Effects of hormonal contraceptives on the female genital tract microbiota in South African adolescents

By CHRISTINA BALLE
BLLCHR014

Dissertation presented for the Degree of
PhD (Med) in Clinical Sciences & Immunology

Supervisor: Dr Heather Jaspan
Co-supervisors: Associate Professor Jo-Ann Passmore
& Dr Katie Lennard

Department of Pathoglogy
Division of Immunology
Faculty of Health Sciences
University of Cape Town

March 2019

Plagiarism Declaration

I, **Christina Balle**, hereby declare that the work on which this dissertation is based on my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I authorise the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

SIGNATURE: Signed by candidate

DATE: 15 March 2019

Acknowledgements

Firstly, I would like to express my gratitude to my supervisor Dr Heather Jaspan for giving me the opportunity to join her team at University of Cape Town. You have always been very supportive and provided me with valuable guidance. Thank you for being a great mentor. To my co-supervisor, Dr Katie Lennard, thank you for your patience and your extremely valuable assistance with the bioinformatics and statistical analyses. To my other co-supervisor, Associate Prof Jo-Ann Passmore, thank you for your always valued inputs, comments and suggestions on my research.

To former and current Jaspan group members including Enock Havyarimana, Shameem Jaumdally, Donald Nyangahu, Jerome Wendoh, Agano Kiravu and Muchaneta Gudza-Mugabe, thank you for your valuable feedback and assistance when needed. To the uCHOOSE team at UCT, particularly Iyaloo Konstantinus, Anna Happel, Rachel Esra and Madeleine Heller, thank you for your assistance in sample processing and for making late nights at the laboratory a bit more bearable.

I would also like to thank the uCHOOSE study team at the Desmund Tutu HIV foundation (DTHF) clinic in Masiphumelele, particularly Tanya Pidwell, Pinky Ngobo, Eve Mendel, Janine Nixon and Keshani Naidoo for all their hard work and assistance. Thank you Prof Linda Gail Bekker for allowing us to be part of the uCHOOSE study at the DTHF clinic. Most importantly, I would like to thank all the young women who kindly participated in the uCHOOSE study and contributed to the research. Without all of you, none of this would be possible.

To Anni, Iris, Liz, Claire, Tine, Katie, Zoë and Petrus, thank you for making Cape Town my home away from home. To all my friends back in Denmark, thank you for all of support you have shown me over the years.

Lastly, I would like to thank my family for always encouraging me to follow my dreams and for being so supportive of my decision to move to South Africa. I would not have been able to do this without the love and support you have shown me and for that I am truly grateful.

Table of Content

Abstract

Background

Young women in sub-Saharan Africa are disproportionally affected by HIV and often rely on injectable hormonal contraception (HC) to prevent unintended pregnancies. However, HC might affect HIV-1 risk through changes in the female genital tract (FGT) microbiota. We examined the impact of three different HC methods on the adolescent female genital tract microbiota and related cytokine and HIV target cell levels at the cervical mucosa in a randomized, crossover trial.

Study design and methods

131 adolescent females aged 15 to 19 from Cape Town were enrolled into a randomized, crossover study. The participants were randomized into three study arms: 1. progestin-only injectable norethisterone enanthate (Net-En), 2. combined oral contraceptive pills (COCPs) or 3. combined contraceptive vaginal ring (CCVR) for 16 weeks. Participants then switched to one of the other HC options for a final four months. Vaginal samples were collected at baseline, crossover and exit. STI testing and Nugent scoring were performed at all study visits. Vaginal microbiota was characterized by 16S rRNA gene amplicon sequencing, cytokine concentrations were measured by Luminex and CD4+ T cells analysed by flow cytometry.

Results

Using fuzzy clustering, three major female genital tract bacterial community types were identified. Two of these were dominated by *Lactobacillus* species (*L. crispatus* and *L. iners*, respectively) and the third was comprised of a diverse group of anaerobic bacteria associated with bacterial vaginosis (BV). In an intention-to-treat analysis at crossover, participants randomized to COCP had a significantly less diverse vaginal microbiota compared to participants randomized to either Net-En or CCVR. The same was observed in an according to protocol analysis at crossover. Using differential abundance testing and random forest analyses, we found that species associated with BV and risk of HIV were

significantly more abundant in, and predictive of, participants on Net-En (e.g. *Prevotella*, *Sneathia* and *Dialister*) or CCVR (e.g. *Prevotella, Mycoplasma* and *Parvimonas*) compared to COCP while *L. iners* was more common in the COCP group. Cytokine concentrations were positively associated with a diverse vaginal community and with specific bacterial taxa associated with BV and increased risk of HIV including species enriched in participants on Net-En and NuvaRing. In contrast, there were no association of the frequencies of CD4+ T cells expressing CCR5+ with the vaginal community or BV status. There was likewise no significant association with BV or diversity with Th17 cell frequency, yet BV-associated bacteria were more abundant in participants with higher frequencies of Th17 cells.

Conclusions

Our data generated from a randomized study suggests that COCPs use may exert a positive influence on genital health through an increase in lactobacilli and a decrease in BV-associated bacterial taxa with an accompanying decrease in overall bacterial diversity, vaginal pH and cytokine levels. In contrast, the vaginal microbiota of participants on Net-En and NuvaRing have increased levels of bacteria associated with BV and HIV risk and increased cytokine levels. We did not observe any association of the frequencies of CD4+ T cells expressing CCR5 or Th17-like cells with the vaginal community, BV status or HC use.

List of Figures

List of Tables

List of Abbreviations

°C	degrees Celsius
ß-ME	Beta-mercaptoethanol
aHR	Adjusted hazards ratio
aOR	adjusted odds ratio
AGYW	Adolescent girls and young women
AUC	Area under the curve
BARC	Bio Analytical Research Corporation
BMI	Body mass index
BV	Bacterial vaginosis
BVAB	BV-associated bacterium
CCVR	Combined contraceptive vaginal rings
CI	Confidence interval
COCP	Combined oral contraceptive pills
Ct	Chlamydia trachomatis
CVL	Cervicovaginal lavage
DMPA	Depot medroxyprogesterone acetate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTHF	Desmond Tutu HIV Foundation
EC	Endocervix
EDTA	Ethylenediaminetetraacetic acid
E2	Oestradiol
FCS	Foetal calf serum
FGT	Female genital tract
FSH	Follicle-stimulating hormone
HC	Hormonal contraception
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HR	Hazards ratio
HSV-2	Herpes simplex virus type 2
H_2O_2	Hydrogen peroxide
IC	Injectable contraceptives
IFN	Interferon
IL	Interleukin
IUCD	Intrauterine contraceptive device
LH	Luteinizing hormone
LW	Lateral wall
Mg	Mycoplasma genitalium
mRA	Mean relative abundance
Net-En	Norethisterone enanthate
NICD	National Institute of Communicable Diseases
Ng	Neisseria gonorrhoea
OR	Odds ratio
PBS	Phosphate buffered saline
PAM	Partitioning around medoids
PCR	Polymerase chain reaction
PCoA	Principal Coordinates Analysis
PID	Pelvic inflammatory disease
PSA	Prostate specific antigen
PSLG	Penicillin-Streptomycin L-Glutamine solution
P4	Progesterone
RCF	Relative centrifugal force
RCT	Randomized clinical trial
RPM	Revolutions per minute

RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal ribonucleic acid
SI	Shannon Index
SSA	Sub-Saharan Africa
STI	Sexually transmitted infection
Th	T helper
Tv	Trichomonas vaginalis
UCT	University of Cape Town
UTI	Urinary tract infection

Chapter 1: Literature review

1.1 HIV prevalence and unintended pregnancies amongst adolescent females in sub-Saharan Africa

1.1.1 HIV prevalence amongst sub-Saharan adolescent females

The population of sub-Saharan Africa (SSA) constitutes less than fifteen percent of the world's population, yet an estimated 25.5 million (70%) of people infected with the human immunodeficiency virus (HIV) live in this area (UNAIDS, 2017) (**Figure 1.1**). Adolescent girls and young women (AGYW) are at particularly high risk of HIV acquisition. More than half of all new HIV infections in SSA occur in women under 25 years of age, and AGYW are more than four times as likely to acquire HIV as young men of the same age group (Cowan & Pettifor, 2009; UNAIDS, 2017). Furthermore, AIDS-related illnesses are the second leading cause of death for young women aged 15–24 years in Africa (WHO, 2017).

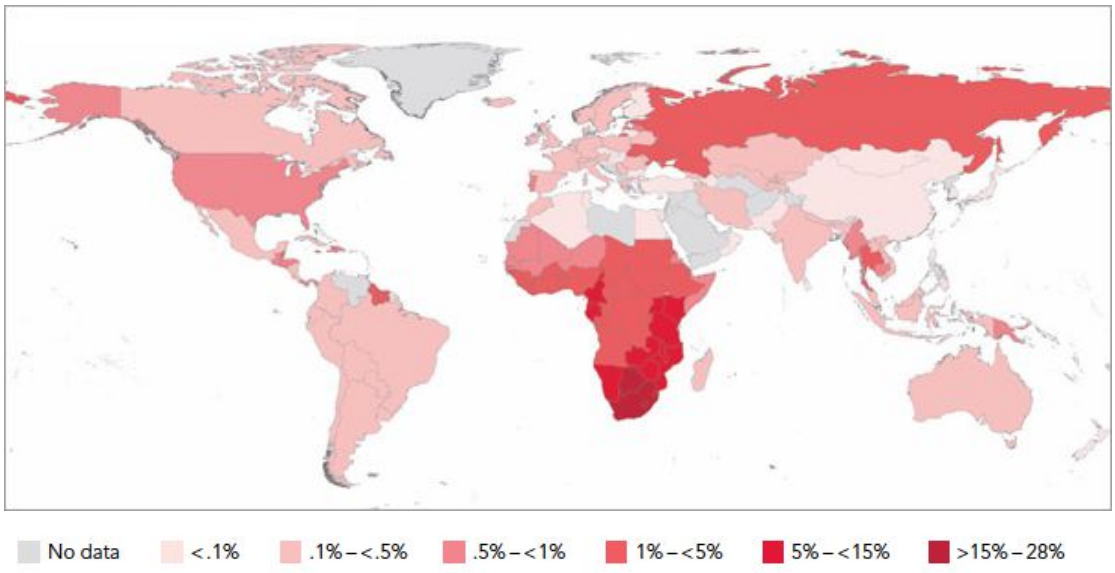


Figure 1.1. Global prevalence of HIV-1 in 2009. Source: UNAIDS Report on the Global AIDS epidemic (2010) (Joint United Nations Programme on HIV/AIDS, 2010).

In developing countries, the main mode of HIV transmission is heterosexual intercourse during which risk of HIV acquisition has been found to be higher for women than for men (UNAIDS, 2016). Between 70 and 90% of all HIV infections among women are due to heterosexual transmission across the genital mucosa(Abdool Karim et al., 2010; Shattock & Moore, 2003). High-risk sexual networks and sexual risk behaviours, including condomless sex, sex with an

older partner (intergenerational sex) and sex in exchange for gift or money (transactional sex), place adolescent women at high risk of HIV exposure (Chesson et al., 2012; Katz & Low-Beer, 2008). However, sexual risk behaviour alone cannot explain the increased risk of HIV acquisition in AGYW in SSA (Katz & Low-Beer, 2008; Pettifor et al., 2011). Emerging evidence suggests that there are biological reasons for the disproportionate risk of HIV acquisition amongst these young females (Jaspan, 2011) and understanding of the biological mechanisms that underlie the high HIV incidence rates in African AGYW is critical for developing new preventative measures.

1.1.2 Unintended pregnancies and hormonal contraception

In addition to the risk of HIV acquisition, AGYW in SSA are also faced with a high risk of unintended pregnancy with up to 35% of pregnancies amongst 15-19 year olds in SSA being unintended (Biddlecom et al., 2007). Unintended pregnancies amongst young women are associated with high maternal mortality and morbidity particularly in developing countries for instance due to unsafe abortions (Aitken et al., 2008; Nove et al., 2014). In a study conducted in South Africa, it was found that 23% of pregnancies in 13–16 year olds and 14.9% in 17–19 year olds ended in abortion (Buchmann et al., 2002). Additionally, young women are more at risk for adverse birth outcomes including obstructed labour and preterm delivery and of giving birth to small for gestational age infants compared to older women (Khashan et al., 2010). Pregnancy-related school dropout amongst adolescents also result in restricted job opportunities for young women (Grant & Hallman, 2008). Some adolescent girls will engage in intergenerational and transactional sex (Krishnan et al., 2008; Longfield et al., 2004). In such relationships the adolescent girl often have little or no negotiating power with their partners to insist on safe sex (e.g. condoms usage), which may result in high risk of unintended pregnancy and contracting sexually transmitted infections (STIs), including HIV (Gregson et al., 2002; Chatterji et al., 2004).

One of the most important successes in reproductive health has been the development of safe, effective and reversible family planning methods and the expansion of their use in low- and middle-income countries (Cleland et al., 2006). As such, hormonal contraceptive methods have become an important tool in

preventing unintended pregnancies and associated sequela and are now used by over 150 million women worldwide (Clifton et al., 2008). Hormonal contraceptives refer to a wide variety of birth control options composed of one or more steroid hormones that act on the endocrine system. The primary mechanism of action of hormonal contraceptives is suppressing the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary gland. The normal menstrual cycle is heavily regulated by complex interaction of hormones, including FSH and LH, which promote ovulation and stimulate the ovaries to produce the female sex hormones oestrogen and progesterone. The menstrual cycle consists of three distinct phases: the follicular phase (before release of the egg), the ovulatory phase (ovulation) and the luteal phase (after egg release) (**Figure 1.2**).

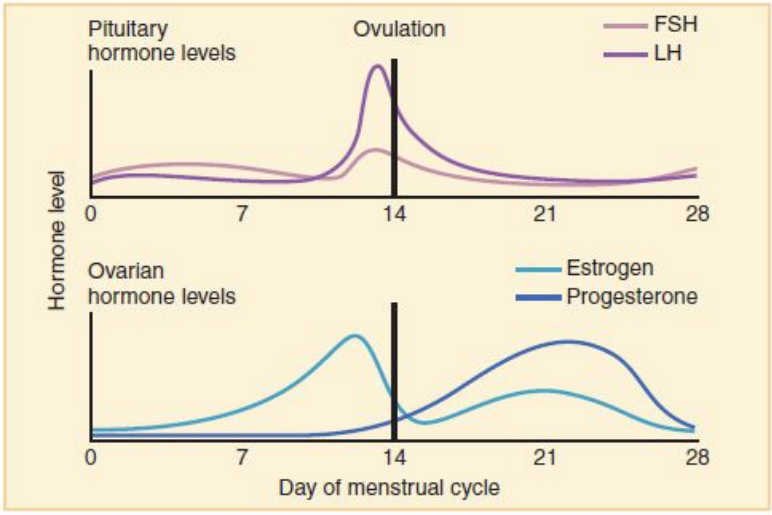


Figure 1.2. Hormone levels during the menstrual cycle. Level of pituitary (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) and ovarian hormones (oestrogen and progesterone) throughout the menstrual cycle. Source: OpenStax, Anatomy & Physiology. OpenStax CNX. Jun 25, 2018 http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@10.1. (OpenStax, 2018).

At the beginning of the follicular phase, the levels of oestrogen and progesterone are low. The top layers of the thickened lining of the uterus (endometrium) break down and are shed during menstruation. The FSH level increases slightly, stimulating the development of several follicles in the ovaries of which each contains an egg. As the level of FSH begin to decrease, only one follicle continues to develop. This follicle produces oestrogen causing an increase in oestrogen levels. This leads to a surge in LH and FSH levels which initiates the ovulatory

phase. The oestrogen level peaks during this surge, and the progesterone level starts to increase. During the luteal phase, LH and FSH levels decrease as a negative feedback response to the increased oestrogen levels. During most of this phase, the oestrogen level is high. Progesterone and oestrogen cause the lining of the uterus to thicken in preparation of potential fertilization. If the egg is not fertilized, the progesterone and oestrogen levels decreases, the top layers of the endometrial lining break down and a new menstrual cycle begins.

Hormonal contraceptives are divided into two main chemical groups: combination contraceptive methods, in which both an oestrogen and a progestin (synthetic analogue of progesterone) are present, and progestin-only methods (without any oestrogen derivatives). Progesterone with oestrogen naturally inhibits secretion of FSH and LH through a negative feedback mechanism (Shaw et al., 2010). This inhibition prevents the rise in FSH that is necessary to initiate follicle maturation and the LH surge needed to trigger ovulation. Another mechanism by which the steroid hormones in hormonal contraception prevent pregnancy is by interfering with the movement of sperm in the FGT by thickening of the cervical mucus thus making it harder for the sperm to reach the egg and by changing the lining of the uterus thereby hindering implantation (Rivera et al., 1999).

1.1.3 Hormonal contraceptive options and preferences in South Africa

There are different available options for effective, reversible hormonal contraception in the public sector of South Africa, including daily-administered combined oral contraceptive pills (COCPs), combined contraceptive vaginal rings (CCVRs) and injectable progestin-only contraceptives. The latter option is the most commonly used hormonal contraceptive method in South Africa (Sibeko et al., 2011; Ross & Agwanda, 2012) and includes depot medroxyprogesterone acetate (DMPA) and norethisterone enanthate (Net-En). These two injectables differ in the type of progestin they contain and the injected doses. Both injectales are given by intramuscular injection, DMPA at a does of 150 mg (in 1 ml) every three months and Net-En at a dose of 200 mg (in 1 ml) every two months. Although DMPA is the method of choice, Net-En use is on the rise in South Africa, especially among young women (Sibeko et al., 2011; Ross & Agwanda, 2012).

COCPs are generally less popular and less effective than the progestin-only injectables due to difficulty with adherence to daily-administered medication (Hooper, 2010; Mansour, 2014). The CCVR, such as the NuvaRing, an etonogestrel/ethinyl oestradiol vaginal ring, is a new alternative, short-term hormonal contraceptive method that has recently been licensed in South Africa. The CCVR is inserted into the vagina for three weeks followed by a one-week break to allow for menstrual bleeding. During the three-week insertion the hormones (oestrogen and progestin) are slowly released to the bloodstream through the vaginal wall. Due to its recent appearance on the market, the acceptance of CCVRs in a South African context is still to be determined and its usage is still very infrequent.

1.2 Hormonal contraception and HIV-1 risk

1.2.1 Injectable hormonal contraception and HIV-risk

The introduction of hormonal contraception for prevention of unintended pregnancies has markedly reduced the risk of maternal morbidity and mortality and of mother-to-child transmission of HIV worldwide (Cleland et al., 2006). However, despite its contraceptive benefits, concerns about the role of hormonal contraception in HIV susceptibility have recently grown. A number of observational studies have suggested that the use of certain HC may increase uninfected women's risk of HIV acquisition and transmission (Baeten et al., 2007; Crook et al., 2014; McCoy et al., 2013; Morrison et al., 2012; Heffron et al., 2012; Morrison et al., 2010; Martin et al., 1998), although the data have been inconsistent (Kleinschmidt et al., 2007; Morrison et al., 2007; Myer et al., 2007; Stringer et al., 2007). In a study of seronegative women of discordant couples, the use of any hormonal contraceptive method was associated with a two-fold risk of HIV acquisition (adjusted hazard ratio (aHR) 1.98, 95% CI 1.06-3.68) (Heffron et al., 2012). In a recent prospective observational cohort study, the HIV-1 incidence in South African women using DMPA and Net-En was 2.93 times higher than the incidence in those not using long-term contraception (Byrne et al., 2015). In other studies, an association with DMPA, but less so or none with Net-En and COCPs, with higher HIV-1 acquisition compared to non-hormonal contraceptive use have been observed (Crook et al., 2014; Lavreys et al., 2004;

Morrison et al., 2010; Wand & Ramjee, 2012; Richardson et al., 2007; Noguchi et al., 2015a). Young women (<25 years) who used DMPA appear to be at especially increased risk of HIV acquisition (aHR 2.76, 95% confidence interval (CI) 1.62-4.72) (Morrison et al., 2012, 2010). Furthermore, women using COCPs or injectables at the time of HIV-1 infection were more likely to become infected with multiple HIV variants (odds ratio (OR) 2.7, 95% CI 1.3–5.6) (Sagar et al., 2004). Infection with multiple HIV variants has been linked to increased viral load set-point and faster disease progression (Sagar et al., 2003). Also, in HIV-infected women, increased cervical shedding of HIV-1 DNA and RNA was found in women using injectables (Heffron et al., 2012; Mostad et al., 1997). Some studies have also suggested that male partners of HIV infected women using HC are faced with an elevated risk of acquiring HIV (aHR 1.97, 95% CI 1.12-3.45) (Heffron et al., 2012).

A number of recent systematic reviews examining the available evidence on hormonal contraception and HIV risk have suggested that progesterone only-injectable use, particularly DMPA, is associated with an increased risk of HIV (Ralph et al., 2015; Polis et al., 2016; Morrison et al., 2015). In one such meta-analysis by Ralph et al. (2015), the effect of DMPA, Net-En and COCP on HIV risk was evaluated in several observational studies all conducted in SSA. A pooled analysis of ten studies showed an elevated HIV acquisition risk in DMPA users as compared to women using non-hormonal or no hormonal contraceptive methods (pooled relative risk (RR) 1.40, 95% CI 1.16-1.69) (Ralph et al., 2015). In contrast, there was no evidence of an increased HIV risk in the ten studies examining COCPs (pooled RR 1.00, 95% CI 0.86-1.16) or in five studies investigating Net-En versus no hormonal contraceptive use (pooled RR=1.10, 95% CI 0.88-1.37) (Ralph et al., 2015). A similar outcome was recently reported in a meta-analyses by Polis et al. (2016) (Polis et al., 2016). In addition, these authors also included the results of two studies directly comparing HIV risk between different hormonal contraceptive options. Both of these reported a statistically significant increased risk of HIV for DMPA use (aHR: 1.32 and 1.41) compared to Net-En use (Polis et al., 2016; Morrison et al., 2015; Noguchi et al., 2015b). One of these studies, an individual participant data meta-analysis by Morrison et al. (2015), also compared each progestin-only injectable against

COCPs. A significantly increased HIV risk for DMPA users versus COCP users (aHR 1.43, 95% CI: 1.23–1.67) and a close to, but non-significant increased risk for Net-En versus COCPs users (aHR 1.30 (0.99–1.71) were reported in this study. The use of CCVRs has only recently been licensed in South Africa and evidence regarding CCVRs and HIV risk is still limited (Kapiga et al., 1998; Mati et al., 1995; Morrison et al., 2009; Polis et al., 2014).

