
Otu238	152	Verrucomicrobia(100);Verrucomicrobiae(100); Verrucomicrobiales(100);Verrucomicrobiaceae(100);
Otu239	73	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiales_incertae_sedis(100);
Otu240	96	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);
Otu241	120	Proteobacteria(100);Betaproteobacteria(100);Rhodocyclales(100); Rhodocyclaceae(100);Dechloromonas(100);
Otu242	206	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Beijerinckiaceae(100);
Otu243	23	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Phyllobacteriaceae(100);
Otu244	44	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Ruminococcus2(100);
Otu245	10	Firmicutes(100);Bacilli(100);Lactobacillales(100); Enterococcaceae(100);Melissococcus(100);
Otu246	10	Firmicutes(100);Bacilli(100);Lactobacillales(100); Streptococcaceae(100);
Otu247	136	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Microbacteriaceae(100);Herbiconiux(100);
Otu248	307	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Xanthomonadaceae(100);Lysobacter(100);
Otu249	219	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiaceae(100);Ralstonia(100);
Otu250	25	Nitrospirae(100);Nitrospira(100);Nitrospirales(100); Nitrospiraceae(100);Nitrospira(100);
Otu251	46	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Gemmiger(100);
Otu252	82	Acidobacteria(100);Acidobacteria_Gp4(100); Acidobacteria_Gp4_unclassified(100); Acidobacteria_Gp4_unclassified(100);
Otu253	115	Acidobacteria(100);Acidobacteria_Gp17(100);Gp17(100); Gp17_unclassified(100);
Otu254	112	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Sphingobacteriaceae(100);Sphingobacterium(100);
Otu255	125	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100); Cryomorphaceae(100);Fluviicola(100);
Otu256	141	Proteobacteria(100);Deltaproteobacteria(100);Myxococcales(100); Phaselicystidaceae(100);Phaselicystis(100);
Otu257	261	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Phyllobacteriaceae(100);Mesorhizobium(100);

Otu258	59	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Geodermatophilaceae(100);Blastococcus(100);
Otu259	196	Proteobacteria(100);Deltaproteobacteria(100); Desulfovibrionales(100);Desulfohalobiaceae(100); Desulfohalobium(100);
Otu260	208	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Methylobacteriaceae(100);
Otu261	103	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Dermacoccaceae(100);Dermacoccus(100);
Otu262	32	Proteobacteria(100);Deltaproteobacteria(100); Desulfovibrionales(100);Desulfovibrionales_unclassified(100);
Otu263	105	Proteobacteria(100);Gammaproteobacteria(100);Chromatiales(100); Chromatiaceae(100);Rheinheimera(100);
Otu264	140	Firmicutes(100);Bacilli(100);Lactobacillales(100); Lactobacillaceae(100);Pediococcus(100);
Otu265	161	Firmicutes(100);Bacilli(100);Bacillales(100);Bacillaceae_2(100); Oceanobacillus(100);
Otu266	96	Proteobacteria(100);Alphaproteobacteria(100);Caulobacterales(100); Caulobacteraceae(100);Caulobacter(100);
Otu267	169	Verrucomicrobia(100);Spartobacteria(100); Spartobacteria_unclassified(100);Spartobacteria_unclassified(100);
Otu268	179	Proteobacteria(100);Deltaproteobacteria(100);Bdellovibrionales(100); Bacteriovoracaceae(100);Bacteriovorax(100);
Otu269	131	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);Chitinophaga(100);
Otu270	9	Actinobacteria(100);Actinobacteria(100);Bifidobacteriales(100); Bifidobacteriaceae(100);
Otu271	80	Verrucomicrobia(100);Verrucomicrobiae(100); Verrucomicrobiales(100);Verrucomicrobiaceae(100); Luteolibacter(100);
Otu272	126	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Propionibacteriaceae(100);Friedmanniella(100);
Otu273	8	Firmicutes(100);Bacilli(100);Lactobacillales(100); Lactobacillaceae(100);Paralactobacillus(100);
Otu274	64	Deinococcus-Thermus(100);Deinococci(100);Deinococcales(100); Trueperaceae(100);Truepera(100);
Otu275	101	Proteobacteria(100);Gammaproteobacteria(100);Pasteurellales(100); Pasteurellaceae(100);Aggregatibacter(100);
Otu276	76	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Clostridium_XVIII(100);
Otu277	173	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiaceae(100);Polynucleobacter(100);
Otu278	102	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Propionibacteriaceae(100);Microlunatus(100);