1.2.2 Consequences of increased HIV risk

The global public health implications of a potential interaction between injectable use and HIV risk have recently been modelled (Jain, 2012; Rodriguez et al., 2012; Butler et al., 2013) (**Figure 1.3**). Based on these models, it was concluded that if use of progestin-only injectable hormonal contraceptives increases HIV acquisition risk by 1.2-2.19-fold (as suggested by observational data), it could result in 27,000–130,000 new HIV infections every year worldwide (Butler et al., 2013). Of these, it was estimated that nearly 90% would occur in SSA (Butler et al., 2013). Yet, these models also showed that discontinuation of progestin-only injectables without an equally effective reversible contraceptive method to replace it with would result in decreased life expectancy due to a significant increase in maternal deaths from unintended pregnancy (Jain, 2012; Rodriguez et al., 2012; Butler et al., 2013). In South Africa alone the increase in maternal deaths was estimated to be 246 per 100,000 women (Rodriguez et al., 2012).

Any false concern about increased HIV risk leading to restricted availability or use of progestin-only injectables was estimated to potentially cause 18,000 or more additional maternal deaths worldwide every year, and likely even greater maternal morbidity (Butler et al., 2013). Taken together, this strongly emphasizes the need to investigate the biological effects of different hormonal contraceptives methods on the FGT mucosa to get a clearer understanding of any interplay between hormonal contraceptive use and HIV risk. The World Health Organization (WHO) have concluded that there is still insufficient evidence to support a change in the current guidelines of no restriction on the use of progestin-only injectable contraceptives among women at high risk for HIV acquisition (World Health Organization, 2017). Yet, the WHO

states that women at high risk of HIV acquisition who are considering progestin-only injectables should be informed about the uncertainty of whether injectables increase HIV risk or not and be provided with access to and guidance on other HIV preventive measures, including male and female condoms.

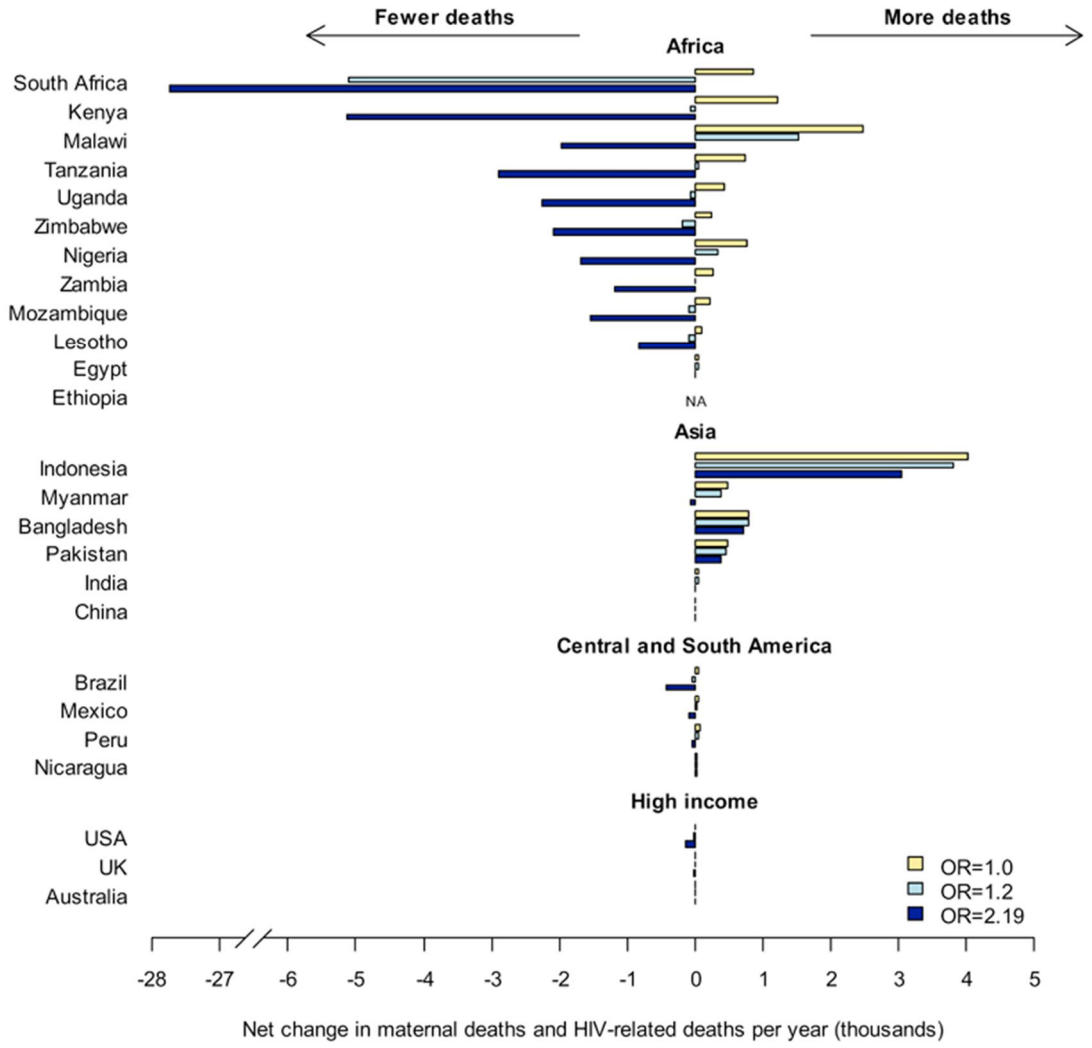


Figure 1.3. Effect on maternal deaths by withdrawing progestin-only injectables from family planning programs. The change in net maternal and HIV-related deaths on cessation of all injectable hormonal contraceptive use, assuming (1) risk ratio (RR)=1.0 (yellow bar), (2) RR=1.2 (light blue bar) and (3) RR=2.19 (dark blue bar), shown for the countries presented in figure. Source: (Butler et al., 2013).

Due to concerns about the impact of particularly progestin-only contraceptives on HIV susceptibility, some countries in SSA are considering withdrawing DMPA from their family planning programs (Ralph et al., 2015), which in conflict with WHO guidelines. However, withdrawing injectables, such as DMPA, is not an option in many countries because no alternative methods are available with

equivalent efficacy. As described before, in the case of South Africa alternative effective contraceptive methods are available. Compared to other SSA countries the HIV incidence is higher, while the detriment of unintended pregnancy is lower. Thus, use of alternative contraceptive methods are potentially beneficial in a South African setting.

1.3 The female genital tract microbiota and HIV risk

Several different biological mechanisms have been proposed by which hormonal contraception could potentially influence HIV-1 acquisition. These include anatomical changes (such as vaginal thinning and cervical ectopy) (Critchlow et al., 1995; Marx et al., 1996; Smith et al., 2004), immunological changes (such as increased genital tract inflammation) (Chandra et al., 2013; Ghanem et al., 2005; Ildgruben et al., 2003), increased co-receptor CCR5 expression (Chandra et al., 2013; Prakash et al., 2005, 2002; Byrne et al., 2015), recruitment of CCR5+ T cells and other HIV target cells to the FGT mucosa (Zang et al., 2002); and/or alterations in the vaginal microbiota (Miller et al., 2000a; van de Wijgert et al., 2013). Indeed, it is becoming increasingly accepted that the microbial composition in the vagina may have a dramatic impact on the FGT mucosal environment and HIV susceptibility (Atashili et al., 2008; Mitchell et al., 2013; Myer et al., 2005b; Taha et al., 1998).

1.3.1 An optimal vaginal microbiota and bacterial dysbiosis

Molecular studies of the vaginal microbiota of healthy, reproductive-age women have described a distinct group of common bacterial community types (Borgdorff et al., 2017; Ravel et al., 2011). In general, two to four low-diversity communities dominated by a single *Lactobacillus* species (*Lactobacillus crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii*) and one to two high-diversity communities, comprised of a mixture of facultative anaerobic bacteria, have been reported in these studies (Ravel et al., 2011; Gajer et al., 2012; Anahtar et al., 2015; Gautam et al., 2015). A vaginal microbiota dominated by *Lactobacillus* species have been considered optimal as lactobacilli are thought to protect the lower FGT from invading pathogenic microorganisms. *Lactobacillus* species restrict the growth of pathogens by competing for nutrients, by secretion of bacteriostatic and

microbicidal compounds and by maintaining an acidic environment (pH <4.5) through lactic acid production (Amabebe & Anumba, 2018). In vitro studies have suggested that the infectivity of cell-free HIV particles is dependent on pH. At an acidic pH, lactic acid could eliminate the negative surface charge on HIV, which may disrupt HIV surface protein structures and/or possibly inactivate the virus, while at neutral pH, HIV was able to maintain its surface charge (Lai et al., 2009). Furthermore, it has been reported that acidic cervicovaginal mucus, obtained from women with *L. crispatus*-dominated vaginal microbiota, efficiently traps HIV (Nunn et al., 2015).

Bacterial vaginosis (BV) is a genital condition characterized by the displacement of protective *Lactobacillus* species in the FGT with various anaerobic and facultative bacteria, including *Gardnerella vaginalis, Atopobium vaginae, Megasphaera, Prevotella* spp*., Sneathia* and BV-associated bacteria (BVAB) 1-3. BV is the most common vaginal disorder among reproductive-age women and the main symptoms are vaginal discharge and fishy vaginal odour. Diagnosis of BV includes clinical examination and microscopic scoring of bacterial morphotypes along with the presence of "clue cells" (vaginal epithelial cells coated with bacteria). The Nugent Score is a Gram stain scoring system of vaginal smears commonly used to identify BV. The Nugent score is determined by assessing the presence of large gram-positive rods (*Lactobacillus* morphotypes; decrease in lactobacilli scored as 0 to 4), small gram-variable rods (*G. vaginalis* morphotypes; scored as 0 to 4), and curved gram-variable rods (*Mobiluncus* species morphotypes; scored as 0 to 2). The total score ranges from 0 to 10 with a score of 0 to 3 being considered BV negative, 4 to 6 BV intermediate and 7 to 10 BV positive (Nugent et al., 1991).

Even though the majority of women affected by BV do not show any symptoms, having BV has been associated with an increased risk of urinary tract infections (UTIs) (Harmanli et al., 2000), pelvic inflammatory disease (PID) (Haggerty et al., 2004) and poor pregnancy outcomes, including preterm delivery, low birth weight and spontaneous abortions (Gupta et al., 2000; Thorsen et al., 2006; Afolabi et al., 2016). BV has also been implicated in increased risk of various STIs, including HIV-1 and *Chlamydia trachomatis* (Myer et al., 2005a; Taha et al., 1998; Wiesenfeld et al., 2003; Borgdorff et al., 2014). In

a meta-analysis including 14,874 participants from Eastern and Southern Africa, women with BV were at significantly increased risk for HIV acquisition compared to women with a *Lactobacillus*-dominated vaginal microbiota (aHR 1.53, CI 1.24-1.89) (Low et al., 2011). Similarly, a meta-analysis including studies in the US suggested that BV increases the risk for HIV-1 acquisition by 60% (CI 21-113) (Atashili et al., 2008). Furthermore, in a prospective study of 463 HIV-1-infected mothers and their infants, BV during pregnancy was associated with a three-fold increased risk (95% CI 1.0-7.0) of in-utero transmission of HIV-1 (Farquhar et al., 2010). In studies describing Molecular-BV, a non-optimal vaginal microbiota lacking lactobacillus species as characterized by molecular methods, have suggested that a larger proportion of asymptomatic women may be at increased risk of HIV (Gosmann et al., 2017; McClelland et al., 2018). As such, a recent prospective study in South Africa have reported that young women colonized with a highly diverse community had a 4.4-fold (95% CI = 1.17–16.61) increased risk of acquiring HIV compared with women with *Lactobacillus crispatus*-dominant microbiota (Gosmann et al., 2017).

1.3.2 Population-based differences in vaginal communities

The distribution and number of vaginal community types and the BV prevalence identified in a population vary depending on ethnicity. As such, high diversity vaginal bacterial communities not dominated by *Lactobacillus* species are more commonly found in African and Hispanic women compared to Caucasians and Asian women (Ravel et al., 2011). In a study conducted on the vaginal microbiota of North American women representing four ethnic groups: white, black, Hispanic, and Asian, Ravel et al. (2011) showed that *Lactobacillus* species were dominant in 89.7 and 80.2% of white and Asian women, respectively, but in only 59.6 and 61.9% of Black and Hispanic women. Similarly, Zhou et al. (2007) found *Lactobacillus* species to be dominant in 91% of white women but only in 68% of Black women (Zhou et al., 2007). These differences in vaginal community composition between women of different ethnicities have also been observed in subsequent studies done on the vaginal microbiota of women in Europe (Borgdorff et al., 2017; MacIntyre et al., 2015).

Recently, a number of studies on the vaginal microbiota of African women

have emerged (Gosmann et al., 2017; Anahtar et al., 2015; Lennard et al., 2017). In a study conducted in a population of reproductive-age South African women only 37% had lactobacilli dominated communities with *L. iners* being the most common species found (Anahtar et al., 2015). Similar results were seen in analyses of the FGT microbiota of young South African females (Gosmann et al., 2017; Lennard et al., 2017). These results have led to the hypothesis that population-based differences in vaginal microbiota may contribute to the disproportionate burden of HIV in African women (Buve A, Jespers V, Crucitti T, 2014; McClelland et al., 2018). In line with this hypothesis, recent studies have suggested that dysbiosis of the vaginal microbiota accounts for 20-30% of the population attributable risk of HIV in African women (Van De Wijgert et al., 2009; Masese et al., 2015).

1.3.3 Specific bacterial species and HIV risk

Due to high recurrence rates and inefficient treatment of BV (Sobel et al., 1993; Bradshaw et al., 2006), clinical interventions focusing on eliminating key FGT bacteria associated with BV have been suggested as an alternative approach for reducing women's susceptibility to HIV infection. However, the specific bacteria underlying the association between BV and HIV remain poorly understood. In a study from South Africa, a higher relative abundance of the BV-associated bacteria *Prevotella melaninogenica, Veillonella montpellierensis, Mycoplasma, Prevotella bivia,* and *Sneathia sanguinegens* was associated with increased risk of HIV acquisition (Gosmann et al., 2017). In a nested case-control study using data from five cohorts of at-risk African women, McClelland et al. (2018) investigated the association between the concentration of specific vaginal bacteria and risk of HIV acquisition (McClelland et al., 2008). They identified seven taxa with significant concentration-dependent associations with increased odds of HIV infection, including *Parvimonas* species type 1 and 2, *Gemella asaccharolytica*, *Mycoplasma hominis*, *Sneathia*, *Eggerthella* species type 1 and *Megasphaera* spp. (McClelland et al., 2008). Interestingly, these studies also showed an association of a lack of non-*iners Lactobacillus* species with increased HIV risk (Gosmann et al., 2017; McClelland et al., 2018). As stated previously, having a vaginal microbiota dominated by different *Lactobacillus* species has been associated

with health and protection against HIV. Recently, data have emerged suggesting that this protection may apply more specifically to non-*iners Lactobacillus*-dominated communities. Interestingly, van Houdt and colleagues recently found that Dutch women who tested positive for *C. trachomatis* infection after having been contact-traced by a *C. trachomatis* positive partner were more likely to have a *L. iners* dominant community type than a *L. crispatus* dominant community type (van der Veer et al., 2017) and that women with *L. iners* dominated microbiota had an increased susceptibility to *C. trachomatis* (van Houdt et al., 2017). As described previously, cervicovaginal mucus obtained from women with *L. crispatus*-dominated vaginal microbiota, were shown to efficiently traps HIV. However, mucus from *L. iners*-dominated communities were shown to trap HIV with intermediate efficiency (Nunn et al., 2015). These results indicate a possible lack of protection of *L. iners* against pathogens in the FGT. It has been suggested that *L. iners* may become a dominant part of the vaginal microbiota when the microbiota is in a transitional stage between normal and dysbiosis (Ferris et al., 2007; Jakobsson & Forsum, 2007) and that *L. iners* may predispose to development of an abnormal vaginal microbiota (Verstraelen et al., 2009; Santiago et al., 2012). Furthermore, re-colonisation of the FGT with *L. iners* after BV may be a risk factor for a recurrence of BV (Verstraelen et al., 2009; Santiago et al., 2012). The genome of *L. iners* contains a number of genes putatively involved in adaptation to the fluctuating vaginal environment (Macklaim et al., 2013; France et al., 2016), suggesting a high degree of niche specialization of this particular *Lactobacillus* species.

1.4 Impact of sex hormones on the vaginal microbiota and HIV risk

1.4.1 Development of the vaginal microbiota

Traditional culturing and microscopic analyses of bacteria in the FGT have shown that the vaginal microbiota is a highly dynamic community, which undergoes several significant changes over the course of a women's lifetime for instance in response to external factors such as sexual behaviour, antibiotic use and intravaginal practices, including douching (Schwebke et al., 1999; Fashemi et al., 2013; Hickey et al., 2013; Mayer et al., 2015). Although still unclear, it appears that the female sex hormones, including oestrogen and progesterone,

and the anatomical features of the vaginal epithelium play a key role in shaping the composition and stability of the vaginal microbiota over time. Oestrogen is associated with vaginal epithelium thickening and glycogen deposition in vaginal epithelial cells, which aid lactic acid metabolism and promote lactobacilli colonization (Farage & Maibach, 2006; Hillier, 1998). Progesterone has the opposite effect on the vaginal epithelium and glycogen deposition thus hindering colonization of lactobacilli (Hickey et al., 2015; Hillier, 1998).

Oestrogen passed on to the infant in utero from the maternal placenta creates favourable environment for *Lactobacillus* species colonization which persists for weeks after birth (Farage & Maibach, 2006). The effects of the maternal oestrogen decline after a few weeks of life and pre-pubertal females produce very little oestrogen, leading to low levels of glycogen and an increase in vaginal pH. The vaginal epithelium prior to puberty is a thin, stratified squamous epithelium covered with a thin layer of mucus. These hormonal and anatomical changes results in the proliferation of several aerobic and facultative anaerobic bacteria which persists throughout childhood (Thoma et al., 2011; Farage & Maibach, 2006; Hill et al., 1995; Hickey et al., 2015). During puberty and menarche, the production of oestrogen rapidly rises with a resultant increase in the level of free glycogen and the vaginal epithelium thickens and becomes covered by a thick layer of mucus. These changes leads to proliferation of *Lactobacillus* species, acidification of the vaginal environment (pH~4.0-4.5) and the displacement of the facultative anaerobic bacteria (Thoma et al., 2011; Alvarez-Olmos et al., 2004; Yamamoto et al., 2009). However, sexual debut which typically occur during adolescence is associated with an inflammatory vaginal reaction (Jespers et al., 2016) and the presence of semen markers has been found to negatively influence the prevalence of *Lactobacillus* species (Fethers et al., 2009; Jespers et al., 2015).

Throughout a woman's reproductive years, the vaginal microbial community is highly affected by the menstrual cycle, which is characterized by fluctuating levels of oestrogen and progesterone. At the beginning of menstruation, the proportion of *Lactobacillus* species decreases and the microbiota becomes less stable with higher levels of *G. vaginalis* (Eschenbach et al., 2000b; Gajer et al., 2012; Hickey et al., 2013; Srinivasan et al., 2010; Santiago

et al., 2012). The high mid-cycle oestrogen levels are associated with increased glycogen production and increased *Lactobacillus* proliferation (Eschenbach et al., 2000b; Gajer et al., 2012; Hickey et al., 2013; Srinivasan et al., 2010). This observation is also seen during pregnancy where oestrogen levels increase dramatically due to additional production by the placenta (Romero et al., 2014; Walther-Antonio et al., 2014; Verstraelen et al., 2009). Oestrogen levels decrease post-partum and concomitant changes in communities have been described, with lactobacilli becoming less dominant (MacIntyre et al., 2015; Doyle et al., 2018). Following menopause, the abundance of *Lactobacillus* species rapidly declines and the vaginal pH once more increases, potentially due to decreased oestrogen levels and reduced glycogen production (Galhardo et al., 2006; Pabich et al., 2003). The vaginal epithelium resembles the pre-puberty stage, with fewer layers and a thinner mucus layer. The changes in the vaginal microbiota and epithelium throughout a woman's lifetime is summarised in **Figure 1.4** (Petrova et al., 2013).

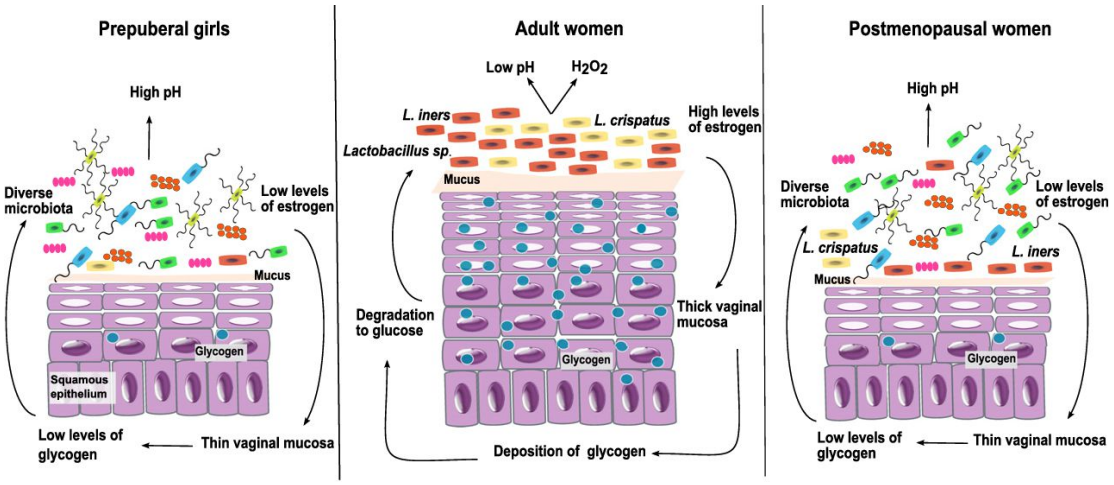


Figure 1.4. Changes in the vaginal microbiota and epithelium during different stages of a woman's lifetime. Figure from (Petrova et al., 2013).

1.4.2 Role of glycogen and amylase in composition of the vaginal microbiota

During exfoliation of the epithelial cells, free glycogen is released into the genital environment. Free glycogen in genital fluid is associated with a genital microbiota dominated by *Lactobacillus* (Mirmonsef et al., 2014) and several *Lactobacillus* species have been shown to grow on small oligomers produced through depolymerisation of glycogen by α-amylase. Vaginal secretions have

been shown to exhibit amylase-like activity and to depolymerize glycogen (Spear et al., 2014). Yet, it is not clear whether the amylase in vaginal secretions originates from the human host, from one or more bacterial populations in vaginal communities, or both (Spear et al., 2014). It was recently reported that *L. iners* differentially expresses genes responsible for breakdown of glycogen under vaginal conditions (Macklaim et al., 2013). Nasioudis et al. (2015) conducted a study to evaluate α-amylase levels in women with BV or vulvo-vaginal candidiasis (Nasioudis et al., 2015). Their results showed the lowest α-amylase levels in women with BV and the highest levels in healthy women without any vaginal condition. The authors did not look into the composition of the vaginal communities; thus, direct association of α-amylase levels with particular *Lactobacillus* species is difficult (Nasioudis et al., 2015).

1.4.3 HIV risk according to fluctuations in endogenous hormones

Recent data, primarily from non-human primate studies have suggested that the susceptibility to HIV/simian immunodeficiency virus (SIV*)* changes over the course of a menstrual cycle with an enhanced risk of infection during the high endogenous progesterone luteal phase compared to the oestrogen-dominant follicular phase (Venkatesh & Cu-Uvin, 2014; Vishwanathan et al., 2011; Kersh et al., 2014; Marx et al., 1996). Pregnancy, a state characterised by increased levels of progesterone, has been associated with two-fold increased risk of HIV acquisition(Mugo et al., 2011). Furthermore, postmenopausal women have a 4–8-fold higher HIV risk when compared to similar premenopausal populations (Aaby et al., 1996; European Study Group on Heterosexual Transmission of HIV., 1992). In a study using *in vitro* explant cultures of human cervical tissue it was shown that HIV more productively infects explants collected during the luteal phase than those collected during the follicular phase (Saba et al., 2013). The mechanisms responsible for the enhanced susceptibility is still poorly understood, however, enhanced recruitment of HIV target cells to the FGT, decreased integrity of the protective vaginal epithelial layers and reduced local innate antiviral and adaptive secretory immune responses during the high-progesterone luteal phase have been suggested (Byrne et al., 2016; Birse et al., 2015; Vishwanathan et al., 2015; Goode et al., 2014).

1.5 Hormonal contraceptive use and the vaginal microbiota

1.5.1 Impact of hormonal contraceptives on the vaginal microbiota

To date, most studies that have investigated the effect of hormonal contraceptives on the vaginal microbiota have primarily been based on either clinical signs of BV, the Nugent score or levels of limited members of the bacterial community as measured by quantitative polymerase chain reaction (qPCR) (Vodstrcil et al., 2013; van de Wijgert et al., 2013). Some of these studies observed subtle shifts and changes in women on hormonal contraceptives including enhancement of *Lactobacillus* species or reduction in total bacterial load and *G. vaginalis* (Roxby et al., 2016; Mitchell et al., 2014; Borgdorff et al., 2015; Kazi et al., 2012). Others have demonstrated a decrease in BV incidence in women using both COCP and DMPA (Bradshaw et al., 2013b; Baeten et al., 2001; Van De Wijgert et al., 2008; Riggs et al., 2007; Koumans et al., 2007; Pettifor et al., 2009). In one study in which Net-En was evaluated as a separate contraceptive group, a reduction in BV prevalence was also found (Pettifor et al., 2009). A small number of prospective-cohort studies have evaluated vaginal microbiota in COCP users in which it was reported that the vaginal microbiota was unchanged one to two months after initiating COCP use (Gupta et al., 2000; Eschenbach et al., 2000a; Donders et al., 2017). Two recent studies conducted in Zimbabwean and Kenyan women found no association between injectable contraceptive usage and changes in microbiota (Birse et al., 2017; Achilles et al., 2018). Yet, two systemic reviews completed by van de Wijgert et al. (2013) and Vodstrcil et al. (2013) concluded that both COCP and DMPA use were associated with a decrease in BV (van de Wijgert et al., 2013; Vodstrcil et al., 2013). Recently, a retrospective study conducted on 16S rRNA gene sequencing data of vaginal samples from 692 participants from the Human Vaginal Microbiome Project who reported using either condoms, COCPs, DMPA or the intrauterine system (LNG-IUS), reported that women using COCs (adjusted Odds Ratio (aOR): 0.29, 95% CI 0.13-0.64) and DMPA (aOR 0.34, 95% CI 0.13-0.89), but not LNG-IUS (aOR 1.55, 95% CI 0.72-3.35), were less likely to be colonized by BV-associated bacteria relative to women who used condoms (Brooks et al., 2017). Furthermore, women using COCs (aOR 1.94, 95% CI 1.25-3.02) were more likely to be colonized by beneficial *Lactobacillus* species compared with women using

condoms (Brooks et al., 2017). Of note, none of these studies were randomized and studies of the effects of contraception are always potentially biased due to sexual behaviour and condom usage.

To date, there is limited data available on the impact of CCVRs on the vaginal bacterial community. In studies using microbial culture and gram staining methods, no significant changes in vaginal bacteria among healthy, sexually active European women using CCVR for up to six months were observed (Davies et al., 1992; Roumen et al., 1996; Roy et al., 1981; Schwan et al., 1983). In two studies assessing the effect of CCVRs and COCPs, in which women were randomized to or requested to receive either CCVRs or COCPs, participants using CCVR compared to COCP users showed an increase of hydrogen peroxide (H_2O_2)-producing lactobacilli species as assessed by colony forming units (CFUs) (Veres et al., 2004; De Seta et al., 2014). However, in a randomized trial of 500 women comparing the vaginal microbiota of women using CCVRs or hormonal contraceptive patches, there was no statistically significant change in vaginal microbiota over time as assessed by Nugent score (Creinin et al., 2008). In a recent study, assessing the impact of a reusable CCVR (up to thirteen cycles) on the vaginal microbiota, the Nugent scores did also not differ over time, yet a non-significant prevalence increase in H_2O_2-positive *Lactobacillus*-dominated vaginal microbiota from 76.7% at baseline to 90.2% at cycle thirteen was observed (Huang et al., 2015b). Furthermore, in a recently published study, the impact of NuvaRing use on the incidence of BV and selected vaginal bacterial species was investigated in a population of Rwandan women with a high BV prevalence followed for 12 weeks after initiating ring use (Crucitti et al., 2018). A mean decrease in Nugent score was observed with ring use and while the concentration of *Lactobacillus* species in vaginal secretions increased significantly, the concentration of *G. vaginalis* and the presence of *A. vaginae* decreased significantly. Like the studies on DMPA and COCPs, these data suggest that use of CCVR may decrease the prevalence of BV, however, these data should be interpreted with the same precautions as mentioned above.

1.6 Impact of the vaginal microbiota on genital tract immunity

Any disturbance to the FGT microbial community induced by hormonal contraception may lead to depletion of certain species and enrichment of others, including enrichment of members with specific virulence, biofilm formation or specific metabolic potential traits that could directly impact an individual's susceptibility to HIV and other STIs. Another way whereby hormonal contraceptive-induced changes to the FGT microbiota could indirectly influence HIV susceptibility is through alterations in the local immune environment of the FGT mucosa. Different mechanisms by which such alterations could affect HIV acquisition risk include increased genital tract inflammation (Chandra et al., 2013; Ghanem et al., 2005; Ildgruben et al., 2003) and increased recruitment and activation of HIV target cells to the FGT mucosa (Byrne et al., 2015; Prakash et al., 2005, 2002; Zang et al., 2002).

1.6.1 Immune cell populations, commensal bacteria and HIV risk

CD4+ T cells represents the primary target cells for initial HIV infection in the FGT (Schacker et al., 2001). HIV infects discrete subsets of CD4+ T cells all of which express phenotypic receptors and co-receptors necessary for HIV to gain intracellular access with CCR5 being the predominant target co-receptor for initial infection (Hladik et al., 2007; Saba et al., 2010). Activated, proliferating CD4+ T cells are particularly susceptible to infection and support viral replication (Grossman et al., 2006; Zhang et al., 1999). Little data is available that directly link the composition of the vaginal microbiota with changes in immune cell populations in the genital tract. However, the level of mucosal activated CD4+ T cells and CD4+ T cells expressing CCR5 have been shown to be increased in women with BV (Thurman et al., 2015; Gosmann et al., 2017). Furthermore, Rebbapragada et al. (2008) reported that when 15 HIV infected women with BV were successfully treated with oral metronidazole, there was a significant decrease in activated CD4+ T cells in cervical cytobrush samples obtained before and after treatment (Rebbapragada et al., 2008). Accordingly, both in vitro and animal models have shown that an acidic environment, characteristic of a BV negative, lactobacilli-dominant microbiota, result in decreased proliferation and

motility of CD4+ T cells (Hill & Anderson, 1992; Olmsted et al., 2005). In a study including women enrolled in the Miami Women Interagency HIV Study (WIHS), the level of gamma delta 1 (GD1) and GD2 T cells in the cervix of HIV negative women was analysed and related to BV status (Alcaide et al., 2016). The level of cervical GD1 T cells was significantly higher in BV negative women compared to BV positive women while the opposite was found for cervical GD2 T cells. GD1 T cells in the FGT play an important role in epithelial barrier protection while GD2 T cells are involved in humoral immunity and have been described as HIV target cells (Alcaide et al., 2016). Thus, the increased level of GD2 T cells and other CD4+ T cells expressing CCR5 among women with a dysbiotic microbiota could predispose women with BV to HIV acquisition (Alcaide et al., 2016).

1.6.2 Th17 cell regulation and HIV

Growing evidence have implicated T helper type 17 (Th17) cells, a distinct lineage of CD4+ T cells enriched in mucosal tissues, as key target cells for HIV infection and replication during sexual transmission (Stieh et al., 2016; Gosselin et al., 2011; McKinnon et al., 2011; Monteiro et al., 2011). Th17 cells are characterized by interleukin-17 (IL-17) production, although they also secrete other cytokines including IL-21 and IL-22 and express surface markers such as CCR4, CCR6 and CD161 (Korn et al., 2009). IL-17 can be pro-inflammatory, inducing the expression of other pro-inflammatory cytokines and chemokines by various cell types (Ouyang et al., 2008). Furthermore, IL-17 serves as a chemoattractant of immune cells including neutrophils (Laan et al., 1999; Miyamoto et al., 2003), increase the production of antimicrobial peptides (Kao et al., 2004; Liang et al., 2006) and support the maintenance of tight junctions and the survival and regeneration of epithelial cells in the gut (Kinugasa et al., 2000). The IL-17 cytokine family includes six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F, of which Th17 cells mainly produce IL-17A and IL-17F. IL-17A and IL-17F both mediate pro-inflammatory responses with certain differences depending on the type and site of inflammation (Ishigame et al., 2009). Although Th17 cells are the major source of IL-17A and IL-17F, recent studies have also identified other contributors to IL-17A and IL-17F production, mainly in the innate arm of the immune system (Cua & Tato,

2010). Th17 cells represent a major immune cell subset in the FGT (Rodriguez-Garcia et al., 2014; McKinnon et al., 2011) and were recently shown to be the primary targets of SIV during vaginal transmission in macaques (Stieh et al., 2016). Th17 cells express high levels of CCR5 (Rodriguez-Garcia et al., 2014; McKinnon et al., 2011), lack HIV-inhibitory RNAses (Christensen-Quick et al., 2016) and have generally been described as highly permissive to HIV infection (Gosselin et al., 2011; Monteiro et al., 2011; Hed et al., 2010). Studies in humans and macaques have shown that Th17 cells are depleted from the gut during HIV and SIV infection (McKinnon et al., 2011; Hed et al., 2010; Cecchinato et al., 2008; Favre et al., 2009). It has been suggested that depletion or loss of function of Th17 cells at mucosal surfaces compromise the mucosal defences leading to translocation of microbial products to the systemic compartment, resulting in immune activation and disease progression (Brenchley et al., 2006; Cecchinato et al., 2008; Ryan et al., 2016).

Commensal bacteria have been shown to play an essential part in the differentiation of intestinal Th17 cells (Sawa et al., 2011; Shaw et al., 2012; Zaph et al., 2008; Ivanov et al., 2008). This is emphasized by the near absence of Th17 cells in germ-free mice, the inhibition of Th17 differentiation in mice treated with selective antibiotics and the acquisition of Th17 cells in mice lacking these cells prior to co-housing with Th17 cell-sufficient mice (Ivanov et al., 2008). Gut homeostasis is largely achieved by a balance between Th17 cells and regulatory T cells (Tregs) (Sehrawat & Rouse, 2017). While the accumulation of tryptophan catabolites facilitate the differentiation of Tregs, it inhibits the development of Th17 cells (Favre et al., 2010). Indoleamine 2,3-dioxygenase (IDO) is a rate-limiting enzyme that mediates the catabolism of tryptophan, and is found in antigen-presenting cells and activated after Toll-like receptor signalling (Favre et al., 2010). In a study of SIV infected rhesus macaques, a depletion of gut-resident *Lactobacillus* correlating with increased IDO activity and Th17 loss was observed during acute and chronic infection (Vujkovic-Cvijin et al., 2015). Supplementing the SIV-infected macaques with a *Lactobacillus*-containing probiotics lead to a decrease in IDO activity and an associated increase in Th17 levels, suggesting that gut-resident *Lactobacillus* species can inhibit primate IDO in favour of Th17 cells (Vujkovic-Cvijin et al., 2015). In another study of antiretroviral (ARV)-

treated, SIV-infected pigtailed macaques, it was demonstrated that probiotic and IL-21 supplementation of ARV was associated with increased Th17 frequencies and reduced microbial translocation compared to infected controls receiving ARVs alone (Ortiz et al., 2016).

Women with bacterial STIs, specifically *C. trachomatis* and *N. gonorrhoea*, have been shown to have higher genital IL-17 concentrations than women without any STIs (Masson et al., 2015b). Similarly in mice models, IL-17 was produced in response to *Neisseria gonorrhoea* and *C. trachomatis* infection and infection was prolonged in the absence of IL-17 responses (Feinen et al., 2010; Scurlock et al., 2011; Vicetti Miguel et al., 2016). Together, these data suggest an important role for Th17 in response to bacterial STIs. Th17 cells also play an essential role in murine defence against fungal pathogens, including *Candida albicans* (Pandiyan et al., 2011; Pietrella et al., 2011). Accordingly, women with *Candida* infections were found to have lower IL-17 concentrations compared with women without (Masson et al., 2015b). Research exploring how changes in the vaginal microbiota impact the numbers, activation status and co-receptor expression of genital immune cell populations, such as HIV-permissive Th17 cells, is important to establish a mechanistic link of how vaginal microbiota changes could potentially affect HIV susceptibility.

1.6.3 The vaginal microbiota and mucosal inflammation

Heterosexual transmission models suggest that HIV in the male ejaculate must overcome numerous innate and adaptive immune factors in the vaginal lumen (Carias et al., 2013). Inflammatory responses are beneficial and often required to effectively eliminate STIs, yet women with elevated genital tract inflammatory markers, including pro-inflammatory cytokines, prior to HIV exposure are at increased risk of acquiring HIV (Masson et al., 2015a). Masson et al. (2015a) observed a threefold increased risk of HIV-1 infection in South African women who had elevated levels of at least five mucosal pro-inflammatory cytokines, including interleukin (IL)-8, IL-1β, IL-1α and TNF-α (Masson et al., 2015a). Elevated cervicovaginal lavage (CVL) concentrations of IL-1β, IL-6, IL-8 and soluble CD40L (sCD40L) have also been associated with increased risk of HIV acquisition in South African women (Mlisana et al., 2012). Furthermore, a higher

level of pro-inflammatory proteins, particularly RANTES and lower secretory leukocyte protease inhibitor (SLPI), were associated with HIV seroconversion in women from Uganda and Zimbabwe (Morrison et al., 2014). Inflammation has been shown to activate HIV replication and to recruit CCR5+ activated HIV target cells thus contributing to the increased risk of HIV acquisition (Klatt et al., 2013).

High-diversity microbial communities have been shown to correlate with genital pro-inflammatory cytokine concentrations (Lennard et al., 2017; Gautam et al., 2015; Anahtar et al., 2015; Gosmann et al., 2017). In a study by Gosmann et al. (2017), *L. crispatus* and, to a lesser degree, *L. iners* were associated with reduced inflammation while *Prevotella*, *Sneathia* and other anaerobes associated with BV and HIV risk were associated with increased inflammation (Gosmann et al., 2017). Similar results were observed by Lennard et al. (2017). Accordingly, women with BV have increased levels of pro-inflammatory cytokines declining with successful treatment and restoration of a lactobacilli-dominant community (Rebbapragada et al., 2008; Losikoff et al., 2007; Yudin et al., 2003). In vitro studies have shown a lack of cytokine release from vaginal epithelial cells stimulated with lactobacilli species, while BV-associated bacteria induce a strong pro-inflammatory response (Eade et al., 2015; Anahtar et al., 2015). Furthermore, genital lactic acid was found to induce an anti-inflammatory response from cervicovaginal epithelial cells through the production of IL-1RA, and the inhibition of IL-6, IL-8, TNF-α, RANTES, and macrophage inflammatory protein (MIP)-3α (Hearps et al., 2017). H_2O_2-producing lactobacilli have also been associated with lower levels of the inflammatory cytokine IL-1β (Mitchell et al., 2015). These data suggest that microbial diversity and specific BV-associated bacteria may be capable of inducing inflammatory responses in the vaginal mucosa, which could potentially modify susceptibility to STIs, including HIV.

1.7 Molecular analysis of microbial communities

1.7.1 Analysing the human microbiome

For decades, the characterization of the microbial communities living in and on the human body, including the FGT, were limited to information gained from culture- and microscopy-based studies. Although the knowledge gained from

many of these studies have markedly increased our understanding of the pivotal role not only pathogens but also commensal microbes play in human health and disease, culture techniques can be very laborious and time-consuming and the majority of human-associated bacterial species have not yet been successfully cultivated. Molecular methods improved our understanding but were limited by the need to cultivable organisms for targeted identification of specific organisms. The development of cost-effective high-throughput next generation sequencing (NGS) technologies has significantly advanced our understanding of what constitutes a normal and a dysbiotic human microbiota and their association with disease. With the application of high-throughput NGS methods it is possible to analyse hundreds of samples in a single run with an unprecedented depth providing large amounts of information on the microbial species present in the human body, including the vagina (Methe, 2012). Because genomic sequencing techniques are capable of analysing DNA isolated directly from environmental samples, bacterial culturing is not necessary for analysis.

Molecular-based characterization of microbial communities is commonly done by sequencing of specific marker genes that have been amplified by polymerase chain reaction (PCR) or by whole-genome shotgun metagenomics in which the full genomes of a community are sequenced. The most widely used approach to study microbial communities is based on the 16S ribosomal ribonucleic acid (rRNA) gene, which is ubiquitous to all bacteria. The 16S rRNA gene is comprised of conserved stretches of sequence interspersed with nine hyper-variable sequence regions (V1-V9). Universal primers targeting the different conserved regions are used to amplify either selected hypervariable regions or the whole 16S rRNA gene for sequencing while the sequences of the hypervariable regions are used to assign taxonomic identity of the microorganisms (Yang et al., 2016). Bioinformatics analysis of marker gene amplicon sequencing data includes correction for chimeras (hybrid products between sequences of separate origin) and other sequencing artefacts in the sequencing data. Sequencing reads are grouped into operational taxonomic units (OTUs), groups of closely sequences, typically at a 97% sequence similarity threshold. The generated OTUs are used as proxies for bacterial species and bacterial taxonomy is typically assigned to these by comparing the sequences to

16S rRNA reference databases such as Greengenes or Silva. An OTU table with taxonomic assignment is used in downstream analyses of the sequencing data.

1.7.2 Downstream analyses of microbiome data

There is a high level of similarity between the community data generated in human microbiome studies and classical ecological community research. Like ecology-type community data, human microbiome data is often non-normal, zero-rich, and highly skewed and special analytical methods are often a requirement for accurate sequencing data analyses. Many of the same analytical approaches commonly applied in classical ecology analyses have therefore been adapted for human microbiome analyses.

Ecological analysis of sequencing data is primarily centred around alpha and beta diversity measures. Alpha diversity is a quantitative measure of the diversity of a bacterial community within a sample and can be compared across sample groups. It is usually expressed by the number of species (i.e. species richness) or the distribution of OTUs (i.e. diversity). The Shannon Diversity Index is a commonly used diversity measure, which describes both the species richness (the number of different taxa) and the species evenness (the proportion of each species) in a single value. A high Shannon Diversity Index can thus both represent microbial community with a high number of taxa of varying proportions or a community with an even distribution of a limited number of taxa. Beta diversity is a measure that describes the degree of similarity (or dissimilarity) of a community feature (e.g. taxa or genes) between samples based on either phylogenetic (e.g. UniFrac (Lozupone & Knight, 2005)) or count-based distance measures (e.g. Bray-Curtis (Bray & Curtis, 1957; Faith et al., 1987)). As such, beta diversity measures compare dissimilarity between each pair of samples, generating a distance matrix of beta diversity distances between all pairs of samples. Quantitative metrics (e.g. Bray–Curtis and weighted UniFrac) include feature abundance in calculations, whereas qualitative metrics (e.g. unweighted UniFrac) only consider the presence or absence of the feature. Principal coordinates analysis (PCoA) is a commonly applied ordination technique for visualization of microbial data based on the beta diversity measure. Each sample is presented as a data point in the PCoA and the spatial

distance between points can then be interpreted as the relative difference in community composition between the samples. Hence, points that are closer to each other represent samples that are more similar in their bacterial composition than samples represented by points further apart in the plot. In PCoA the samples are typically projected into a two-dimensional coordinate system for visualization purposes.

Cluster analysis refers to an unsupervised machine learning method that partitions the observations in a data set into a limited number of discrete groups, or clusters, where each observation belongs to one cluster. The goal of cluster analysis is to cluster observations into subsets based on their similarity so that data points assigned to the same cluster are more similar to each other than to data points within other clusters. Cluster analyses can be achieved by various algorithms that differ in their definition of a cluster and how they each cluster is generated and assigned observations. Two of the most commonly applied clustering approaches include hierarchical clustering (Glynn, 2005) and *k*-mediod clustering (Kaufman & Rousseeuw, 1987). Hierarchical clustering of samples or taxa is done by aggregating individual units into progressively larger clusters producing a dendrogram of the similarities based on the chosen distance measure (e.g. Bray-Curtis or UniFrac) between clusters at each step. In contrast, *k*-medoid clustering aims to partition the observations in a dataset into a distinct number of clusters, *k*, in which each observation belongs to the cluster for which it has the closest distance to an observation designated as the centre of that cluster. The most common version of *k*-medoid clustering is Partitioning Around Medoids (PAM) (Kaufman, 1990). PAM clustering can determine the optimal configuration of *k* clusters in data points for which distances have been measured by for instance Bray-Curtis or UniFrac. The optimal cluster configuration occurs when the distances between data points within clusters are as small as possible. Selection of *k* can be done by iterating of the PAM clustering algorithm with different *k*´s. Evaluation of optimal *k* is then performed by silhouette plots, in which the length of the silhouettes reflects how well each individual data point is assigned to the clusters within each iteration. Fuzzy clustering is another form of *k*-medoid clustering in which each observation can belong to more than one cluster with differing probabilities. For each

observation, a membership grades indicating the degree to which that observations belong to each cluster, are assigned. As for PAM clustering, selection of *k* can be done by iterating the Fuzzy clustering algorithm with different *k´*s and evaluating of the resultant silhouette widths.