Otu279	48	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Nocardioidaceae(100);
Otu280	29	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Xanthomonadales_unclassified(100);
Otu281	33	Actinobacteria(100);Actinobacteria(100);Solirubrobacterales(100); Conexibacteraceae(100);Conexibacter(100);
Otu282	54	Verrucomicrobia(100);Opitutae(100);Opitutales(100); Opitutaceae(100);Opitutus(100);
Otu283	75	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Mycobacteriaceae(100);Mycobacterium(100);
Otu284	31	Deinococcus-Thermus(100);Deinococci(100);Thermales(100); Thermaceae(100);Thermus(100);
Otu285	100	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Pseudonocardiaceae(100);Pseudonocardia(100);
Otu286	121	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Dermacoccaceae(100);Kytococcus(100);
Otu287	76	Acidobacteria(100);Acidobacteria_Gp5(100);Gp5(100); Gp5_unclassified(100);
Otu288	124	Proteobacteria(100);Betaproteobacteria(100);Rhodocyclales(100); Rhodocyclaceae(100);Uliginosibacterium(100);
Otu289	32	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Dorea(100);
Otu290	57	Firmicutes(100);Clostridia(100);Clostridia_unclassified(100); Clostridia_unclassified(100);
Otu291	158	Proteobacteria(100);Alphaproteobacteria(100);Rhodospirillales(100); Acetobacteraceae(100);Acetobacter(100);
Otu292	131	Fusobacteria(100);Fusobacteriia(100);Fusobacteriales(100); Leptotrichiaceae(100);Leptotrichia(100);
Otu293	89	Proteobacteria(100);Alphaproteobacteria(100);Rhodospirillales(100); Rhodospirillales_unclassified(100);
Otu294	42	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Erythrobacteraceae(100);
Otu295	26	Planctomycetes(100);Planctomycetia(100);Planctomycetales(100); Planctomycetaceae(100);Aquisphaera(100);
Otu296	59	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Kandleria(100);
Otu297	54	Proteobacteria(100);Epsilonproteobacteria(100); Campylobacterales(100);Helicobacteraceae(100);Sulfuricurvum(100);
Otu298	63	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Lachnospira(100);
Otu299	61	Deinococcus-Thermus(100);Deinococci(100);Deinococcales(100); Deinococcaceae(100);Deinococcus(100);
Otu300	69	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Saprospiraceae(100);Haliscomenobacter(100);

Otu301	67	Proteobacteria(100);Gammaproteobacteria(100); Methylococcales(100);Methylococcaceae(100);
Otu302	9	Chlamydiae(100);Chlamydiia(100);Chlamydiales(100); Parachlamydiaceae(100);Neochlamydia(100);
Otu303	81	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Sanguibacteraceae(100);Sanguibacter(100);
Otu304	67	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Hyphomicrobiaceae(100);Devosia(100);
Otu305	82	Proteobacteria(100);Gammaproteobacteria(100); Cardiobacteriales(100);Cardiobacteriaceae(100); Cardiobacterium(100);
Otu306	5	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiaceae_1(100);Anaerobacter(100);
Otu307	6	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Brucellaceae(100);
Otu308	13	Proteobacteria(100);Betaproteobacteria(100);Neisseriales(100); Neisseriaceae(100);Neisseria(100);
Otu309	11	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Moraxellaceae(100);Alkanindiges(100);
Otu310	65	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiales_Incertae_Sedis_XI(100);Finegoldia(100);
Otu311	163	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Xanthomonadaceae(100);Dokdonella(100);
Otu312	104	Firmicutes(100);Negativicutes(100);Selenomonadales(100); Veillonellaceae(100);Mitsuokella(100);
Otu313	61	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Micromonosporaceae(100);
Otu314	26	Actinobacteria(100);Actinobacteria(100);Coriobacteriales(100); Coriobacteriaceae(100);Enterorhabdus(100);
Otu315	4	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Corynebacteriaceae(100);
Otu316	67	Actinobacteria(100);Actinobacteria_unclassified(100); Actinobacteria_unclassified(100);Actinobacteria_unclassified(100);
Otu317	19	Proteobacteria(100);Gammaproteobacteria(100);Vibrionales(100); Vibrionaceae(100);Vibrio(100);
Otu318	108	Spirochaetes(100);Spirochaetia(100);Spirochaetales(100); Spirochaetaceae(100);Treponema(100);
Otu319	35	Bacteroidetes(100);Cytophagia(100);Cytophagales(100); Cytophagaceae(100);Flectobacillus(100);
Otu320	11	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);Odoribacter(100);

Otu321	89	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Microbacteriaceae(100);Leucobacter(100);
Otu322	93	Firmicutes(100);Negativicutes(100);Selenomonadales(100); Veillonellaceae(100);Megamonas(100);
Otu323	22	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);Terrimonas(100);
Otu324	25	Firmicutes(100);Bacilli(100);Lactobacillales(100); Aerococcaceae(100);Dolosicoccus(100);
Otu325	4	Proteobacteria(100);Deltaproteobacteria(100); Desulfovibrionales(100);Desulfohalobiaceae(100);
Otu326	47	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Clostridium_XlVb(100);
Otu327	4	Verrucomicrobia(100);Verrucomicrobia_unclassified(100); Verrucomicrobia_unclassified(100); Verrucomicrobia_unclassified(100);
Otu328	21	Firmicutes(100);Bacilli(100);Bacillales(100);Paenibacillaceae_1(100);
Otu329	179	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);Erwinia(100);
Otu330	96	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100); Flavobacteriaceae(100);Myroides(100);
Otu331	74	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Erythrobacteraceae(100); Altererythrobacter(100);
Otu332	11	Firmicutes(100);Bacilli(100);Lactobacillales(100);Aerococcaceae(100)
Otu333	4	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Diaphorobacter(100);
Otu334	22	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Coprobacillus(100);
Otu335	14	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Xanthomonadaceae(100); Thermomonas(100);
Otu336	15	Firmicutes(100);Clostridia(100);Clostridiales(100); Peptococcaceae_1(100);Peptococcus(100);
Otu337	26	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Porphyromonadaceae(100);Porphyromonas(100);
Otu338	12	Proteobacteria(100);Alphaproteobacteria(100);Caulobacterales(100); Caulobacteraceae(100);Asticcacaulis(100);
Otu339	25	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Lachnoanaerobaculum(100);
Otu340	15	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Sinobacteraceae(100);Nevskia(100);

Otu341	44	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiales_incertae_sedis(100);Tepidimonas(100);
Otu342	20	Proteobacteria(100);Betaproteobacteria(100);Rhodocyclales(100); Rhodocyclaceae(100);Ferribacterium(100);
Otu343	96	Proteobacteria(100);Alphaproteobacteria(100);Rhodospirillales(100); Rhodospirillaceae(100);
Otu344	3	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);Rugamonas(100);
Otu345	17	Firmicutes(100);Negativicutes(100);Selenomonadales(100); Veillonellaceae(100);Psychrosinus(100);
Otu346	58	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Hyphomicrobiaceae(100);
Otu347	3	Firmicutes(100);Clostridia(100);Clostridiales(100); Peptostreptococcaceae(100);Sporacetigenium(100);
Otu348	3	Proteobacteria(100);Betaproteobacteria(100);Methylophilales(100); Methylophilaceae(100);Methylotenera(100);
Otu349	24	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Geodermatophilaceae(100);Modestobacter(100);
Otu350	33	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Methylobacteriaceae(100);Microvirga(100);
Otu351	26	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Intrasporangiaceae(100);Ornithinimicrobium(100);
Otu352	23	Acidobacteria(100);Acidobacteria_Gp10(100);Gp10(100); Gp10_unclassified(100);
Otu353	29	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Pseudomonadaceae(100);Rhizobacter(100);
Otu354	12	Proteobacteria(100);Deltaproteobacteria(100);Myxococcales(100); Cystobacteraceae(100);
Otu355	26	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Kineosporiaceae(100);Kineosporia(100);
Otu356	9	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Anaerotruncus(100);
Otu357	3	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100); Flavobacteriales_unclassified(100);
Otu358	63	Firmicutes(100);Clostridia(100);Clostridiales(100); Peptostreptococcaceae(100);Acetoanaerobium(100);
Otu359	9	Proteobacteria(100);Epsilonproteobacteria(100); Campylobacterales(100);Helicobacteraceae(100);Helicobacter(100);
Otu360	8	Acidobacteria(100);Acidobacteria_unclassified(100); Acidobacteria_unclassified(100);Acidobacteria_unclassified(100);

Otu361	71	Firmicutes(100);Bacilli(100);Bacillales(100);Paenibacillaceae_1(100); Paenibacillus(100);
Otu362	5	Chlamydiae(100);Chlamydiia(100);Chlamydiales(100); Parachlamydiaceae(100);Parachlamydia(100);
Otu363	3	Firmicutes(100);Bacilli(100);Bacillales(100);Bacillaceae_2(100); Cerasibacillus(100);
Otu364	90	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Cellulomonadaceae(100);Cellulomonas(100);
Otu365	46	Acidobacteria(100);Acidobacteria_Gp1(100); Acidobacteria_Gp1_unclassified(100); Acidobacteria_Gp1_unclassified(100);
Otu366	9	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiaceae_2(100);Alkaliphilus(100);
Otu367	223	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Brucellaceae(100);Ochrobactrum(100);
Otu368	16	Firmicutes(100);Bacilli(100);Bacillales(100); Thermoactinomycetaceae_1(100);Hazenella(100);
Otu369	54	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Hyphomicrobiaceae(100);Hyphomicrobium(100);
Otu370	26	Chloroflexi(100);Thermomicrobia(100); Thermomicrobia_unclassified(100); Thermomicrobia_unclassified(100);
Otu371	65	Bacteroidetes(100);Cytophagia(100);Cytophagales(100); Cytophagaceae(100);Adhaeribacter(100);
Otu372	58	Firmicutes(100);Clostridia(100);Clostridiales(100); Peptococcaceae_1(100);Desulfosporosinus(100);
Otu373	19	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Sunxiuqinia(100);
Otu374	25	Proteobacteria(100);Deltaproteobacteria(100);Bdellovibrionales(100); Bacteriovoracaceae(100);Peredibacter(100);
Otu375	52	Proteobacteria(100);Betaproteobacteria(100);Neisseriales(100); Neisseriaceae(100);
Otu376	2	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Dermabacteraceae(100);
Otu377	103	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Xanthomonadaceae(100);Luteimonas(100);
Otu378	4	Firmicutes(100);Bacilli(100);Lactobacillales(100); Leuconostocaceae(100);Leuconostoc(100);
Otu379	14	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Nocardioidaceae(100);Marmoricola(100);
Otu380	25	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Geodermatophilaceae(100);Geodermatophilus(100);
Otu381	17	Acidobacteria(100);Acidobacteria_Gp1(100);Gp1(100); Gp1_unclassified(100);