Random forest analysis is another machine learning method applicable to analysis of sequencing data. Random forest is a type of data mining algorithm that can select from among a large number of variables, e.g. bacterial taxa, those that are most important in predicting the response variable of interest. Another common analysis approach in microbiome analyses is to look at differentially abundant features (e.g. microorganisms) in two or more comparison groups of interest. Permutational multivariate analysis of variance (PerMANOVA) can be used for assessing significant beta diversity clustering between groups. PerMANOVA is a statistical test used to test the null-hypothesis of no-difference between the bacterial communities according to category of interest. PerMANOVA is a permutation-based version of the multivariate analysis of variance (Anderson, 2005). It is a non-parametric test, which uses the distances between samples to partition variance and randomizations or permutations of the data to produce the p-value for the hypothesis test.

1.8 Study aims and Objectives

1.8.1 Aims

The specific aims of this study were 1) to describe the genital tract microbiota of South African females during adolescence and in the context of endogenous hormone levels; 2) to determine the impact of hormonal contraceptive use on the diversity and composition of the female genital tract microbiota; and 3) to assess whether hormonal contraceptive-induced changes to the vaginal microbiota affect the numbers, activation status and co-receptor expression of genital immune cell populations and the inflammation status of the genital tract mucosa to establish a biological basis of how hormonal contraception and the vaginal microbiota potentially alters HIV susceptibility.

1.8.2 Objectives

The specific objectives of the research were: 1) to characterize the genital tract microbiota of South African adolescent females using 16S rRNA amplicon sequencing and to relate this to endogenous hormone levels and the presence of sexually transmitted infections; 2) to describe any changes in diversity and composition of the adolescent female genital tract microbiota in response to three different hormonal contraceptive options; combined oral contraceptive pills (Nordette or Triphasil), a progestin-only injectable (Net-En), and a combined contraceptive vaginal ring (NuvaRing) in a randomized cohort; and 3) to relate any changes in the female genital tract microbiota with differences in inflammatory cytokine levels and CD4+ T cell frequencies and activation patterns in the female genital tract mucosa.

1.8.3 Hypotheses

I expected the microbial communities of the female genital tract of South African adolescents to be characterized by a low lactobacilli abundance and high bacterial diversity. I hypothesized that the use of a progestin-only hormonal contraceptive options would alter the female genital tract microbiota resulting in microbial dysbiosis and that progestogen-induced microbial dysbiosis in return would cause an increase in local mucosal inflammation and an increase in

activation and recruitment of HIV target cells. I further expected that the use of combined hormonal contraception would have less effect on the female genital tract microbiota due to the presence of oestrogen, resulting in lower levels of genital inflammation and activated HIV target cells within the cervix of adolescent females.

Chapter 2: Materials and Methods

2.1 Study cohorts and sample collection

For this PhD dissertation, data and samples from two different adolescent cohorts were used. One cohort, WISH (2.1.3) was used to address the part of the first research question, to characterize the genital tract microbiota, both vaginal and endocervical, of South African adolescent females using 16S rRNA amplicon sequencing and to relate this to the presence of STI, particularly *C. trachomatis*. In this study, there was no intervention being tested, which could have confounded the relationship between *C. trachomatis* and vaginal microbiota. The second cohort, uCHOOSE-A-Star (2.1.1), was used to address the second and third objectives to describe any changes in diversity and composition of the adolescent female genital tract microbiota in response to three different hormonal contraceptive options; and to relate any changes in the female genital tract microbiota with differences in inflammatory cytokine levels and CD4+ T cell frequencies and activation patterns in the female genital tract mucosa. The rational behind this was based on the study design of each cohort and the samples available for analysis.

2.1.1 uCHOOSE-A-Star study design

The participants for the uCHOOSE-A-Star (uCHOOSE add on study to access risk) cohort were recruited from uCHOOSE, an open-label, randomized crossover study with the purpose of evaluating the feasibility of different hormonal contraceptive options among adolescent females attending the Desmond Tutu HIV Foundation (DTHF) Youth Centre in Masiphumelele, Cape Town. Masiphumelele is a low income, high-density area with a high HIV prevalence (30%) placing the local youth at high risk of HIV. Recruitment of participants took place from the family planning clinic and other clinics housed within the community centre. Potentially eligible study participants came in for a screening visit at which all participants 18 years or older provided informed consent, while informed assent from the participant and informed consent from a parent or a legal guardian were obtained for those younger than 18 years. Eligibility criteria for enrolment included being HIV-negative, non-pregnant, either contraception naive or on a short-term hormonal contraceptive option (within 30 days or less of needing a new supply of contraception) and willing to change method, no

symptomatic STIs within the prior 40 days, no known sensitivity to any of the study products, and no intentions of becoming pregnant throughout the study period. Furthermore, the adolescents should be willing to abstain from inserting any non-study products or objects into the vagina throughout the duration of study. If eligible, the participants returned to the clinic for enrolment within 40 days of the screening visit. At enrolment participants were randomly assigned in a 1:1:1 ratio to one of three study arms described below:

- **Arm 1/Group A:** Participants assigned to this group were supplied with one of two combined oral contraceptive pills (COCPs: Triphasil or Nordette; both containing ethinyl oestradiol and levonorgestrel) and were required to take a daily tablet for 21 days each month and a placebo tablet for 7 days (day 22-28) each month, for a 16-week period.
- **Arm 2/Group B:** Participants assigned to this arm received a combined contraceptive vaginal ring (NuvaRing; containing etonogestrel/ethinyl oestradiol, which delivers 0.120mg/0.015mg per day) to be inserted once every 28 days (and removed after 21 days of each 28 day insertion) for a 16-week period.
- **Arm 3/Group C:** Participants assigned to this group received a progestin-only injectable (Net-En; containing 200mg of the progestogen norethisterone enantate) once every 8 weeks for a 16-week period.

After 16 weeks, the participants returned for a crossover visit at which they switched over to another hormonal contraceptive option for the final four months of the study. Participants initially assigned to Net-En or the daily COCPs switched over to the NuvaRing, while participants receiving the NuvaRing as the first method were given the choice between either Net-En or the daily COCPs as their second method. After a total of 32 weeks the participants returned for a final visit at the clinic and subsequently exited the study.

At all study visits (screening, enrolment, crossover and exit), a rapid HIV test was performed at the clinic. If a participant seroconverted during the duration of the study she was counselled and referred for HIV management while allowed to continue her participation in the parent study albeit mucosal

sampling would no longer be performed. A pregnancy test was also performed at all visits. If a participant became pregnant during the study, she would be discontinued from the study and referred to appropriate counselling and care. A detailed interviewer-assisted questionnaire assessing medical/health history, sexual behaviour, last menstrual cycle, adherence to study product, intra-vaginal practices, adverse experiences and antibiotic use was completed at all visits. Participants were enrolled in the uCHOOSE study between July 2015 and July 2017 with follow up visits completed in February 2018. A total of 180 participants were screened of which 156 provided mucosal samples and 131 were enrolled and randomized. The DTHF clinic staff performed recruitment and enrolment. The overview of the study design can be found in **Figure 2.1**.

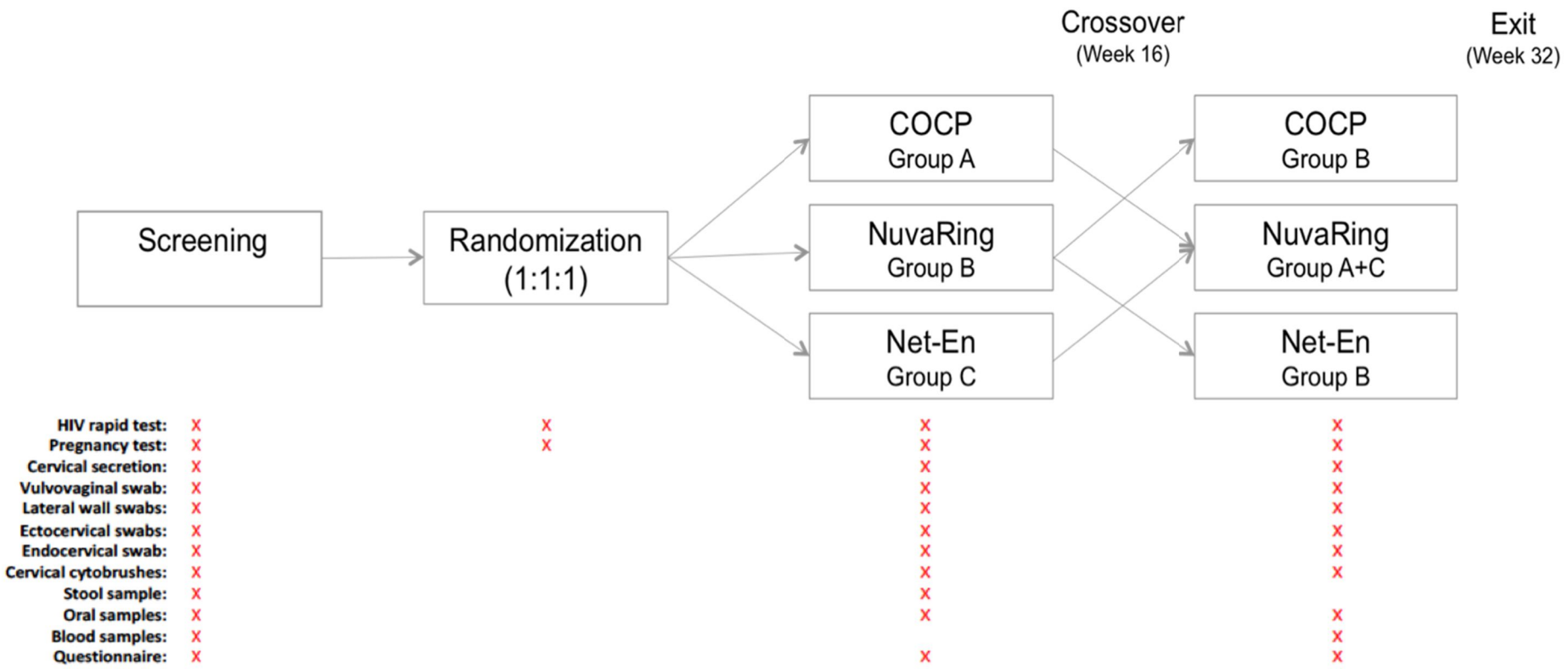


Figure 2.1. uCHOOSE study design and sample collection. Potential study participants came in for a screening visit and if eligible, the participants returned to the clinic for a randomization visit. The participants were assigned in a 1:1:1 ratio to one of three study arms and received either Net-En, NuvaRing or combined oral contraceptive pills (COCP) for the first 16 weeks of the study. The participants subsequently returned for a crossover visit at which they switched over to another hormonal contraceptive option for the final four months of the study. Participants initially assigned to Net-En or COCPs switched over to the NuvaRing, while participants receiving the NuvaRing as the first method were given the choice between either Net-En or COCPs as their second method. The participants returned to the clinic for a final visit (after 32 weeks total) and exited the study.

2.1.2 uCHOOSE-A-Star sample collection and STI screening

The following genital tract samples were collected (in the indicated order) from each adolescent female at the screening, crossover and exit study visits by a nurse at the DTHF clinic: 1) cervical secretion collected with a Softcup® menstrual cup inserted for 30 minutes for cytokine analysis, 2) a vulvovaginal dry swab for STI screening, 3) a vaginal lateral wall flocked dryswab for BV and Candida screening and pH measurement, 4) a vaginal lateral wall digene swab for microbiota analysis; 5) a vaginal lateral wall anaerobic swab for culturing of vaginal bacteria; 6) an ectocervical digene swab for microbiota analysis; and 7) two cervical cytobrushes for analysis of cervical immune and epithelial cells. One cytobrush was placed in 3 ml R10 (10% heat-inactivated fetal calf serum (FCS) in RPMI supplemented with L-glutamine (50 ng/µl), anti-fungin (0.8 ng/µl), penicillin (50 U) and streptomycin (50 ng/µl)) for RNA extraction and sequencing, and the other in 3 ml R10 for *ex vivo* flow cytometry analysis. At the screening and exit visits, one vial of blood was obtained for peripheral blood mononuclear cell (PBMC) isolation (9 ml heparin tube) and another for hormone level measurement (yellow top tube with acid citrate dextrose additives (ACD)). Tubes for stool collection was provided at the screening and crossover visits for the adolescents to bring home and return to the clinic the following day. Stool samples, genital tract swabs, plasma and cervical secretions were stored at -80°C and PBMCs on liquid nitrogen at University of Cape Town. Cervicovaginal mucosal sampling was postponed if the participant was menstruating, but performed as soon as possible, within the visit window.

Vulvo-vaginal swabs were collected for testing for the following STIs: *Chlamydia trachomatis, Neisseria gonorrhoea, Trichomonas vaginalis* and *Mycoplasma genitalium* by multiplex PCR at the National Institute of Communicable Diseases (NICD) as previously described (Lewis et al., 2012). Blood was obtained for HIV rapid test, serum hormone level measurement (oestradiol (E2), luteinizing hormone (LH), follicle-stimulating hormone (FSH)) and for herpes simplex virus 2 (HSV-2) serological testing. Hormone level analysis and HSV-2 serology were performed at a contract laboratory in Mowbray, Cape Town (Bio Analytical Research Corporation, BARC). Serological tests for HSV-2 were directed against antibodies to HSV-2 glycoproteins G-1 and

G-2. A vaginal lateral wall swab was collected for BV testing by Gram staining and Nugent scoring (BV negative (Nugent 0–3), intermediate (Nugent 4–6) or positive (Nugent 7–10)) and Candidiasis screening by microscopy (candida hyphae and spores), which was performed at the NICD. Vaginal pH was measured using pH-indicator strips (McolorpHast™, pH 2.0-9.0, Merck Millipore) at the DTHF youth centre clinic and documented.

2.1.3 WISH study design

The Women's Initiative in Sexual Health (WISH) study population consisted of 149 16-22-year-old women from Masiphumelele, Cape Town. Participants were enrolled in the WISH study between November 2013 and December 2014. Detailed procedures and characteristics of the cohort have previously been described (Barnabas et al., 2017). All participants 18 years or older provided informed consent, while informed assent from the participant and informed consent from a parent or a legal guardian were obtained for those younger than 18 years. Females were enrolled if they were HIV-negative, in good health, not pregnant or menstruating at the time of sampling, if they had not had condomless sex or douched in the last 48 hours, nor taken antibiotics in the prior two weeks. The women were followed longitudinally every two months if they were using norethisterone enanthate (Net-En) injectable contraceptive, combined oral contraceptives or barrier contraception only, or every three months if they were using depot medroxyprogesterone acetate (DMPA) injectable contraceptive, for a total of three visits. Study visits were scheduled two weeks after injection for participants on injectable contraceptives, or otherwise during the luteal phase of their menstrual cycles (between day 14–28) if they were not using hormonal contraceptives or if they were using oral contraceptives. Prior to any specimen collection, an HIV rapid test (Alere Determine™ HIV-1/2 Ag/Ab Combo, Alere, Waltham, MA), a pregnancy test (U-test Pregnancy strip, Humor Diagnostica, Pretoria, South Africa) and a general physical examination was performed.

2.1.4 WISH sample collection and STI screening

At each visit, vulvovaginal swabs for STI testing and Nugent scoring were obtained, as well as swabs from the lateral vaginal wall and endocervix for microbiota analyses. Vulvovaginal swabs were tested for *C. trachomatis, N. gonorrhoea, T. vaginalis, M. genitalium,* herpes simplex virus type 1 (HSV-1) and HSV-2, *Haemophilus ducreyi, Treponema pallidum* by multiplex PCR at the NICD in Johannesburg (Lewis et al., 2012). Blood was obtained for HIV rapid testing and HSV-2 serology (performed at BARC). Endocervical swabs were collected for human papillomavirus (HPV) detection and genotyping by Roche Linear Array (Mbulawa et al., 2015). Posterior fornix swabs were collected for Nugent scoring (at NICD) and vaginal pH was measured using colour-fixed indicator strips at the clinic (Macherey-Nagel, Düren, Germany).

2.1.5 Ethics

Approval for the two studies was obtained from the Human Research Ethics Committee at the University of Cape Town. All aspects of the studies were conducted according to the International Conference on Harmonization (ICH) Good Clinical Practices (GCP), and with approval by all relevant Institutional Review Boards (IRB) in Cape Town and the USA. In addition, the studies complied with the South African National Health Act (NHA, No. 61, 2003) and the South African Good Clinical Practice Guidelines (DOH 2006).

2.2 16S rRNA amplicon sequencing

2.2.1 DNA extraction from uCHOOSE-A-Star vaginal swabs

I, Christina Balle, together with Master's student Rachel Esra, Division of Immunology, University of Cape Town, completed the DNA extraction for the uCHOOSE-A-Star study. Aliquots from the uCHOOSE-A-Star lateral wall vaginal swabs (digene Female Collection Swab kit, QIAGEN) from all three sample visits were thawed and treated with an enzyme cocktail consisting of mutanolysin (25kU/ml, Sigma Aldrich), lysozyme (450kU/ml, Sigma Aldrich), and lysostaphin (4kU, Sigma Aldrich) for 1 hour at 37°C. The microbial DNA was extracted using the *Quick*-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research) following the

manufacturer's protocol. Mechanical disruption was performed in the Qiagen TissueLyser LT for 5 minutes at 50 oz.

2.2.2 DNA extraction from WISH vaginal swabs

For the WISH study, Enock Havyarimana, Division of Immunology, University of Cape Town, performed the DNA extraction for the lateral wall swabs, while I, Christina Balle, extracted DNA from the endocervical swabs. Samples from participants with matched lateral wall and endocervical swabs (digene Female Collection Swab kit, QIAGEN) from the first WISH sample visit were thawed and treated with an enzyme cocktail as described in section 2.2.1. This was followed by mechanical disruption using the Thermo Savant FastPrep 120 Cell Disrupter system for 3x30 seconds at speed setting 5.5 m/s. Microbial DNA was extracted using the PowerSoil DNA Isolation kit (Mo Bio Laboratories Inc., Germantown, MD, USA) following the manufacturer's protocol.

2.2.3 16S rRNA sequencing libraries

I, Christina Balle, performed the 16S rRNA sequencing library preparation for both WISH and uCHOOSE-A-Star. 16S rRNA sequencing libraries were generated following the same protocol for both the WISH and the uCHOOSE cohorts. The V4 hypervariable region of the bacterial 16S rRNA genes was amplified by PCR using a high fidelity DNA polymerase (Thermo Scientific™ Phusion™) and the modified universal primary primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Pearce et al., 2014) with overhang adaptors specific for Illumina sequencing adaptors. For each reaction 1 µl forward primer (5 µM), 1 µl reverse primer (5 µM), 12.5 µl, KAPA Hotstart ReadyMix, template DNA (10-20 ng/µl) and nuclease-free H_2O was used for a final volume of 25 µl. PCR was performed in a thermal cycler (Geneamp PCR system 9700, Applied Biosystems) using the following program: 95°C for 3 minutes, 35 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and 72°C for 5 minutes. The samples were purified using Agencourt AMPure XP beads (Beckman Coulter) to remove free primers and primer dimer species according to the manufacturer's protocol. A 1:1 volume (25 µl) of AMPure XP beads was used per sample. The purified amplicons were stored in -

20°C freezer until barcoding PCR.

Illumina sequencing adapters and dual-index barcodes were added to the purified amplicon products using limited cycle PCR and the Nextera XT Index Kit (Illumina). Eight forward and twelve reverse secondary primers were used in unique combinations for multiplexing of up to 96 samples per sequencing library (primer sequences and combinations can be found in Appendix I). Each of the secondary primers consisted of the Illumina P5 (forward) or P7 (reverse) sequencing adaptors (allowing the amplicons to bind to the flow cell), a 8 bp index sequence (for de-multiplexing), a primer binding site (allowing the sequencing primers to bind), and the 515F or 806R primer sequences (**Figure 2.2**).

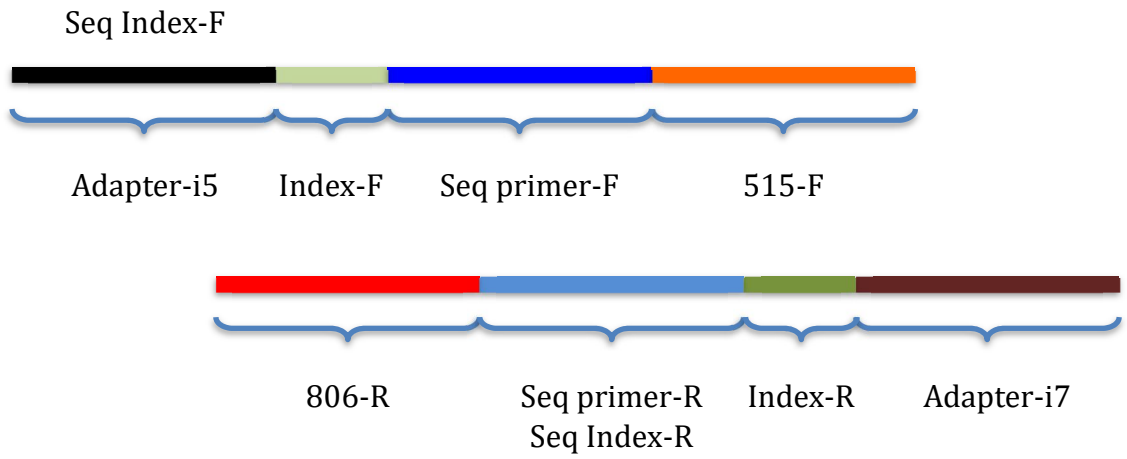


Figure 2.2. Forward and reverse index (secondary) primers for paired-end amplicon sequencing of the 16S rRNA V4 gene region. Seq Index-F: binding site for forward index primer; Adapter-i5: Illumina P5 (forward) sequencing adaptor; Index-F: forward index sequence; Seq primer-F: binding site for forward sequencing primer; Index-F: forward index sequence; 515-F: V4 forward primary amplification primer; 806-R: reverse primary amplification primer; Seq primer-R: binding site for reverse sequencing primer; Seq Index-R: binding site for reverse index primer; Index-R: reverse index sequence; Adapter-i7: illumina P7 (reverse) sequencing adaptor.

For each reaction 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM), 25 µl, KAPA Hotstart ReadyMix, 5 µl template DNA (amplicon products from 1st PCR) and 10 µl H_2O was used giving a final volume of 50 µl. PCR was performed in a thermal cycler using the following program: 95°C for 3 minutes, 8 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and 72°C for 5 minutes. The amplicon products were purified using AMPure XP beads to remove free primers and primer dimer species according to the manufacturer's protocol. A 1:1 volume (50 µl) of AMPure XP beads was used per sample. The purified

amplicons were stored at -20°C until library quantification and pooling. Amplicons from 96 samples and controls were pooled in equimolar amounts and the resultant libraries purified by gel extraction (Qiagen) and quantified using the Qubit dsDNA HS Assay Kit (Life Technologies). The controls included a DNA extraction control, a first and second PCR negative water control and a positive control using a mock community of known bacterial DNA (Cell Mock Community PBS version 2, BEI resources). The libraries were sequenced on the Illumina MiSeq platform (300 bp paired-end) with the v3 chemistry at Seattle Children's Research Institute. Raw sequence data for 16S rRNA gene amplicon sequences are available at http://www.ebi.ac.uk/ under project number PRJEB30774.