Otu382	100	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100);Rhizobiales_incertae_sedis(100);Bauldia(100);
Otu383	2	Planctomycetes(100);Planctomycetia(100);Planctomycetia_unclassified(100);Planctomycetia_unclassified(100);
Otu384	5	Proteobacteria(100);Gammaproteobacteria(100);Pseudomonadales(100);Moraxellaceae(100);Moraxella(100);
Otu385	34	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100);Microbacteriaceae(100);Leifsonia(100);
Otu386	3	Proteobacteria(100);Gammaproteobacteria(100);Enterobacteriales(100);Enterobacteriaceae(100);Morganella(100);
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Otu395	3	Acidobacteria(100);Acidobacteria_Gp4(100);Blastocatella(100);Blastocatella_unclassified(100);
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Otu397	6	Proteobacteria(100);Alphaproteobacteria(100);Rhodobacterales(100);Rhodobacteraceae(100);Gemmobacter(100);
Otu398	2	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100);Sphingobacteriaceae(100);
Otu399	2	Proteobacteria(100);Gammaproteobacteria(100);Oceanospirillales(100);Oceanospirillaceae(100);Marinomonas(100);
Otu400	2	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100);Flavobacteriaceae(100);Epilithonimonas(100);
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Otu402	51	Proteobacteria(100);Gammaproteobacteria(100); Alteromonadales(100);Psychromonadaceae(100); Psychromonas(100);
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Otu407	14	Chloroflexi(100);Dehalococcoidetes(100);Dehalogenimonas(100); Dehalogenimonas_unclassified(100);
Otu408	14	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Xanthomonadaceae(100);Rudaea(100);
Otu409	2	Acidobacteria(100);Acidobacteria_Gp22(100);Gp22(100); Gp22_unclassified(100);
Otu410	3	Firmicutes(100);Bacilli(100);Lactobacillales(100); Carnobacteriaceae(100);Alloiococcus(100);
Otu411	14	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);Flavisolibacter(100);
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Otu413	2	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Burkholderiales_incertae_sedis(100);Paucibacter(100);
Otu414	21	Proteobacteria(100);Deltaproteobacteria(100); Syntrophobacterales(100);Syntrophaceae(100);Desulfomonile(100);
Otu415	13	Firmicutes(100);Clostridia(100);Clostridiales(100); Lachnospiraceae(100);Robinsoniella(100);
Otu416	2	Proteobacteria(100);Gammaproteobacteria(100); Enterobacteriales(100);Enterobacteriaceae(100);Kluyvera(100);
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Otu418	17	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Pseudoflavonifractor(100);
Otu419	25	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Rhodobiaceae(100);Parvibaculum(100);
Otu420	42	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Oxalobacteraceae(100);Noviherbaspirillum(100);
Otu421	2	Proteobacteria(100);Deltaproteobacteria(100);Myxococcales(100); Cystobacteraceae(100);Anaeromyxobacter(100);

Otu422	20	Bacteroidetes(100);Cytophagia(100);Cytophagales(100); Cytophagaceae(100);Leadbetterella(100);
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Otu444	1	Actinobacteria(100);Actinobacteria(100);Coriobacteriales(100); Coriobacteriaceae(100);Collinsella(100);
Otu445	1	Proteobacteria(100);Gammaproteobacteria(100); Pseudomonadales(100);Moraxellaceae(100);Perlucidibaca(100);
Otu446	1	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Malikia(100);
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Otu449	1	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Saprospiraceae(100);
Otu450	1	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Cellulomonadaceae(100);
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Otu453	1	Proteobacteria(100);Alphaproteobacteria(100); Sphingomonadales(100);Sphingomonadaceae(100); Sandaracinobacter(100);
Otu454	1	Fusobacteria(100);Fusobacteriia(100);Fusobacteriales(100); Fusobacteriaceae(100);
Otu455	10	Bacteroidetes(100);Flavobacteriia(100);Flavobacteriales(100); Flavobacteriaceae(100);Bergeyella(100);
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Otu459	1	Bacteroidetes(100);Sphingobacteriia(100);Sphingobacteriales(100); Chitinophagaceae(100);Parasegetibacter(100);
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Otu462	10	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100); Comamonadaceae(100);Simplicispira(100);
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Otu465	1	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Nocardiaceae(100);
Otu466	1	Proteobacteria(100);Epsilonproteobacteria(100); Campylobacterales(100);Helicobacteraceae(100);
Otu467	1	Firmicutes(100);Negativicutes(100);Selenomonadales(100); Veillonellaceae(100);
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Otu471	23	Proteobacteria(100);Gammaproteobacteria(100); Aeromonadales(100);Aeromonadaceae(100);
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Otu477	1	Proteobacteria(100);Alphaproteobacteria(100);Rhizobiales(100); Bradyrhizobiaceae(100);Afipia(100);
Otu478	111	Proteobacteria(100);Deltaproteobacteria(100);Myxococcales(100); Polyangiaceae(100);
Otu479	24	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Sporichthyaceae(100);Sporichthya(100);
Otu480	1	Actinobacteria(100);Actinobacteria(100);Acidimicrobiales(100); Acidimicrobiaceae(100);
Otu481	1	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Sinobacteraceae(100);Steroidobacter(100);
Otu482	42	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Nocardiopsaceae(100);Nocardiopsis(100);
Otu483	1	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100); Erysipelotrichaceae(100);Holdemania(100);

Otu484	1	Proteobacteria(100);Gammaproteobacteria(100); Alteromonadales(100);Alteromonadaceae(100);Haliea(100);
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Otu487	1	Firmicutes(100);Negativicutes(100);Selenomonadales(100); Veillonellaceae(100);Selenomonas(100);
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Otu489	5	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Microbacteriaceae(100);Clavibacter(100);
Otu490	3	Firmicutes(100);Clostridia(100);Clostridiales(100); Clostridiaceae_1(100);Sarcina(100);
Otu491	1	Proteobacteria(100);Deltaproteobacteria(100);Desulfobacterales(100); Desulfobacteraceae(100);
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Otu493	1	Proteobacteria(100);Alphaproteobacteria(100);Caulobacterales(100); Caulobacterales_unclassified(100);
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Otu497	1	Proteobacteria(100);Alphaproteobacteria(100);Rhodobacterales(100); Rhodobacteraceae(100);Rhodobacter(100);
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Otu499	1	Proteobacteria(100);Gammaproteobacteria(100); Xanthomonadales(100);Sinobacteraceae(100);Singularimonas(100);
Otu500	1	Actinobacteria(100);Actinobacteria(100);Rubrobacterales(100); Rubrobacteraceae(100);Rubrobacter(100);
Otu501	1	Proteobacteria(100);Alphaproteobacteria(100); Alphaproteobacteria_incertae_sedis(100);Geminicoccus(100);
Otu502	4	Proteobacteria(100);Betaproteobacteria(100);Neisseriales(100); Neisseriaceae(100);Iodobacter(100);
Otu503	2	Firmicutes(100);Clostridia(100);Clostridiales(100); Ruminococcaceae(100);Clostridium_III(100);

Otu504	2	Proteobacteria(100);Gammaproteobacteria(100); Aeromonadales(100);Aeromonadaceae(100);Oceanisphaera(100);
Otu505	12	Actinobacteria(100);Actinobacteria(100);Actinomycetales(100); Propionibacteriaceae(100);Tessaracoccus(100);
Otu506	1	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100); Prevotellaceae(100);Hallella(100);
Otu507	4	Chloroflexi(100);Thermomicrobia(100);Sphaerobacterales(100); Sphaerobacteraceae(100);Sphaerobacter(100);