2.2.4 Bioinformatics analysis of 16S rRNA sequencing data

I, Christina Balle, performed bioinformatics analyses using a pipeline developed by University of Cape Town's Computational biology department. Pre-processing of sequencing reads, OTU generation and taxonomic annotation of sequencing data from both the WISH and the uCHOOSE cohorts were performed as previously described in detail (Lennard et al., 2017). Briefly, following de-multiplexing, raw reads were pre-processed by merging forward and reverse reads using usearch7 (Edgar, 2010), allowing a maximum of three mismatches. The resultant merged reads were quality filtered using usearch7 (reads with E scores >0.1 were discarded). Primer sequences were removed using a custom python script and the sequences truncated at 250bp (corresponding to the length of the 16S rRNA V4 region). Sequences were then de-replicated whilst recording the level of replication for each sequence using usearch7. De-replicated sequences were sorted by abundance (highest to lowest) and clustered *de novo* into operational taxonomic units (OTUs) at 97% similarity using usearch7. Chimeric sequences were detected (against the Gold database) using UCHIME (Edgar et al., 2011) and removed. Individual sequences were assigned to the specific identifiers using a 97% similarity threshold.

Taxonomic assignment was performed in QIIME 1.8.0 (Caporaso et al., 2010) using the RDP classifier (using the default confidence level of 0.5) against the GreenGenes 13.8 reference taxonomy for 97% identity. To increase species-level resolution, we used the usearch_global command implemented in VSEARCH

(Rognes et al., 2016) to search the de novo picked OTUs' representative sequences against our own Custom Vaginal 16S Reference Database previously described (Lennard et al., 2017). All hits with ≥ 97% identity were accepted. The remaining OTUs were manually curated using both BLAST on NCBI's nucleotide database (excluding uncultured organisms) and with usearch_global against the Vaginal 16S rRNA Reference Database developed by Fettweis and colleagues (Fettweis et al., 2012). OTUs that mapped to two or three species (with the same identity score) would be annotated as follows: Genus speciesA_speciesB or Genus speciesA_speciesB_speciesC, respectively. If an OTU mapped to more than three species but one species was clearly associated with vaginal microbiota (based on prior knowledge) the OTU was named Genus species_cluster, where 'species' was selected based on the majority of hits (Lennard et al., 2017). These results were assigned to the phyloseq object in R (R Core Team, 2016). Any chloroplast-derived or mitochondrial sequences were excluded before proceeding with analysis. Samples with ≥ 5000 reads were selected for downstream analyses. The OTU table was normalized (i.e. transformed to relative abundance*median sample read depth), and filtered so that each OTU had to have at least 10 counts in at least 20% of samples or have a relative abundance of at least 0.001%. Computations were performed using facilities provided by the University of Cape Town's ICTS High Performance Computing team: http://hpc.uct.ac.za.

2.3 Statistical analysis

I, Christina Balle, performed statistical analyses with input and assistance from my co-supervisor Katie Lennard. All downstream statistical analyses were performed in RStudio using the packages phyloseq (McMurdie & Holmes, 2013) for beta diversity analyses using PCoA with weighted UniFrac distances, metagenomeSeq (Paulson et al., 2013) for differential abundance testing, vegan (Oksanen et al., 2016) for alpha diversity estimates (Shannon Index), ordinations and redundancy analysis, and pheatmap (Kolde, 2015) and NMF (Gaujoux, 2014) for annotated heatmaps. Microbiota community clusters were established by Fuzzy clustering using the R package 'cluster' (Maechler et al., 2017) with optimal k, a membership exponent of 1.25 and weighted UniFrac as

the dissimilarity measure. Sample communities with a probability of less than 60% of belonging to any of the three clusters were excluded from downstream analyses. Alluvial diagrams were created using the R package 'alluvial' (Bojanowski & Edwards, 2016). Data analysis Shapiro-Wilk test for normality was performed to determine the distribution of variables within the dataset. Differences in study population characteristics were tested using Pearson´s Chi-squared test or Fisher's exact test (when the expected value was <5) for count data. Paired or unpaired Students t-tests were a used to test differences in mean (parametric data) and unpaired Mann-Whitney U-Wilcoxon Rank sum test or paired Wilcoxon Signed Rank test were applied for differences in medians (non-parametric data) with correction for multiple comparisons using Benjamini-Hochberg (BH) method. Friedman's test was used for paired, non-parametric longitudinal data with more than two time points. For repeated measured analysis, linear mixed-effect model analysis was performed using the "lme4" (Bates et al., 2015) package in R.

The overall difference in microbial composition between groups was determined by permutational multivariate analysis of variance (PERMANOVA) using distance matrices test with 999 permutations using the adonis2 functions from the vegan package in R and weighted UniFrac as the distance measure. The assumption for PERMANOVA of homogeneity of variance between groups was assessed using the betadisper function also from the vegan package in R. The Spearman's rank test was applied to test for correlation between nonparametric data. Non-parametric assessments of variation between groups was carried out through the Kruskal-Wallis test with Dunn's post-test being applied to test for the effect of multiple comparisons using the Benjamini-Hochberg (BH) method and with Analysis of Variance (ANOVA) with Tukey's post hoc test for parametric data. Specific differences in microbial composition between groups of interest were assessed using metagenomeSeq's MRfulltable function with a custom filter to determine significance. Merged taxa were deemed significantly different if they exhibited a fold change (beta coefficient) of ≥ 1.25, had an adjusted p-value of ≤ 0.05 and if at least one of the two groups being compared had ≥ 20% of samples with the given OTU/taxa OR the Fisher's exact test result was significant (after multiple testing correction (MTC) by Benjamini-Hochberg (BH) method.

OTUs were first merged at the lowest available taxonomic level using a custom script (Lennard et al., 2017). Differential abundance analysis was also performed using DESeq2 (Differential expression analysis based on the Negative Binomial distribution) (Love et al., 2014) with an alpha of 0.05 or 0.01. OTUs (merged at the lowest taxonomic level) were considered significantly differentially abundant between classes if their adjusted p-value was <0.05 and if the estimated fold change was >1.5 or <1/1.5. Random Forests analyses were conducted on merged OTUs using the R package randomForest (Liaw & Wiener, 2002). For random forest analyses, the data was randomly divided into training and test sets, comprising one third and two thirds of the data, respectively. The top five predictive taxa were used to calculate the error rates. Multi state model analyses was performed using the "msm" package in R (Jackson, 2011).

2.4 uCHOOSE-A-Star immunology experiments

2.4.1 Cytokine measurements

Cytokine measurements were performed on cervical secretions collected for the uCHOOSE-A-Star cohort by myself in collaboration with doctoral candidate Iyaloo Konstantinus, Division of Medical Virology, University of Cape Town and Shameem Jaumdally, Division of Immunology, University of Cape Town. The concentrations of IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L and TNF-α in Softcup® cervical secretions collected in the uCHOOSE-A-Star study was measured by Luminex Milliplex assay using the Bio-Plex Pro Human Th17 Cytokine Panel. After removal of the softcup from the participant the softcup was placed in a 50 ml tube and transported to the laboratory within 4 hours at room temperature. Upon arrival of the softcup at the laboratory the tube was centrifuged at 1500 revolutions per minute (rpm) for 10 minutes to collect cervical secretion. The weight of the secretion was determined and diluted 1:4 in phosphate buffered saline (PBS). 50 µl was taken for bacterial culturing analysis and the rest was aliquoted into three 2 ml cryovial and stored at -80°C. For Luminex analysis, samples were thawed overnight at 4°C and centrifuged at 4100rpm for 5 minutes to separate mucous from the cervical fluid. After centrifugation, the supernatant was removed and passed through sterile Costar® Spin-X® polypropylene filters (cellulose acetate

membranes, pore size 0.22 μm, Sigma Aldrich) to remove any residual mucus before addition of the samples to the assay plates.

The assay plates were read using a Bio-Plex Suspension Array Reader (Bio-Rad Laboratories Inc., USA) and Bio-plex manager software version 4 (Bio-Rad Laboratories, Inc.). A 5-parameter logistic regression formula was used to calculate sample concentrations from the standard curves. For cytokine values below limits of detection, half of the lowest detectable value for the specific cytokines was assigned. For values above detection level, the highest detectable value was applied. A total of 5 assay plates were run. Specimens from 6 participants were run across all plates (inter-plate controls) and samples from 6 participants were duplicated on each set of plates (intra-plate controls) for quality control measures. Spearman's rank test was used to measure intra-assay and inter-assay correlation coefficients to determine assay reliability and reproducibility.

2.4.2 CD4 T cell activation and Th17 analysis

Flow cytometry analysis was performed on cervical cytobrushes collected for the uCHOOSE-A-Star cohort by doctoral candidate Iyaloo Konstantinus, Division of Medical Virology, University of Cape Town. The resulting data was shared with me for downstream analysis with the 16S rRNA sequencing data. Cervical mucosal mononuclear cells were collected using a digene cervical sampler (*DNAPap Cervical sampler,* QIAGEN). The cervical cytobrush was inserted into the endocervical os of the participant, rotated through 360 degrees and placed in a 15ml tube containing 3ml R10 and transported to the laboratory within 4 hours in a Nalgene bench-top cooler (Rochester, NY, USA) at approximately 4°C.

Upon arrival of the cytobrush at the laboratory, the cytobrush was gently rotated against the side of the tube to dislodge the cells. Using a sterile Pasteur pipette, the transport medium (R10) was used to flush the cytobrush bristles 30 times. The cytobrush was gently scraped with the tip of the Pasteur pipette to remove any remaining cells and the cytobrush was removed from the tube. The tubes were centrifuged at 1200 rpm for 10 minutes to pellet cells. Using a Pasteur pipette, the supernatant fraction (leaving about 50μl) was transferred to two cryovials for future assays. The cell pellet was re-suspended by vortexing and

transferred to a 96 well plate. The cells were phenotyped using the following monoclonal antibodies: CD3, CCR6, CCR5 (BD Biosciences, San Jose, CA), CD4, CD19, CD14 (Invitrogen), CD38 (eBioscience, San Diego, CA), CD8, CCR10 and HLA-DR (Biolegend). The flow panel is shown in **Table 2.1**.

Viable cells were identified using the live/dead marker Vivid (Invitrogen). 50µl of Vivid was added to the sample and the plate incubated at room temperature for 20 minutes in the dark. The cells were washed twice with PBS (with 100µl and 150µl, respectively) by centrifuging at 1000 RCF at 4^{o}C for 5 minutes. A staining cocktail was prepared using pre-titrated antibody volumes of antibodies for CCR5, CCR10 and CCR6 in PBS. 50µl of the antibody cocktail was added to and mixed with the cells and incubated at 37°C for 30 minutes in the dark. The cells were then washed twice with PBS, as described above. A new staining cocktail was prepared containing pre-titrated volumes of antibodies for CD14, CD19, CD3, CD4, CD8, HLA-DR and CD38. 50µl of the staining cocktail was added to the cells and incubated in the dark at room temperature for 20 minutes. The cells were washed twice with PBS, as described above. The supernatant was discarded and the cell pellet dislodged. The cells were re-suspended in 150µl Cellfix to fix the cells and the suspension transferred to a 5 ml FACS tube. The wells were rinsed with an additional 150µl 1x Cellfix (BD Biosciences), and transferred to the FACS tube. The tubes were wrapped in aluminium foil (to keep it in the dark) and stored at 4°C, until acquisition (within 48 hours of staining) on the BD LSRFortessa™ (BD Immunocytometry Systems, San Jose, CA). FlowJo v9.9.3 (FlowJo, LLC, Ashland, OR) was used for data analysis.

Compensation tubes were prepared for each acquisition. Ten compensation tubes were prepared including one 'unstained' (no antibodies) control and a single-stained tube for each of the nine fluorochromes in the panel (**Table 2.1**). 100µl of PBS, followed by one drop of positive BD CompBeads™ (BD Biosciences) vortexed for 1 minute (to avoid clumping of the beads) was added to 5 ml FACS tubes followed by pre-titrated volume of each antibody to each of the respective compensation tube. For the dump channel (used to exclude dead cells, B-cells and monocytes), the CD14 antibody was used. The tubes were incubated in the fridge (4°C) for 20 minutes before washing of the beads by addition of 1 ml of PBS and centrifugation at 1200 rpm for 5 minutes.

The supernatant was discarded and 100ul of Cellfix added. The tubes were wrapped in aluminium foil and kept at 4^{o}C until acquisition. The gating strategy can be found in Appendix II.

Table 2.1. Antibody panel for flow cytometry.

Marker	Fluorochrome	Laser	Function
CD14 CD19 ViVid	Pacblue	Violet	Dump channel
CD3	APC-H7	Red	T-cell marker
CD4	PE-Cy5.5	Green	CD4+ T cell marker
CD8	BV711	Violet	CD8+ T cell marker
CD38	PE-Cy7	Green	Activation marker
HLA-DR	Alexa 700	Red	Activation marker
CCR5	APC	Red	Co-receptor
CCR6	BV605	Violet	Th17 marker
CCR10	PE	Green	Memory like skin-resident T cells

2.4.3 Statistical analyses

All downstream statistical analyses were performed in RStudio as described in Chapter 2.3 (p. 55-56). In addition, unsupervised clustering of the cytokine data was performed by partitioning around medoids (PAM) clustering available in the R package 'cluster' (Maechler et al., 2017) with optimal k (the k producing the maximum average silhouette width).

Chapter 3: Endocervical and vaginal microbiota in South African adolescents with asymptomatic *Chlamydia trachomatis* infection

Parts of this chapter (including **Table 3.1**, **Figure 3.2**, **Figure 3.3**, **Figure 3.4** and **Figure 3.5A,B**) were published in Scientific Reports, volume 8, Article number: 11109 (2018) on July 23rd, 2018 (Balle et al., 2018).

3.1 Introduction

Chlamydia trachomatis is an obligate intracellular pathogen and the most common cause of bacterial STIs worldwide. Young women, particularly adolescents, are at highest risk (Newman et al., 2015). Infections with *C. trachomatis* remain highly prevalent despite both sensitive diagnostic tests and effective antibiotic treatments being available (Newman et al., 2015). The majority of *C. trachomatis* infected women are asymptomatic, however if left untreated, *C. trachomatis* can lead to serious sequela such as PID, infertility and ectopic pregnancy. Molecular studies of the vaginal microbiota of healthy, reproductive-age women have described a number of common bacterial community types, which vary according to population (Borgdorff et al., 2014; Ravel et al., 2011). These vaginal community types consist of low-diversity communities dominated by a single *Lactobacillus* species (*Lactobacillus crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii*) and one to two high-diversity communities, comprised of a mixture of anaerobic bacteria associated with bacterial vaginosis (BV) (Ravel et al., 2011; Gajer et al., 2012; Anahtar et al., 2015; Gautam et al., 2015). BV is a condition characterized by the displacement of *Lactobacillus* species in the female genital tract (FGT) with various anaerobic and facultative bacteria, including *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera*, *Prevotella*, and BVAB1–3. Several studies have described BV as an independent risk factor for STIs acquisition, including *C. trachomatis*, *Neisseria gonorrhoea* and HIV (Wiesenfeld et al., 2003; Myer et al., 2005a; Atashili et al., 2008; Brotman et al., 2010). Recent molecular studies have shown that women with *C. trachomatis* infection are more likely to have *L. iners*-dominated or dysbiotic vaginal communities compared to those without (van der Veer et al., 2017; van Houdt et al., 2017; Borgdorff et al., 2014; Parolin et al., 2018; Tamarelle et al., 2018). The relationship between the composition of the endocervical microbiota and STIs have been less well studied despite the fact that the cervix is the main site of infection for several pathogens including *C. trachomatis* and *N. gonorrhoea* (Filardo et al., 2017). Whereas the vagina has stratified squamous epithelium, *C. trachomatis* infects the columnar epithelial cells of the cervix. Differing immune and physiological environments (including different oxygen levels) could impact the microbial composition in different regions of the lower FGT. Molecular

studies characterizing bacterial communities at different anatomical sites throughout the FGT have generated varying results with regards to microbial homogeneity (Huang et al., 2015a; Kim et al., 2009; Smith et al., 2014). Yet, distinct microbial communities in cervix that differ from that of the vagina have been described (Chen et al., 2017). It is therefore of great importance to characterize endocervical microbial communities when studying the role that commensal bacteria play in *C. trachomatis* infection. To our knowledge, only a few molecular studies have evaluated the relationship between prevalent *C. trachomatis* infections and the composition of cervicovaginal microbiota and none have focused on African adolescents, a group at extreme risk for STIs. Hence, we compared the endocervical and vaginal microbiota and evaluated their association with *C. trachomatis* infection in young females from a high-risk community in South Africa.

3.2 Materials and methods

For this analysis, we included 77 HIV-uninfected adolescent girls enrolled in the WISH study described in Chapter 2.1.3 (p. 50) from whom matched vaginal lateral wall (LW) and endocervical (EC) swabs at baseline were available. The study procedures were approved by the Human Research Ethics Committee of the University of Cape Town, and informed consent or assent (if <18 years) was obtained from all participants before initiation of the study. Informed consent was obtained from a parent or legal guardian of participants younger than 18 years. Demographic and behavioural data were also collected. As described in Chapter 2.1.4 (p. 51), vulvovaginal swabs were collected for BV and STI testing and vaginal pH determination and swabs from the lateral vaginal wall and endocervix were obtained for microbiota analyses. DNA extraction from lateral wall (LW) and endocervical (EC) swabs, 16S rRNA sequencing library generation and bioinformatics pre-processing of sequencing reads were performed as described in Chapter 2.2.2, 2.2.3 and 2.2.4 (p. 51-55), respectively. All downstream statistical analyses were performed in RStudio as described in Chapter 2.3 (p. 55-56). In addition, any association between community type or any demographic or behavioural characteristic with *C. trachomatis* infection status was analysed by performing univariate and multivariate logistic

regression analyses. Furthermore, the ecologic distances of samples within-subject (lateral wall versus endocervical) was compared with between-subject distances by comparing all within-subject pair-wise distances (using weighted UniFrac) with all between-subject pair-wise distances using Student's t-test.

3.3 Results

3.3.1 Study population characteristics

We conducted 16S rRNA gene sequencing on 154 genital swabs (77 lateral vaginal wall (LW) and 77 endocervical (EC) samples) collected from 77 participants with both LW and EC samples available. Of these, 149 samples passed the sequencing and quality control measures (≥ 5000 reads/sample) with three EC and two LW samples failing. Downstream analyses were conducted on 72 participants for whom both the LW and matched EC samples passed quality control measures. Of those 72 participants, 30 (42%) were *C. trachomatis* positive and 42 (58%) were *C. trachomatis* negative. Nugent scores for BV determination were available for all 72 participants. Nearly half of the participants (49%, n=35) were BV positive (Nugent score 7-10), while 5% were BV intermediate (Nugent score 4-6, n=4) and 46% were BV negative (Nugent score 0-3, n=33) (**Table 3.1**). This was similar to the overall WISH cohort as described by Barnabas et al. (2017). The *C. trachomatis* infected and uninfected participants were similar in their demographic and behavioural characteristics including age, body mass index (BMI), intravaginal practices and HC use. Participants with BV were almost twice as likely to be infected with *C. trachomatis* compared to BV negative participants, but these observations did not reach statistical significance (OR=1.8; 95% CI: 0.71-4.98, p=0.21). Adolescents infected with *C. trachomatis* were less likely to report multiple sexual partners and regular condom use (over the past year) and reported fewer lifetime sexual partners than *C. trachomatis* negative participants (**Table 3.1**). Participants infected with *C. trachomatis* were, however, significantly more likely to be co-infected with *N. gonorrhoea* than *C. trachomatis* uninfected participants (23% versus 0.5%, p=0.03) and there was a higher prevalence of HPV in *C. trachomatis* positive participants (**Table 3.1**).

Table 3.1. Study population characteristics.

	C. trachomatis negative (n=42)(58%)	*C. trachomatis* positive (n=30)(42%)	P-value*
Age, median years (IQR)	18 (18-20)	18.5 (17-20)	0.80
Hormonal contraception			0.73
Injectable	36 (86%)	27 (90%)	
Non-injectable[a]	6 (14%)	3 (10%)	
No (none)	0 (0%)	0 (0%)	
BMI, median (IQR)[b]	25.4 (21.8-28.2)	25.2 (21.0-27.0)	0.35
Intra-vaginal practices[c]			
Douching	3 (8%)	0 (0%)	0.31
Washing w. water	31 (97%)	26 (93%)	0.91
Washing w. soap	22 (67%)	17 (63%)	0.98
Lifetime partners, median (IQR)	3 (2-4)	2 (2-3)	0.20
Multiple sexual partners[d]	22 (61%)	10 (36%)	0.32
Regular condom use[e]	25 (76%)	15 (56%)	0.17
BV prevalence			0.10
BV positive	17 (40%)	18 (60%)	
BV intermediate	4 (10%)	0 (0%)	
BV negative	21 (50%)	12 (40%)	
Endocervix alpha diversity (median SI, IQR)	1.1 (0.3-2.1)	1.8 (1.1-2.1)	0.28
Vaginal pH, mean (±sd)	4.7 (4.1-5.2)	4.8 (4.2-5.3)	0.41
***N. gonorrhoea* positive**	2 (0.5%)	7 (23%)	**0.02**
HPV positive[f]	17 (49%)	15 (75%)	0.09

**Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups and unpaired Mann–Whitney-Wilcoxon test for comparison of medians. Unpaired Student's t-test for alpha diversity. BMI, body mass index; BV, bacterial vaginosis, IQR, interquartile range; HPV, human papillomavirus; sd, standard deviation; SI, Shannon Index.*
BV; bacterial vaginosis, SI; Shannon Index, IQR, interquartile range.
[a] Non-injectable category include combined oral contraceptives and the implant Implanon.
[b]Information missing from 5 participants.
[c] Information missing from 11 participants.
[d] Information missing from 15 participants.
[e] Information missing from 12 participants.
[f] Information missing from 17 participants
Table published in (Balle et al., 2018).