7.2.3 Microbiota analysis of gut samples over time

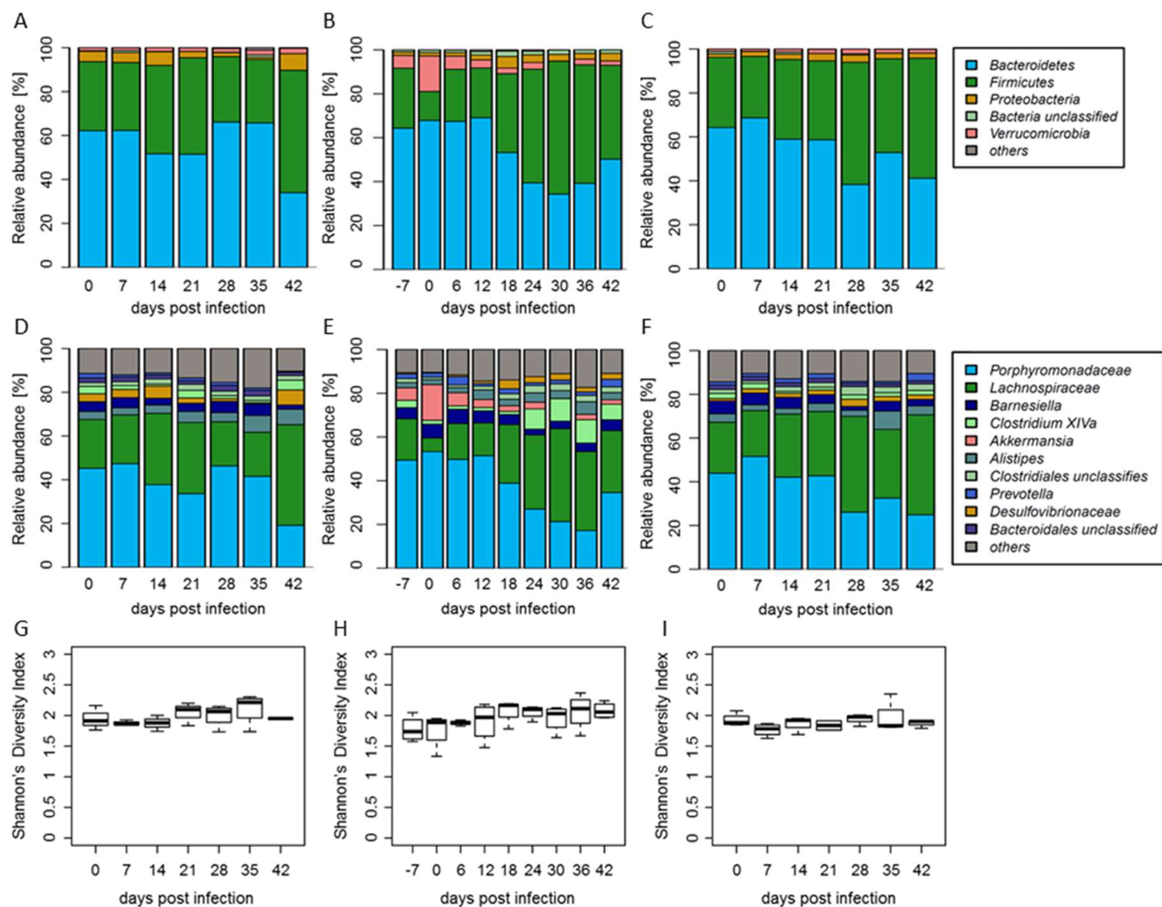

Figure 20: Gut microbiota of hormone-treated and untreated mice over time. (A-C) Gut microbiota on phylum level. **(A)** Gut microbiota of untreated mice showed slight fluctuations over time in abundance of Bacteroidetes and Firmicutes. **(B)** Progesterone-treated mice were similar to untreated mice with a high abundance of Firmicutes. **(C)** β-estradiol had no influence on the composition of gut microbiota. **(D-F)** Gut microbiota on genus level. **(D)** No significant changes in the composition of gut microbiota were observed. **(E)** Progesterone treatment did not significantly alter the proportions of microbiota over time. **(F)** If treated with β-estradiol, gut microbiota did not further differ than underlying natural fluctuations. **(G-I)** Alpha diversity of gut microbiota. **(G)** Gut microbiota of untreated mice did not fluctuate in alpha diversity over time. **(H)** Progesterone treatment did not induce changes to Shannon's diversity index. **(I)** Despite β-estradiol treatment alpha diversity did not change over time. (n=1-4, subsampled to 5000 reads)