3.3.2 Comparison of vaginal and endocervical microbial composition

For this study, a total of 365 OTUs were generated and assigned to 332 different bacterial taxa. Using Fuzzy clustering with optimal number of clusters (k) and data from all samples (LW and EC), we identified three major community types (denoted C1-3) (**Figure 3.2-A-C**) in agreement with previous data from this cohort (Lennard et al., 2017). Optimal k was determined by iteration of k's from 2-10 and evaluation of the resulting silhouette widths. For this cohort, optimal k was determined to be three corresponding to an average silhouette width of 0.429 (**Figure 3.1**)

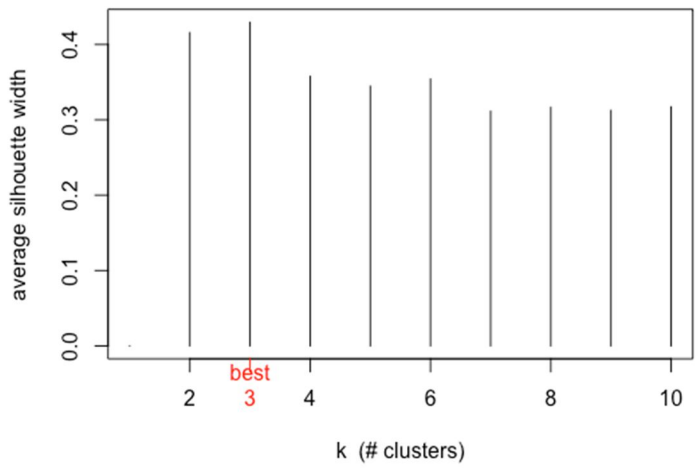


Figure 3.1. Average silhouette width with different k's for the WISH cohort. Barplot depicting the average silhouette width when applying different k's (2-10) for Fuzzy clustering. Optimal k (k=3) determined based on the highest average silhouette width (highlighted in red).

The three identified community types consisted either predominantly of a mix of diverse anaerobic bacteria (C1, n=73; 51%), *L. crispatus* (C2, n=27; 19%) or *L. iners* (C3, n=40; 28%), respectively. The most abundant species in C1 was *G. vaginalis* followed by BVAB1-2, *Prevotella amnii*, *L. iners*, *Sneathia sanguinegens*, *Prevotella timonensis*, *A. vaginae*, *Dialister* spp., *Prevotella pallens* and *Megasphaera* spp. The majority of participants with a C1 community type were BV positive according to Nugent scoring (82%) whereas participants with C2 and C3 community types were mostly BV negative (89% and 73%, respectively) (**Figure 3.2A-B**). To assess any differences in overall bacterial community composition (measured as beta diversity) between sampling sites, a PCoA analysis using weighted UniFrac distances was performed. The samples clearly grouped into three distinct clusters corresponding to the three community types described above (**Figure 3.2B**). Any difference in microbial composition between sampling sites (when adjusted for community type), was determined by permutational multivariate analysis of variance (PERMANOVA) using phylogenetic distance matrices (weighted UniFrac). Within the three community clusters the samples were found to separate according to anatomical site ($p=0.002$, $R2=0.015$) (**Figure 3.3A**). Furthermore, LW samples had significantly higher species richness and alpha diversity compared to EC samples ($p<0.001$) (**Figure 3.3B**). Yet, for 82% of the participants, the LW and EC samples were assigned to the same community cluster and the within-participant variability was significantly less than the variability of samples from same site from

different participants based on mean weighted UniFrac distances (p<0001 for both LW and EC samples) (**Figure 3.3C**).

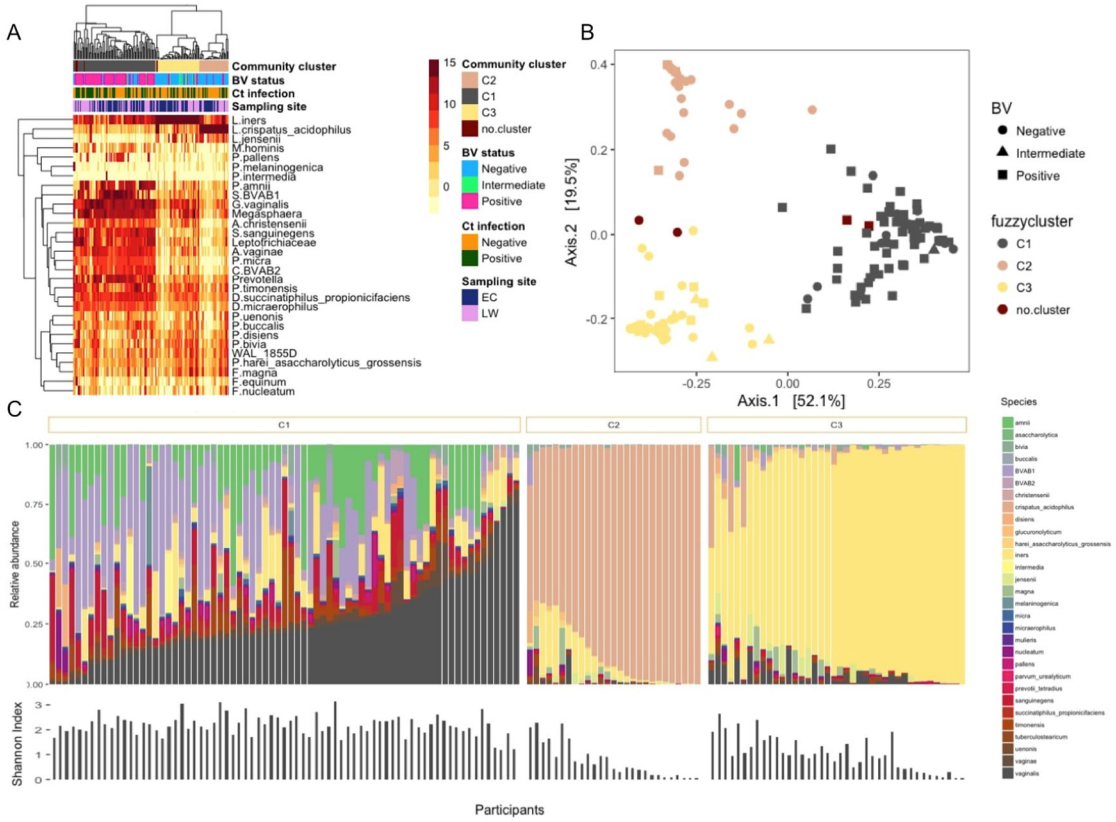


Figure 3.2. Composition of the vaginal lateral wall and endocervical microbiota in adolescents. A) Heatmap of the 30 most abundant taxa (rows) identified by 16S rRNA microbiota profiling using unsupervised hierarchical clustering with weighted UniFrac distances in all samples (columns). Unsupervised hierarchical clustering of 144 matched endocervical and vaginal lateral wall samples from 72 participants. The dendrogram was generated using average linkage clustering with weighted UniFrac distance, based on the relative abundance of taxa (merged at lowest taxonomic level) in each sample. Log2-transformed standardized read counts are illustrated by the colour key. Annotation bars above the heatmap depict community type cluster (top bar), bacterial vaginosis (BV) status based on Nugent scoring (upper middle bar), *Chlamydia trachomatis* (Ct) infection status (lower middle bar) and anatomical sampling site (bottom bar). Samples that did not meet the minimum probability of ≥60% of belonging to any of the three clusters were excluded from downstream analyses (n = 4, "no.cluster" in figure). B) Principal Coordinates Analysis (PCoA) of samples coloured by compositional subtypes generated using Fuzzy clustering with weighted UniFrac distances. Samples are coloured by compositional subtype (C1, C2, C3), with BV status displayed as shapes. Samples that did not meet the minimum probability of ≥60% of belonging to any of these clusters were excluded from downstream analyses (n = 4, "no.cluster" in figure). C) Barplot of the 30 top most abundant taxa identified by 16S rRNA microbiota profiling. Samples are grouped by microbial compositional subtype (C1, C2, C3) established using Fuzzy clustering with weighted UniFrac distances and ordered based on the abundance of the most dominant species in each community type (*G. vaginalis*, *L. crispatus* and *L. iners*, respectively). Samples that did not meet the minimum probability of ≥60% of belonging to any of tree clusters (n = 4) were excluded from the figure. Shannon diversity Index for each sample is depicted below the barplot. Figure published in (Balle et al., 2018).

To identify differentially abundant bacterial taxa between anatomical sites (EC versus LW), metagenomeSeq analysis was applied. The relative abundances of 31 taxa (OTUs merged at lowest taxonomic level) were significantly different between LW and EC samples (**Figure 3.3E** and **Table 3.3**). These included *Achromobacter spanius_cluster*, *Gordonia terrae*, *Methylobacterium aerolatum*, *Enterococcus faecium, C. trachomatis* and *N. gonorrhea*, which were more abundant in the EC samples, and *Peptoniphilus* spp., *Anaerococcus* spp., *Corynebacterium* ssp., *Megasphaera*, *Morganella morganii*, *Finegoldia magna*, *Elizabethkingia meningoseptica*, *Staphylococcus*, *Actinomyces radingae*, *Prevotella bergensis*, *Dialister, Fusobacterium nucleatum*, *Peptococcus niger*, *Varibaculum cambriense* and *Lactobacillus crispatus* which were more abundant in LW samples. Random forest analysis was additionally applied to identify the bacterial taxa most predictive of anatomical site. In agreement with the metagenomeSeq results, the most influential taxa in differentiating EC from LW samples included *A. spanius_cluster*, *E. faecium, M. aerolatum*, *Peptoniphilus harei_asaccharolyticus_grossensis* and *Megasphaera* which were more prominent in EC samples, and *Anaerococcus*, *F. magna*, *Dialister* and *Corynebacterium* ssp., which were more common in LW samples (area under the curve (AUC)=0.98, sensitivity=0.96 and specificity=0.90 for the test set and a validation predicted error rate of 6.25%) (**Figure 3.3D** and **Table 3.2**).

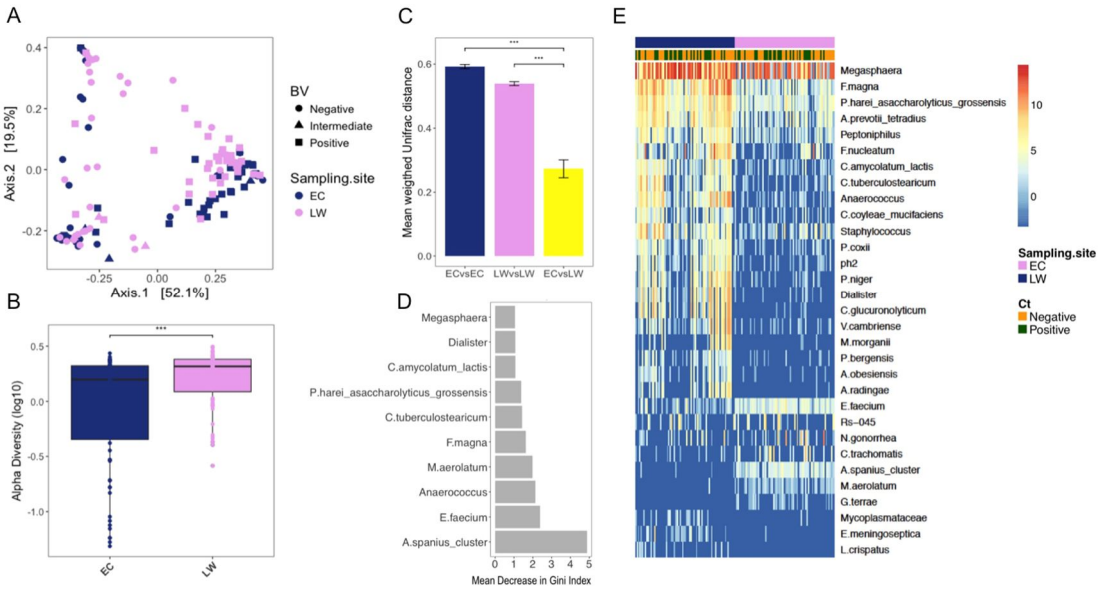


Figure 3.3. Taxa significantly different between vaginal lateral wall and endocervical microbiota. A) Principal Coordinates Analysis (PCoA) of the vaginal and endocervical microbiota using weighted UniFrac distances. Individual samples are coloured by anatomical sampling site (EC, endocervical; LW, vaginal lateral wall) with bacterial vaginosis (BV) status displayed as shapes. B) Boxplot depicting the alpha diversity of the vaginal lateral wall (LW) and endocervical (EC) microbiota. C) Barplot depicting within-subject pair-wise distances (weighted UniFrac) between sampling sites (ECvsLW) and the mean between-subject pair-wise distances (weighted UniFrac) between either endocervical samples (ECvsEC) or vagainal lateral wall samples (LWvsLW). D) The top ten most influential taxa by random forest analysis in predicting anatomical site. The x-axis indicates the mean decrease in Gini Index (length of bar represents predictive ability of each taxon). E) Taxa significantly differentially abundant and/or frequent by anatomical site category by metagenomeSeq (FDR ≤0.05, coefficient ≥1.25, taxa present in ≥20% of samples in at least one of the two groups being compared). Unsupervised clustering of samples (columns) by Bray-Curtis distance; heatmap scale: log2-transformed standardized counts. Figure published in (Balle et al., 2018).

Table 3.2. Random forest analysis of vaginal lateral wall and endocervical microbiota.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_129	11.86	4.884	*Achromobacter spanius_cluster*
OTU_61	8.834	2.385	*Enterococcus* faecium
OTU_33	7.734	2.138	*Anaerococcus*
OTU_262	8.100	1.985	*Microbacterium aerolatum*
OTU_15	6.880	1.628	*Finegoldia magna*
OTU_58	5.837	1.430	*Corynebacterium* tuberculostearicum
OTU_36	4.398	1.384	*Peptoniphilus harei_asaccharolyticus_grossensis*
OTU_76	5.335	1.073	*Corynebacterium amycolatum_lactis*
OTU_45	5.337	1.071	*Dialister*
OTU_3	6.195	1.054	*Megasphaera*

Training set size: 96 samples with 44 and 52 samples per class. Test set size: 48 samples with 28 and 20 samples per class. Validation predicted error: 6.25% (using top five predictive taxa).

Table 3.3. Differentially abundant taxa between the vaginal lateral wall and endocervical microbiota.

	Coeff	P adj.	Family	Genus	Species
EC vs. LW	-2.772	6.4e-10	Alcaligenaceae	*Achromobacter*	*spanius_cluster*
	-2.731	1.5e-20	Enterococcaceae	*Enterococcus*	*faecium*
	-1.857	3.0e-10	Methylobacteriaceae	*Methylobacterium*	*aerolatum*
	-1.789	9.0e-20	Gordoniaceae	*Gordonia*	*terrae*
	-1.577	2.0e-20	Rs-045	NA	NA
	-1.453	0.004	Neisseriaceae	*Neisseria*	*gonorrhea*
	-1.387	0.026	[Weeksellaceae]	*Elizabethkingia*	*meningoseptica*
	1.263	4.1e-06	Corynebacteriaceae	*Corynebacterium*	*coyleae_mucifaciens*
	1.275	0.0001	Staphylococcaceae	*Staphylococcus*	NA
	1.287	0.004	[Tissierellaceae]	*Peptoniphilus*	NA
	1.356	1.5e-05	[Tissierellaceae]	*Peptoniphilus*	*coxii*
	1.378	5.9e-07	Corynebacteriaceae	*Corynebacterium*	*amycolatum_lactis*
	1.387	2.7e-05	Prevotellaceae	*Prevotella*	*bergensis*
	1.400	8.9e-09	[Tissierellaceae]	*Anaerococcus*	*obesiensis*
	1.411	5.2e-07	Fusobacteriaceae	*Fusobacterium*	*nucleatum*
	1.447	0.010	[Tissierellaceae]	*Peptoniphilus*	*harei_asaccharolyticus_grossensis*
	1.545	9.6e-07	Veillonellaceae	*Megasphaera*	NA
	1.546	0.065	[Tissierellaceae]	*Anaerococcus*	*prevotii_tetradius*
	1.589	2.7e-05	Mycoplasmataceae	*NA*	NA
	1.659	1.3e-14	[Tissierellaceae]	*ph2*	NA
	1.764	1.9e-07	Enterobacteriaceae	*Morganella*	*morganii*
	1.924	0.0003	Corynebacteriaceae	*Corynebacterium*	*tuberculostearicum*
	1.960	1.9e-07	Actinomycetaceae	*Varibaculum*	*cambriense*
	2.072	2.2e-05	Lactobacillaceae	*Lactobacillus*	*crispatus*
	2.199	5.0e-23	Actinomycetaceae	*Actinomyces*	*radingae*
	2.259	1.8e-07	Peptococcaceae	*Peptococcus*	*niger*
	2.426	9.6e-08	[Tissierellaceae]	*Anaerococcus*	NA
	2.539	7.9e-10	[Tissierellaceae]	*Finegoldia*	*magna*
	2.587	3.9e-10	Veillonellaceae	*Dialister*	NA
	2.958	2.9e-08	Corynebacteriaceae	*Corynebacterium*	*glucuronolyticum*

*Note: Proteobacteria only annotated at phylum level.

3.3.3 Relationship between genital tract microbiota and C. trachomatis infection

The association between *C. trachomatis* infection and microbiota was assessed separately for LW and EC samples. The alpha diversity of EC microbiota tended to be higher in *C. trachomatis* positive participants compared to *C. trachomatis* negative participants, although not significantly so (1.8 versus 1.1, p=0.28) (**Table 3.1**); this was also the case in LW samples (p=0.32, data not shown). In univariate analysis, participants with a community type dominated by diverse anaerobic bacteria (C1) or *L. iners* (C3) were more likely to be infected with *C. trachomatis* (OR= 2.98; 95% CI: 0.76-15.0 and OR=2.50; 95% CI: 0.56- 13.6, respectively), compared to those having an *L. crispatus*-dominated community, although not significantly. None of the study participant demographic or behavioural factors listed in **Table 3.1** were associated with *C. trachomatis* in univariate or multivariate analyses. On the other hand, differential abundance testing (using metagenomeSeq) revealed bacterial taxa significantly more abundant in *C. trachomatis* positive versus negative women, including as expected *N. gonorrhea*, but also *Sutterella sanguinus_morbirenis* and *Porphyromonas somerae* (**Figure 3.3C** and **Table 3.4**). Using random forest analysis, these same species, as well as *G. vaginalis*, *Aerococcus christensenii*, *Dialister* spp., *Megasphaera*, *A. vaginae* and *Prevotella disiens* were found to be predictive of *C. trachomatis* infection (AUC=0.46, sensitivity=0.60, specificity=0.42 for the test set) (**Figure 3.3A** and **Table 3.5**). However, the model had an AUC of 0.46 and error rate of 54.17%, suggesting that this model do not have any predictive power likely due to the small sample size. In agreement with this, no overall differences in EC microbiota composition (beta diversity) were found between *C. trachomatis* positive and *C. trachomatis* negative participants (p=0.27) (**Figure 3.3C**).

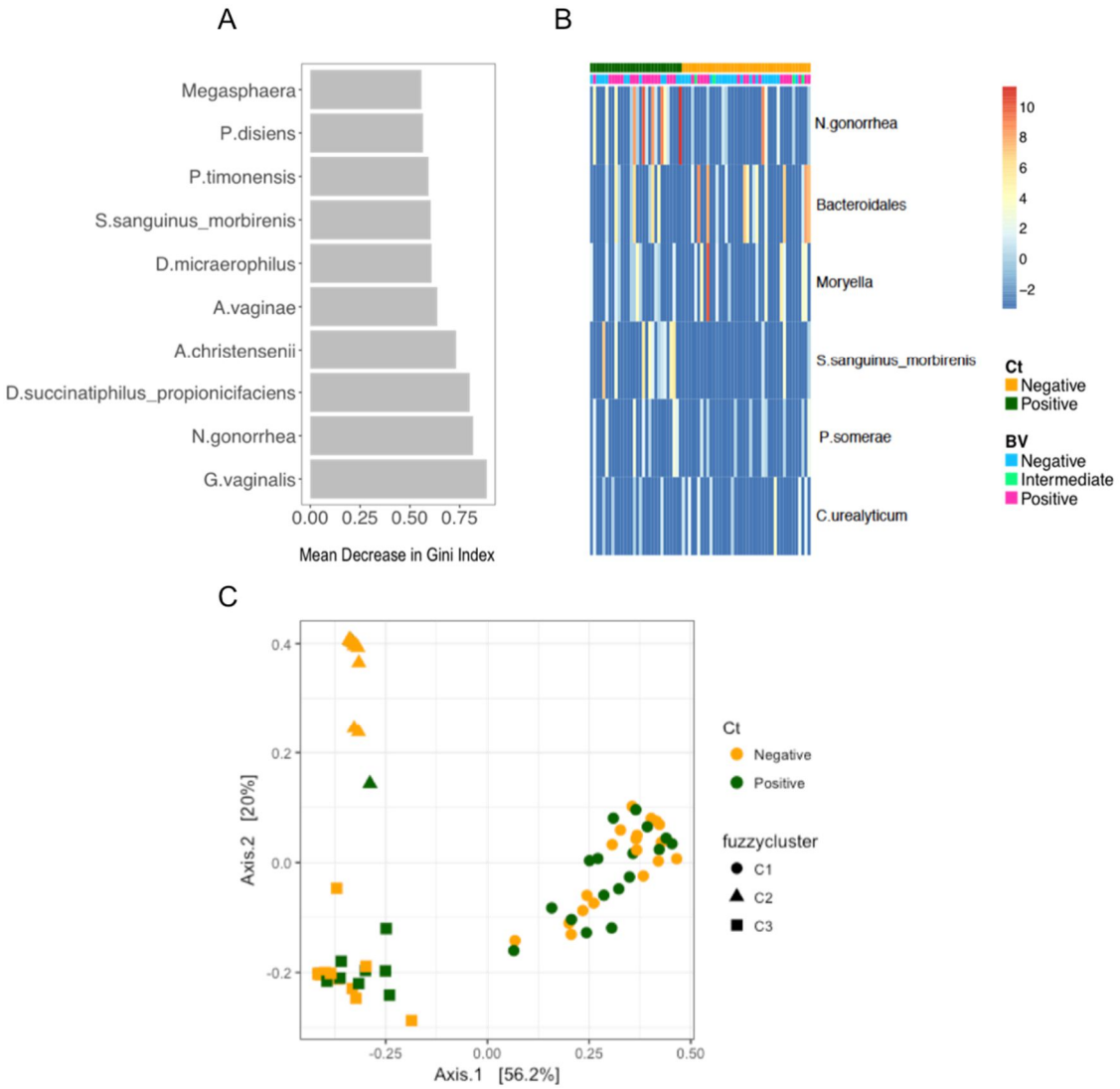


Figure 3.4. Taxa significantly different in *C. trachomatis* infected versus uninfected individuals in the endocervical microbiota. A) Taxa significantly differentially abundant and/or frequent by *C. trachomatis* (Ct) category in the endocervical (EC) microbiota by metagenomeSeq (FDR ≤0.05, coefficient ≥1.25, taxa present in ≥20% of samples in at least one of the two groups being compared). Unsupervised clustering of samples (columns) by Bray-Curtis distance; heatmap scale: log2-transformed standardized counts. The OTU assigned C. trachomatis taxonomy was excluded for this analysis. B) The top 20 most influential taxa by random forest analysis in predicting *C. trachomatis* infection. The x-axis indicates the mean decrease in Gini Index (length of bar represents predictive ability of each taxon), where a larger index indicates greater predictive power. Taxa that were significantly differentially abundant and/or frequent in *C. trachomatis* infected versus uninfected individuals (FDR ≤0.05, coefficient ≥1.25, taxa present in ≥20% of samples in at least one of the two groups being compared); hierarchical clustering (Bray-Curtis distance); heatmap scale: log2-transformed standardized counts. The OTU assigned C. trachomatis taxonomy was excluded for this analysis. C) Principal Coordinates Analysis (PCoA) of the endocervical microbiota using weighted UniFrac distances. Individual samples are coloured by *C. trachomatis* infection status with community cluster (C1, C2, C3) generated by Fuzzy clustering displayed as shapes. Figure published in (Balle et al., 2018).