7.2.4 Relative abundance of vaginal microbiota on genus level

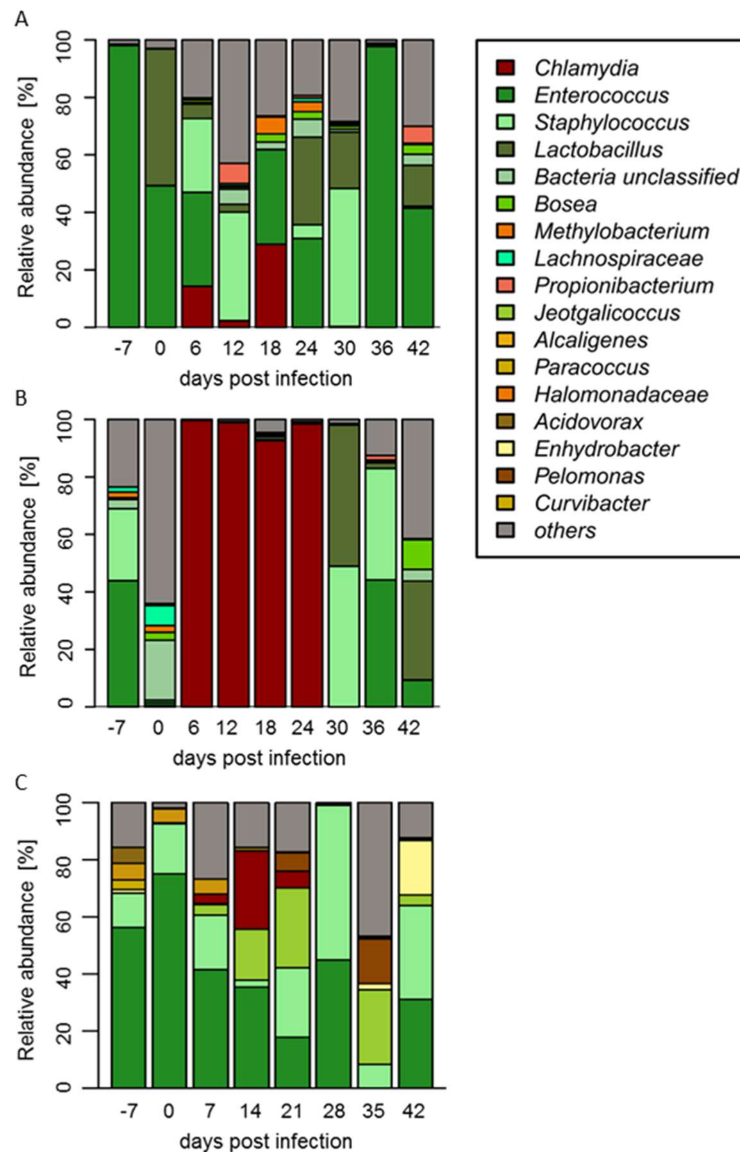

Figure 21: Vaginal microbiota during chlamydial infection under different hormonal conditions. Vaginal microbiota on genus level. **(A)** If untreated, microbiota only slightly changed and post infection chlamydial reads were barely found. **(B)** If progesterone-treated, enterococci were reduced and *Chlamydia* were high after infection. **(C)** Administration of β-estradiol did not induce changes within the vaginal microbiota. Chlamydial reads were low. (n=4, subsampled to 1000 reads, Wilcoxon Rank Sum test with Continuity Correction and correction for multiple testing)

7.2.5 Effect of antibiotic pretreatment on vaginal microbiota on genus level

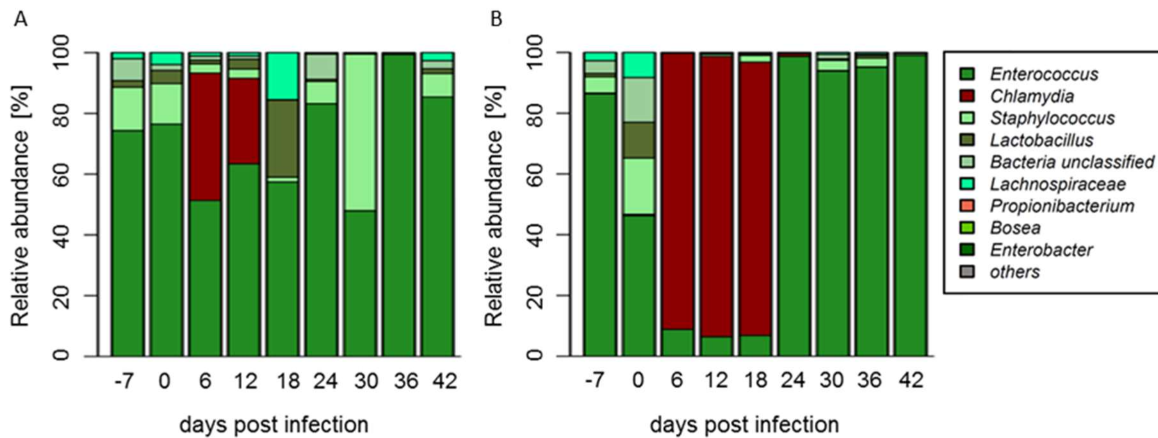

Figure 22: Influence of doxycycline on vaginal microbiota and chlamydial infection. Relative abundance of vaginal microbiota on genus level. **(A)** Doxycycline pretreatment had no influence on the composition of microbiota. *Chlamydia* was barely found after infection. **(B)** Simultaneous administration of progesterone and doxycycline led to a reduction of enterococci and after infection high chlamydial reads. (n=4, subsampled to 1000 reads, Wilcoxon Rank Sum test with Continuity Correction and correction for multiple testing)