Table 3.4. Differentially abundant taxa in endocervical and vaginal lateral wall microbiota between *C. trachomatis* positive and negative participants.

	Coeff	P adj.	Family	Genus	Species
Endo-cervical	-2.536	0.001	Bacteroidales*	NA	NA
	-1.577	0.018	Lachnospiraceae	*Moryella*	NA
	-1.400	2.3e-05	Corynebacteriaceae	*Corynebacterium*	*urealyticum*
	1.304	0.001	Porphyromonadaceae	*Porphyromonas*	*somerae*
	2.561	0.001	Alcaligenaceae	*Sutterella*	*sanguinus_morbirenis*
	3.368	0.002	Neisseriaceae	*Neisseria*	*gonorrhea*
Lateral wall	-1.609	0.049	Actinomycetaceae	*Varibaculum*	*cambriense*
	-1.312	0.012	Streptococcaceae	*Streptococcus*	*anginosus*
	-1.290	0.049	[Tissierellaceae]	*Anaerococcus*	NA
	1.271	6.01e-06	[Mogibacteriaceae]	*Mogibacterium*	NA
	1.293	4.3e-05	Bacteroidetes*	NA	NA
	1.311	0.015	Ruminococcaceae	NA	NA
	1.456	0.008	Ruminococcaceae	*Ruminococcus*	*bromii*
	1.540	0.017	Neisseriaceae	*Neisseria*	*gonorrhea*
	1.624	2.8e-05	Lactobacillaceae	*Lactobacillus*	*ruminis*
	1.801	0.011	Lachnospiraceae	*Eubacterium*	*rectale*
	1.907	0.003	Veillonellaceae	*Megasphaera*	*elsdenii*
	2.081	2.9e-06	Erysipelotrichaceae	*Catenibacterium*	NA
	2.083	0.0004	Veillonellaceae	*Mitsuokella*	NA
	2.188	0.003	Rs-045	NA	NA
	2.309	0.001	Ruminococcaceae	*Faecalibacterium*	*prausnitzii*
	2.637	0.001	Succinivibrionaceae	*Succinivibrio*	NA
	2.906	3.7e-07	Alcaligenaceae	*Sutterella*	*sanguinus_morbirenis*
	3.238	2.3e-06	Lachnospiraceae	*Coprococcus*	*eutactus*

*Note: Bacteroidales only annotated at order level and Bacteroidetes at phylum level.

Table 3.5. Random forest analysis of lateral wall microbiota from *C. trachomatis* positive versus negative participants.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_5	3.463	0.756	*Gardnerella vaginalis*
OTU_	1.467	0.611	*Neisseria gonorrhoea*
OTU_	-0.890	0.607	*Dialister succinatiphilus_propionicifaciens*
OTU_22	-2.580	0.587	*Aerococcus christensenii*
OTU_	3.390	0.482	*Atopobium vaginae*
OTU_26	0.993	0.456	*Dialister micraerophilus*
OTU_	-0.912	0.452	*Sutterella sanguinus_morbirenis*
OTU_	3.059	0.439	*Prevotella timonensis*
OTU_14	1.150	0.426	*Prevotella disiens*
OTU_3	0.026	0.418	*Megasphaera*

Training set size: 48 samples with 23 and 25 samples per class. Test set size: 24 samples with 19 and 5 samples per class. Validation predicted error: 54.17% (using top five predictive taxa).

N. gonorrhoea and *Sutterella sanguinus_morbirenis* also had a significantly higher relative abundance in the LW of *C. trachomatis* positive versus negative participants according to metagenomeSeq analysis. Additionally, a group of gut-associated bacteria (e.g. *Ruminococcus, Lactobacillus ruminis, Eubacterium rectale, Faecalibacterium* and *Coprococcus*) were also more abundant in the *C. trachomatis* positive participants. *Prevotella* ssp. and *Megasphaera* were identified as the strongest predictors of *C. trachomatis* infection status in LW samples according to random forest analysis (**Figure 3.5A-B**, **Table 3.4** and **Table 3.6**); however this model did not have a predictive power with an error rate of 66.67% and AUC of 0.29 for the test set (sensitivity=0.69, specificity=0.69 for the test set). As with the EC samples, no overall differences in LW microbiota composition (beta diversity) were found between *C. trachomatis* positive and *C. trachomatis* negative participants (p=0.58) (**Figure 3.5C**).

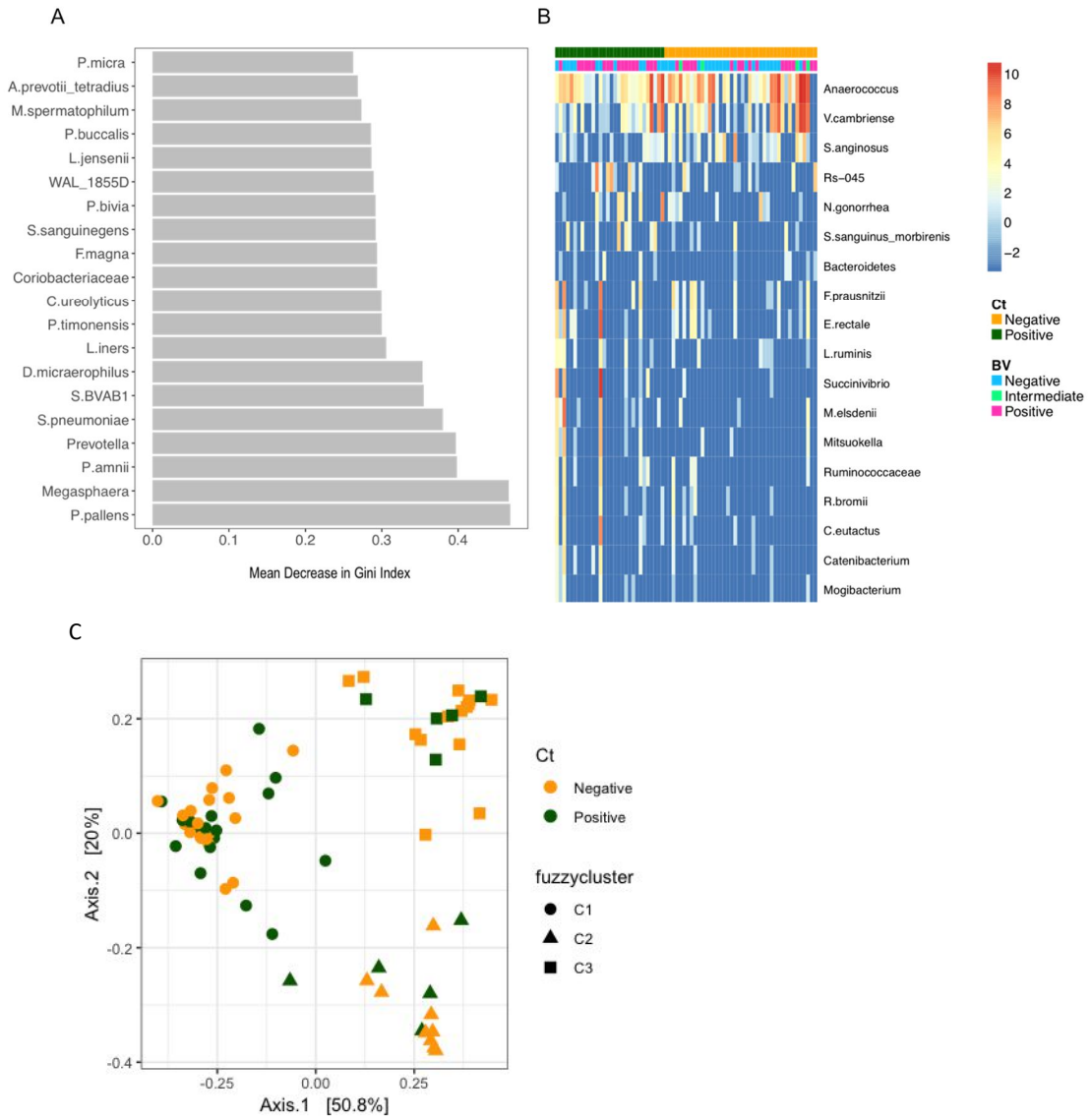


Figure 3.5. Taxa significantly different in C. trachomatis infected versus uninfected individuals in the vaginal microbiota. A) The top 20 most influential taxa by random forest analysis. The x-axis indicates the mean decrease in Gini Index (length of bar represents predictive ability of each taxon). B) Taxa significantly differentially abundant and/or frequent by *C. trachomatis* category in the vaginal lateral wall (LW) microbiota by metagenomeSeq (FDR ≤ 0.05, coefficient ≥ 1.25, taxa present in ≥ 20% of samples in at least one of the two groups being compared). Unsupervised clustering of samples (columns) by Bray-Curtis distance; heatmap scale: log2-transformed standardized counts. The OTU assigned *C. trachomatis* taxonomy was excluded for this analysis. C) Principal Coordinates Analysis (PCoA) of the lateral wall microbiota using weighted UniFrac distances. Individual samples are coloured by *C. trachomatis* infection status with community cluster (C1, C2, C3) generated by Fuzzy clustering displayed as shapes.

Table 3.6. Random forest analysis of lateral wall microbiota from *C. trachomatis* negative versus positive participants.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_17	0.647	0.468	*Prevotella pallens*
OTU_3	2.486	0.466	*Megasphaera*
OTU_6	-0.030	0.398	*Prevotella amnii*
OTU_7	-0.313	0.397	*Prevotella*
OTU_95	2.577	0.380	*Streptococcus pneumoniae*
OTU_4	0.647	0.355	BVAB1
OTU_26	2.279	0.353	*Dialister micraerophilus*
OTU_1	-1.976	0.306	*Lactobacillus iners*
OTU_9	-2.090	0.300	*Prevotella timonensis*
OTU_48	-0.811	0.376	*Campylobacter ureolyticus*
OTU_28	-0.974	0.300	*Coriobacteriaceae*
OTU_15	-0.592	0.294	*Finegoldia magna*
OTU_104	-0.977	0.294	*Sneathia sanguinegens*
OTU_13	-1.022	0.292	*Prevotella bivia*
OTU_27	-1.891	0.289	WAL_1855D
OTU_98	-1.393	0.287	*Lactobacillus jensenii*
OTU_24	1.486	0.286	*Prevotella buccalis*
OTU_101	0.587	0.273	*Mycoplasma spermatophilum*
OTU_30	1.609	0.269	*Anaerococcus prevotii_tetradius*
OTU_19	-1.386	0.262	*Parvimonas micra*

Training set size: 48 samples with 23 and 25 samples per class. Test set size: 24 samples with 19 and 5 samples per class Validation predicted error: 66.67% (using top five predictive taxa).

3.4 Discussion

To our knowledge, this is the first study to describe the relationship between FGT microbial communities and asymptomatic *C. trachomatis* in adolescents. Furthermore, few studies have examined this relationship in African women, who have very different vaginal microbiota than their European counterparts. Finally, most if not all, studies assessing the relationship between *C. trachomatis* and genital tract microbiota have focused only on the vagina and not on the cervix, which the site of *C. trachomatis* infection.

3.4.1 Differences in vaginal and endocervical microbiota

Although the overall bacterial community compositions were found to be more similar between the EC and LW samples within participants than each site

between participants, we found significantly different beta diversity between EC and LW samples within community clusters. Furthermore, consistent with the results of others (Ling et al., 2011), we found the within sample diversity in the vagina to be higher than in the endocervix. Although previous studies have suggested that the microbiota in different anatomical sites within the FGT of adults is similar in composition (Huang et al., 2015a; Smith et al., 2014; Kim et al., 2009), we identified multiple taxa that were significantly more abundant in the vagina. These included anaerobes, such as *Peptoniphilus*, *Anaerococcus* and *Fusobacterium* spp. The endocervix had higher relative abundances of *E. faecium*, the source of which is likely the gastrointestinal tract. These findings are surprising, since the endocervix is further from the external (aerobic) environment and the rectum, than the vagina. In Chinese women (Chen et al., 2017), *L. crispatus* was notably more abundant in the vagina compared to the endocervix, which may be due to epithelial cell characteristics. *L. crispatus* expresses an adhesion molecule which mediates adherence to stratified squamous epithelium (found in the vagina) but not to columnar epithelium which constitutes the cervical epithelium (Edelman et al., 2012). Therefore, factors other than geography likely play a role in microbial ecosystems in the FGT. Since adolescence is a time of change in the cervix, it is possible that the differences we see here are unique to this age group. Alternatively, differences in sample collection technique between LW and EC swabs may have occurred.

3.4.2 The endocervical microbiota and C. trachomatis infection

No studies have explored the relationship between specific endocervical bacterial taxa and *C. trachomatis* status in adolescents. Our findings suggest the endocervical microbiota diversity is not grossly altered in women with *C. trachomatis*, although the sample size was small and the study underpowered. However, we found some taxa differentially abundant between *C. trachomatis* infected and uninfected women, including *G. vaginalis*, *A. vaginae*, *Dialister ssp.*, *Prevotella spp.* and *Megasphaera*, all BV-associated bacteria. Several studies have reported an increased risk of *C. trachomatis* infection among women with BV (Wiesenfeld et al., 2003; Gallo et al., 2012; Brotman et al., 2010). In a longitudinal study among Dutch women, microbiota dominated by *L. iners* was an

independent risk factor for later *C. trachomatis* acquisition (van Houdt et al., 2017), while *L. crispatus* may have been protective (van Houdt et al., 2017; van der Veer et al., 2017; Filardo et al., 2017; Borgdorff et al., 2014). Women with a *L. iners* dominated community are generally classified as BV-negative. Findings based on BV diagnostics may therefore not be optimal in describing the association between the vaginal microbiota and *C. trachomatis*. The correlation between lactobacilli and vaginal health, including protection against pathogens, is often attributed to the production of lactic acid resulting in low vaginal pH (Breshears et al., 2015; Nardini et al., 2016; Gong et al., 2014). Importantly, *L. crispatus* produces significantly more D-lactate than *L. iners*, and this difference has been shown to be important in trapping of pathogens (Nunn et al., 2015). Of note, *L. iners* is far more prevalent than other lactobacilli in African women, often co-exists with BV (Atashili et al., 2008; Ling et al., 2011), and may be one of the reasons why women of African descent are at high risk for STIs. Differences in sexual behaviour can also affect ones risk of acquiring STIs. In this study, *C. trachomatis* positive participants were less likely to report multiple sexual partners and reported fewer lifetime sexual partners than *C. trachomatis* negative participants, both of which is associated with decreased risk. However, regular condom use was higher amongst the *C. trachomatis* negative participants, suggesting that this may have had an influence on *C. trachomatis* infection outcome. Future studies on larger cohorts taking sexual risk behaviour into account should be conducted.

3.4.3 Limitations

One limitation of this study is its cross-sectional nature, which prevents definitive conclusions regarding whether specific taxa directly increase risk of *C. trachomatis* infection. Prospective longitudinal cohort studies will allow comparison of microbial communities prior to and after infection with *C. trachomatis*, will thus provide a deeper understanding of the role of microbiota including *L. iners* in modifying *C. trachomatis* risk in adolescents. Should genital tract colonization with specific bacteria be associated with increased risk of *C. trachomatis* infection, targeting these microbes could be considered as a potential risk reduction strategy.

3.4.3 Conclusions

We observed significant differences between endocervical and vaginal microbiota with the vaginal microbiota being more diverse and harbouring higher relative abundances of multiple taxa. We further identified associations between BV-associated bacteria in the cervix and *C. trachomatis* infection in African adolescents. There is thus a potential of altered functional profiles of the bacterial communities between different anatomical sites, which could alter mucosal susceptibility and should be taken into account when analysing the vaginal microbiota in relation to susceptibility of pathogens such as *C. trachomatis* and *N. gonorrhoea*.

Chapter 4: The female genital tract microbiota in South African adolescents at risk of HIV

Parts of this Chapter (**Figure 4.4** and **Figure 4.5A,B**) have been presented at the HIVR4P Conference in Madrid, Spain, on Oct 23rd, 2018.

4.1 Introduction

Adolescent girls and young women (AGYW) are at extremely high risk of sexually transmitted infections (STIs), particularly in sub-Saharan Africa. The commensal microbes in the female genital tract (FGT) are believed to play an important role in susceptibility to various STIs, including *Chlamydia trachomatis* and HIV-1 (Atashili et al., 2008; Mitchell et al., 2014; Myer et al., 2005a). Molecular-based studies of the FGT microbiota of healthy, reproductive-age women have described a number of distinct bacterial community types often consisting of a one to four low-diversity communities dominated by a single *Lactobacillus* species (*Lactobacillus crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii*) and one to two high-diversity communities, comprised of a mixture of anaerobic bacteria associated with bacterial vaginosis (BV) (Gajer et al., 2012; Ravel et al., 2011; Anahtar et al., 2015; Gautam et al., 2015). BV is a condition characterized by the displacement of lactobacilli in the FGT by a diverse group of facultative anaerobic bacterial species including *Gardnerella vaginalis, Atopobium vaginae, Megasphaera, Prevotella, Sneathia* and BV-associated bacteria (BVAB) 1-3. The distribution of the various community types amongst women varies according to population (Borgdorff et al., 2017; Ravel et al., 2011). Non-*iners Lactobacillus* species are recognized as an important component of the vaginal mucosal defence against invading pathogens due to their production of antimicrobial compounds, hydrogen peroxide (H_2O_2) and lactic acid. The latter maintains an acidic environment (pH<4.5), hostile to pathogens. Conversely, studies have shown an association of BV and *L. iners* dominated communities, both frequent in African women, with increased risk of STI acquisition (Myer et al., 2005b; Atashili et al., 2008; van Houdt et al., 2017; Lennard et al., 2017; Gosmann et al., 2017). The FGT microbiota is a highly dynamic community and undergoes several significant changes over the course of a women's lifetime, even during the course of a menstrual cycle (Gajer et al., 2012; Hickey et al., 2013; Srinivasan et al., 2010). Data suggest that fluctuations in female sex hormones play a crucial role in the alterations of the vaginal microbiota over time and that external factors such as douching, hormonal contraceptive use, sexual behaviour and antibiotic usage can affect the composition of the microbiota (Mayer et al., 2015; Hickey et al., 2013; Fashemi et al., 2013; Schwebke et al., 1999). The dynamic

structure of the vaginal microbiota emphasizes the importance of characterizing the vaginal microbial community at different biological stages throughout a women's life. Yet, most of the molecular-based data published to date on the vaginal microbiota have focused on adult women. During puberty, women undergo dramatic hormonal and anatomical changes, which could affect the microbiota and hence susceptibility to STIs. Furthermore, adolescence is often the time of sexual debut and initial exposure to a range of pathogenic bacteria and viruses. In this study, we aimed to characterize the composition and stability of the vaginal microbiota in South African adolescents, a group at high risk of STIs, and to evaluate the impact of endogenous hormone levels on the vaginal microbiota.

4.2 Methods

For this analysis, we included 151 adolescent girls enrolled in the uCHOOSE-A-Star study described in Chapter 2.1.1 (p. 46-48). The study procedures were approved by the Human Research Ethics Committee of the University of Cape Town, and informed consent or assent (if <18 years) was obtained from all participants before initiation of the study. Informed consent was obtained from a parent or legal guardian of participants younger than 18 years. Information on demographics, medical and reproductive history and sexual risk behaviours was collected. As described in Chapter 2.1.2 (p. 49-50), vulvovaginal and lateral wall swabs were collected for STI, BV and *Candida* testing and lateral vaginal wall and ectocervical swabs were obtained for microbiota analyses. DNA extraction from swabs, 16S rRNA gene sequencing library generation and pre-processing of sequencing reads, including generation of OTU table and taxonomic annotation, were performed as described in Chapter 2.2.1, 2.2.3 and 2.2.4 (p. 51-55), respectively. All downstream statistical analyses were performed in RStudio as described in Chapter 2.3 (p. 55-56).

4.3 Results

4.3.1 uCHOOSE-A-Star study participant characteristics

Out of 156 adolescent girls who were screened for the uCHOOSE-A-Star study, 151 were eligible for enrolment. Of these, 131 were randomized to one of the three study arms (41 to combined oral contraceptive pills (COCPs), 45 to the Net-En injection and 45 to the NuvaRing) while five failed screening (due to hypertension, > grade 2 anaemia, elevated liver function tests, concomitant tuberculosis medication and age (14 years old)), four declined participation and 16 were lost to follow up (LTFU). 107 participants also completed the crossover visit (81.7% retention rate) and 92 participants completed all three visits (70.2% retention rate) (**Figure 4.1**).