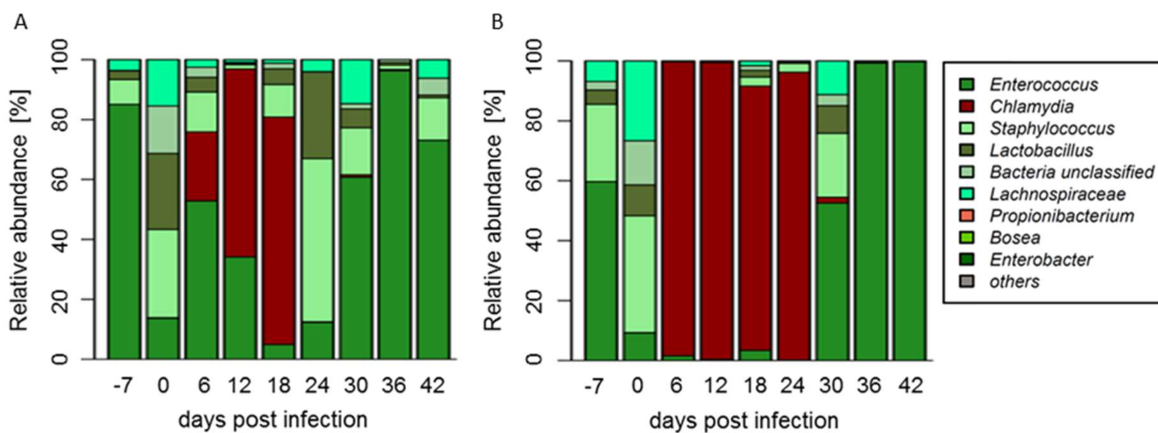

Figure 23: Influence of ampicillin on vaginal microbiota and chlamydial infection. Vaginal microbiota on genus level. **(A)** Ampicillin altered the vaginal microbiota of hormone untreated mice. Chlamydial reads were found. **(B)** Progesterone treatment and ampicillin had an equal effect on the reduction of *Enterococci*. Abundance of *Chlamydia* was high. (n=4, subsampled to 1000 reads, Wilcoxon Rank Sum test with Continuity Correction and correction for multiple testing)

7.3 Publications

Loeper N, Graspeuntner S, Rupp J. Microbiota changes impact on sexually transmitted infections and the development of pelvic inflammatory disease. Microbes and Infection 2018 Feb 13. doi: 10.1016/j.micinf.2018.02.003. (ePub ahead of print)

Graspeuntner S, Loeper N, Künzel S, Baines JF, Rupp J. Selection of validated hypervariable regions is crucial in 16S-based microbiota studies of the female genital tract. Scientific Reports 2018, Volume 8, Article number: 9678.

Shima K, Ledig S, Loeper N, Schiefer A, Pfarr K, Hoerauf A, Graspeuntner S, Rupp J. Effective inhibition of rifampin-resistant *Chlamydia trachomatis* by the novel DNA-dependent RNA-polymerase inhibitor Corallopyronin A. International Journal of Antimicrobial Agents 2018 Aug 6, doi: 10.1016/j.ijantimicag.2018.07.025. (ePub ahead of print)

Loeper N, Graspeuntner S, Ledig S, Kaufhold I, Hoellen F, Schiefer A, Pfarr K, Hoerauf A, Shima K, Rupp J. Corallopyronin A exhibits high efficacy against *Chlamydia trachomatis in vitro* and *ex vivo*. (Submitted to Frontiers in Microbiology section Infectious Diseases Research, 01.10.2018)

7.4 Conference Contributions

7.4.1 Talks

Loeper N, Graspeuntner S, Künzel S, Baines JF, Rupp J. Influence of variations within the vaginal microbiome on murine *Chlamydia muridarum* infections *in vivo.* 15th German Chlamydia Workshop, Jena, TH, Germany, 02/2017.

Loeper N, Graspeuntner S, Ledig S, Künzel S, Baines JF, Rupp J. The role of microbiome-induced metabolic and inflammatory changes on urogenital-tract infections. On-site-review RTG1743, Kiel, SH, Germany, 07/2016.

7.4.2 Posters

Loeper N, Ledig S, Graspeuntner S, Pieper M, Künzel S, Baines JF, König P, Rupp J. Chlamydial infection in female mice is promoted by progesterone altering vaginal microbiota and immune response. 2nd International RTG1743 Symposium, Kiel, SH, Germany, 06/2018.

Loeper N, Ledig S, Graspeuntner S, Pieper M, Künzel S, Baines JF, König P, Rupp J. Chlamydial infections *in vivo* – a perspective on vaginal microbiota and pathology formation. International Cluster Symposium Precision Medicine in Chronic Inflammation, Cluster of Excellence Inflammation at Interfaces, Hamburg, HH, Germany, 03/2018.

Loeper N, Graspeuntner S, Ledig S, Kaufhold I, Hoellen F, Schiefer A, Pfarr K, Hörauf A, Shima K, Rupp J. A novel antibiotic for the treatment of chlamydial infections. 5th Translational DZIF-School, Lübeck, SH, Germany, 11/2017.

Loeper N, Ledig S, Graspeuntner S, Künzel S, Baines JF, Rupp J. Influence of an altered microbiome on infections with *Chlamydia muridarum in vivo*. 68th Annual Meeting of the German Society for Hygiene and Microbiology, Ulm, BW, Germany, 09/2016.

Loeper N, Ledig S, Graspeuntner S, Rupp J. Characterization of host-pathogen interactions in a recently established mouse model of chlamydial infection. HZI/DZIF Summer School on Infection Research, Eiterfeld, HE, Germany, 06/2016.

7.5 Curriculum vitae

8. Acknowledgements