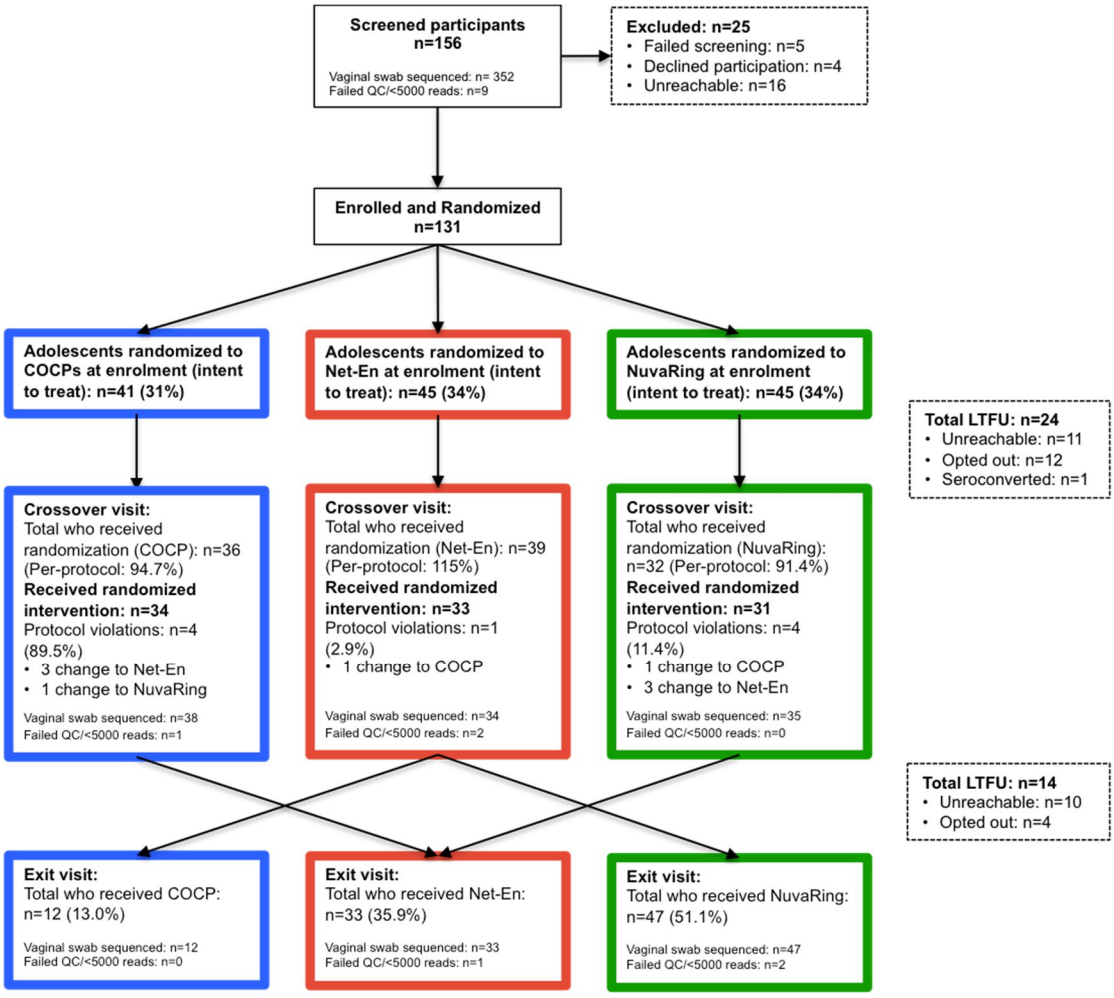


Figure 4.1. Study overview and randomization. The figure shows the number of participants that completed each study visits and the distribution of participants on each of the hormonal contraceptive method analysed: combined oral contraceptive pills (COCP), the Net-En injection and the vaginally inserted NuvaRing at crossover and exit. Note that at crossover some participants could choose their next contraceptive method if assigned to NuvaRing as their first method. LTFU: Lost to follow up.

An overview of the baseline characteristics (at screening) of the study population is shown in **Table 4.1**. Of the 151 participants screened, the median age was 17 years (interquartile range (IQR) 16-18), the median body mass index (BMI) was 25.1 (IQR 21.9-29.0) and all participants had reached menarche prior to study participation (**Table 4.1**). Among the screened participants, 57.0% (n=86) were STI negative while the remaining participants were positive for one or more bacterial or protozoan STIs (43.0%, n=65) (**Table 4.1** and **Figure 4.2**) with *C. trachomatis* being the most common at 33.1% (n=50). Nearly as many (27.2% (n=41)) tested positive for HSV-2 serology.

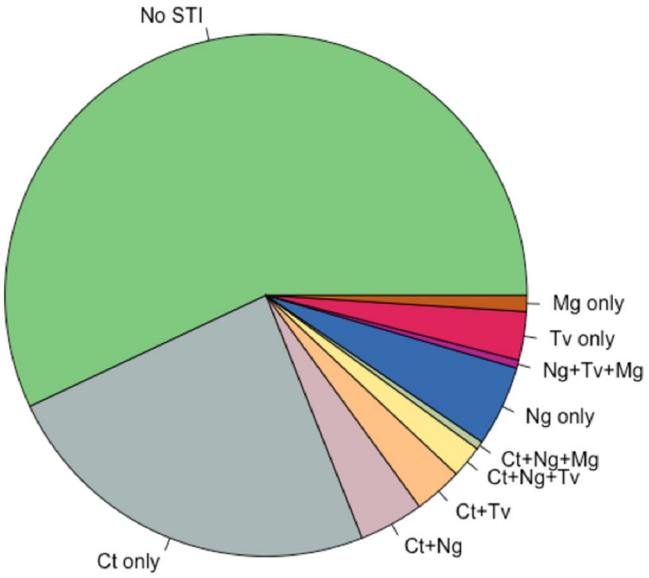


Figure 4.2. Bacterial STI prevalence at screening. Pie chart showing the prevalence of bacterial (Ct, Chlamydia trachomatis, Ng, Neisseria gonorrhoea, Mg, Mycoplasma genitalium) and protozoan (Tv, Trichomonas vaginalis) sexually transmitted infections (STIs) at the screening visit (n=151).

Nugent scores were available for all participants at all visits. At screening 43.0% of the participants were BV positive (n=63, Nugent score 7-10), 11.3% were BV intermediate (n=19, Nugent score 4-6) and the remaining 45.7% were BV negative (n=69, Nugent score 0-3) (**Table 4.1**). Despite a high BV and STI prevalence, none of the participants reported any symptoms of vaginal infection at screening. Vaginal pH was generally high in the cohort with a mean of 4.92 (±sd 4.33-5.51) at screening. At baseline, approximately one fourth of the participants were either hormonal contraceptive (HC) naive (4.8%, n=7) or not currently using any HC method (19.9%, n=29) (**Table 4.1**).

Table 4.1. Baseline characteristics of uCHOOSE participants.

	Screened (n=151)
Age in years at screening, median (IQR)	17 (16-18)
BMI, median (IQR)	25.1 (21.9-29.0)
STI prevalence	
Any STI(s)	65 (43.0%)
Ct	50 (33.1%)
Ng	18 (11.9%)
Tv	13 (8.6%)
Mg	4 (2.6%)
BV prevalence	
BV positive	65 (43.0%)
BV intermediate	17 (11.3%)
BV negative	69 (45.7%)
Vaginal pH, mean (sd)	4.92 (4.33-5.51)
HSV-2 serology[1]	41 (27.2%)
Yeast cells present	23 (15.2%)
Age in years at menarche, median (IQR)	13 (12-14)
Antibiotics past three months	
Any	8 (5.3%)
Endocrinology[2]	
E2 (pmol/l)	100.5 (75.3-140.8)
FSH (U/l)	4.9 (3.6-6.0)
LH (IU/l)	4.3 (2.0-6.0)
Use of hormonal contraception[3]	
Naive	7 (4.8%)
Not currently	29 (19.9%)
Net-En	78 (53.4%)
COCP	9 (6.2%)
DMPA	20 (13.7%)
Implanon	3 (2.1%)
Parity	
Previously pregnant	17 (11.5%)
Vaginal delivery	10 (6.8%)
Caesarean section	5 (3.4%)
Abortion	2 (1.4%)
Intra-vaginal practices[4]	
Douching	1 (0.7%)
Washing with water	19 (12.8%)
Washing with soap	13 (8.8%)
Cloth	4 (2.7%)
Drying	1 (0.7%)
Medication	7 (4.7%)
Tampon use	8 (5.4%)
Herbs	1 (0.7%)
Sexual risk behaviour	
Median age of sexual debut, years (IQR)[5]	15 (14-16)
Sexual partners past 12 months, median, n (IQR)[6]	1 (1-2)
New sexual partners past 12 months, median, n (IQR)[6]	0 (0-1)
General condom use[7]	
Never	12 (9.4%)
Almost never	13 (10.2%)
Not sure	15 (11.7%)
Almost always	46 (35.9%)
Always	42 (32.8%)
Condom use during last PV intercourse [7]	
Yes	78 (61.4%)
PV sex acts per week, median (IQR)	1 (1-2)
Intergenerational sex with older partner (≥5 years)[6]	
No	50 (39.1%)
Unsure	52 (40.6%%)
Yes	26 (20.3%)
Transactional sex[6]	1 (0.8%)

Penile-anal intercourse[6]	4 (3.1%)
Education[6]	
School attendance	114 (85.7%)
Highest grade, median (IQR)	10 (8-11)
Tertiary attendance	6 (4.5%)

BMI, body mass index; BV; bacterial vaginosis; Ct, Chlamydia trachomatis*; E2, oestradiol; FSH, follicle-stimulating hormone, HSV-2, herpes simplex virus type-2 seropositive; IQR, interquartile range; LH, luteinizing hormone; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. One equivocal result.
2. Missing values from two participants.
3. Missing data from five participants.
4. Missing data from four participants.
5. Missing data from six participants.
6. Missing data from 23 participants.
7. Missing data from 24 participants.

Participants who had not been on any HC for ≥2 months (i.e. ≥5 months since last DMPA injection, ≥4 months since last Net-En injection or ≥2 months since last using COCPs or removal of a contraceptive implant (Implanon)) were defined as not on HC and combined for downstream visualization purposes and statistical analyses. In figures and tables, this group is referred to under HC as "None". At baseline, the Net-En injectable was the most commonly used HC method among the screened study participants (53.4%, n=78) followed by the DMPA injectable (13.7%, n=20) and COCPs (6.2%, n=9). Just over ten percent (n=17) of participants had previously been pregnant. The median age of sexual debut in the cohort was 15 years (IQR 14-16) and the median number of self-reported sexual partners over the past year leading up to the study was one (IQR 1-2) (**Table 4.1**). A total of 34 participants reported having one or more new sexual partners over the past 12 months while no new sexual partner was reported by 83 participants. Only one participant reported having had transactional sex before the study. About a fifth of the participants reported having had intergenerational sex with an older partner (five or more years older), whereas forty percent reported not to have had intergenerational relations. The remaining forty percent were unsure. Four participants reported having had penile-anal intercourse. Nearly 70% of the participants reported use of condoms during intercourse always or nearly always (n=88, 68.7%) while almost one fifth reported to never or almost never use condoms during sex (n=25, 19.6%). Condom usage during the last penile-vaginal sexual act before the baseline visit was reported by 61.4% of participants with available data. In regard to

intravaginal practices, washing the vagina with either water (12.8%) or soap (8.8%) were the most common practices reported, yet, overall very few participants reported performing any form of intra-vaginal practices (**Table 4.1**). In terms of education, the majority of the participants were attending school during the study (85.7%) with grade ten being the median grade completed (IQR 8-11). There were no biases observed in the characteristics of successfully screened participants that went on to be randomized and those that were not randomized (Appendix III).

4.3.2 Vaginal community clusters in South African adolescents

Out of the 352 lateral vaginal wall samples collected from 150 uCHOOSE participants and analysed by 16S rRNA sequencing (one sample collected at screening is missing), 343 samples passed the sequencing and quality control (QC) measures (including ≥ 5000 sequencing reads per sample). Of the nine samples that did not pass QC, three were screening samples, three were samples from crossover visits and the remaining three were exit visit samples. Hence, a total of 147 samples from screened participants including 127 from participants randomized to one of the three study arms, 104 from crossover visits and 89 from exit visits were available for downstream analysis (**Figure 4.1**). Sequencing data was available for all three visits for 85 participants. All PCR water controls and DNA extraction controls generated 431 or fewer reads per sample (median 84, IQR 42-152, range 0-431) (Appendix IV). These low read counts suggest limited contamination and the samples were excluded from downstream analysis. The 16S rRNA gene sequences were grouped into operational taxonomic units (OTUs), groups of closely related sequences, at a 97% sequence similarity threshold. The generated OTUs are used as proxies for bacterial species in subsequent analyses. For this dataset, a total of 571 OTUs with at least 10 counts in at least 20% of samples or a relative abundance of at least 0.001% were generated and assigned to 546 different taxa. When merging OTUs at lowest annotated taxonomic level, 341 taxa were generated. For an overall characterization of the vaginal microbiota in adolescents, we first applied an unsupervised clustering approach using Fuzzy clustering with optimal k and weighted UniFrac distances to the sequencing data from all samples passing QC

(n=343). Optimal k was determined by iteration of k's from 2-10 and evaluation of the resulting silhouette widths. For this analysis optimal k was determined to be three with an average silhouette width of 0.479 (**Figure 4.3**).

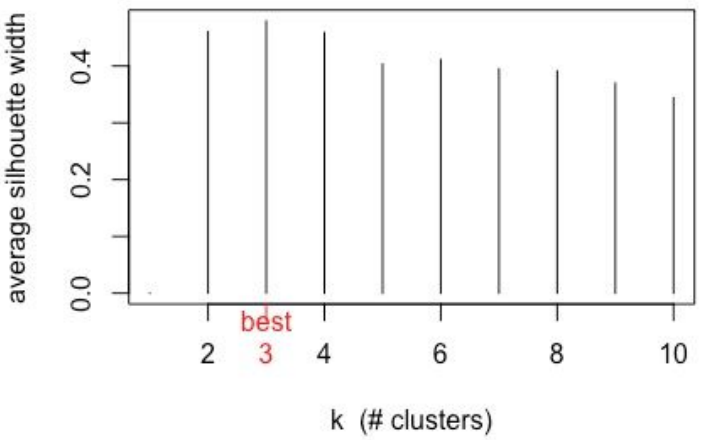


Figure 4.3. Average silhouette width with different k's for the uCHOOSE-A-Star cohort. Barplot depicting the average silhouette width when applying different k's (2-10) for Fuzzy clustering. Optimal k (k=3) determined based on the highest average silhouette width (marked in red).

Using this clustering approach, we identified three major vaginal community types, which we named C1, C2 and C3 in accordance with Lennard et al. (2017) (Lennard et al., 2017). Using Principal Coordinates Analysis (PCoA) of the beta-diversity using weighted UniFrac distances, the samples clearly grouped into three distinct clusters associated with the three community types generated by Fuzzy clustering (**Figure 4.4**). The first community type, C1 (n=153; 44.6%), was a high diversity community comprised of a diverse group of anaerobic bacteria associated with BV with the most abundant species being *Gardnerella vaginalis* (mean relative abundance (mRA) 20.2%) followed by BVAB1 (mRA 16.6%), *Megasphaera* spp. (mRA 11.4%), *L. iners* (mRA 8.3%), *Prevotella* spp. (including *P. amnii* (mRA 7.3%), *P. timonensis* (mRA 3.4%) and *P. bivia* (mRA 1.7%)), *Atopobium vaginae* (mRA 3.5%), *Sneathia* (mRA 3.3%) and *Aerococcus christensenii* (mRA 1.3%) (**Figure 4.4**, **Figure 4.5C** and **Table 4.2**). The latter two communities, C2 and C3, were low diversity communities dominated by *L. crispatus* (n=76; 22.2% with mRA of 82.1%) and *L. iners* (n=103; 30.0% with mRA of 84.0%), respectively (**Figure 4.4**, **Figure 4.5C** and **Table 4.2**). Overall, *L. iners* was the most abundant bacterial species (mRA 32.5%) identified in the cohort followed by *L. crispatus* (mRA 20.7%) and *G. vaginalis* (mRA 10.0%).

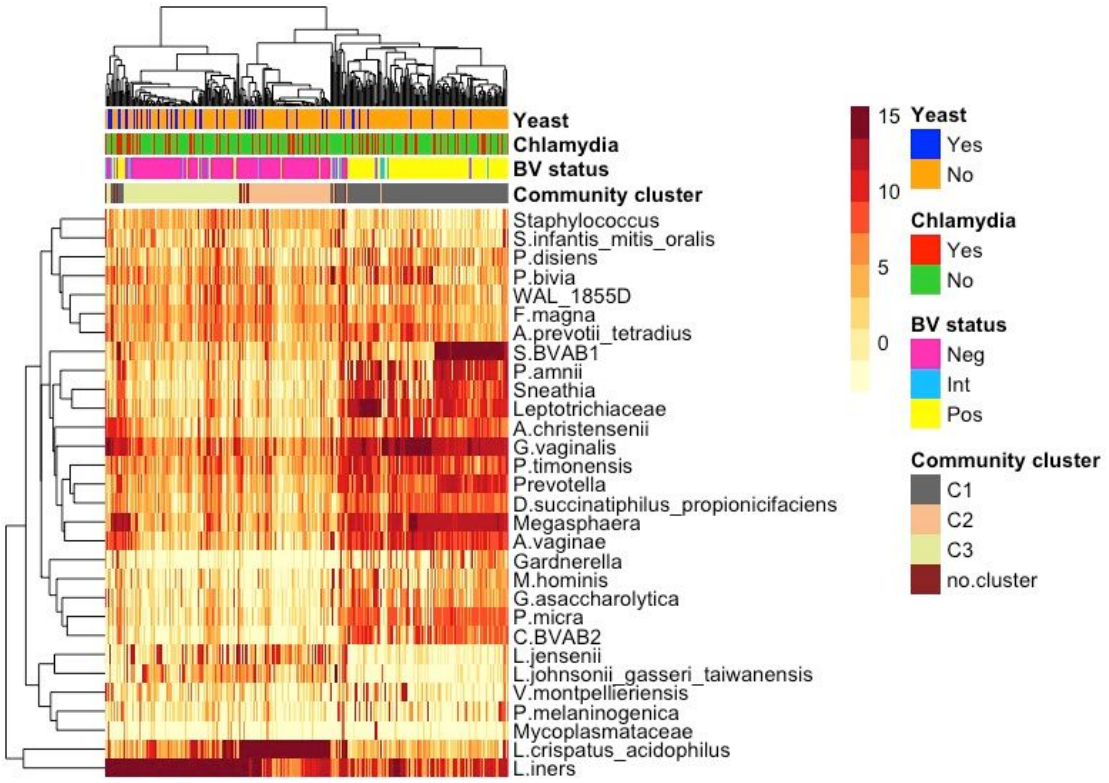


Figure 4.4. The vaginal microbiota of South African adolescents. Heatmap of the top 30 most abundant taxa (rows) in the uCHOOSE-A-Star cohort identified by 16S rRNA microbiota profiling of 343 vaginal samples (columns) from 151 participants using unsupervised hierarchical clustering with weighted UniFrac distances. The dendrogram was generated using average linkage clustering with weighted UniFrac distances, based on the relative abundance of taxa (merged at lowest taxonomic level) in each sample. Log2-transformed standardized read counts are illustrated by the colour key. Annotation bars above the heatmap depict community cluster identified using Fuzzy clustering using weighted UniFrac distances (lower bar), Bacterial vaginosis (BV) status based on Nugent scoring (lower middle bar), Chlamydia trachomatis infection (upper middle bar) and yeast infection (top bar). Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from downstream analyses involving Fuzzy clusters (n=11, "no.cluster" in figure).

Eleven samples were comprised of communities with less than 60% probability of belonging to any of the three clusters (C1-C3) described above and these samples were excluded from any downstream analyses involving community clusters (referred to as "no.cluster" in figures). The vast majority of participants with a C1 vaginal community type were BV positive according to Nugent scoring (87.6%) whereas participants with C2 and C3 community types were most commonly BV negative (94.7% and 87.4%, respectively) (**Figure 4.4**, **Figure 4.5B** and **Table 4.3**). Accordingly, the alpha diversity measured using Shannon Index (SI) was significantly higher in the C1 communities compared to both the C2 (p=7.4e-31) and C3 (p=9.6e-34) communities while no difference in diversity was observed between the two lactobacilli-dominated communities (p=0.322) (**Figure 4.5A** and **Table 4.3**).

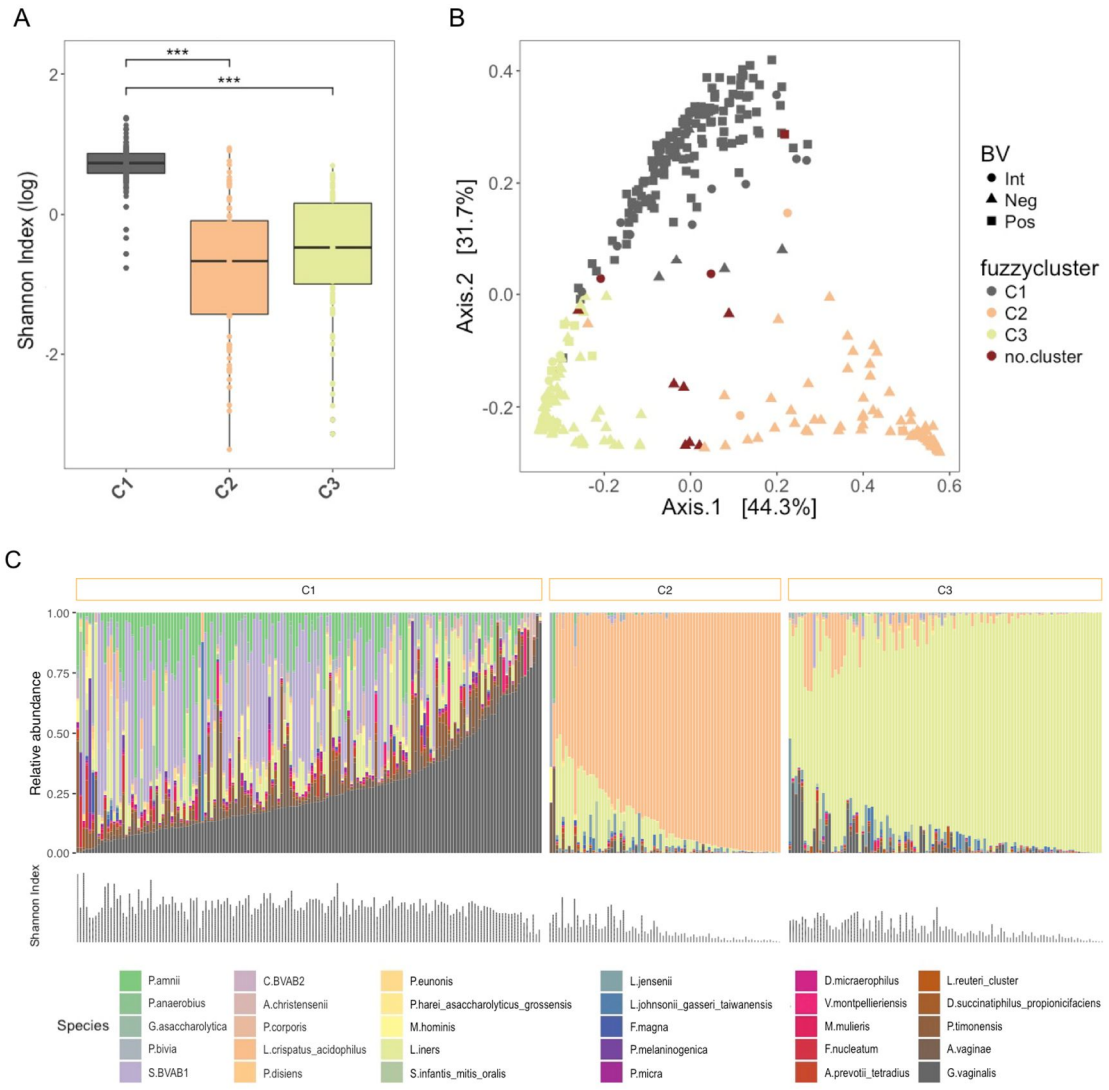


Figure 4.5. Vaginal community clusters in a South African adolescent cohort. A) Boxplot depicting the alpha diversity measured using Shannon Index (log transformed values) of the bacterial communities of 332 vaginal samples from 151 participants according to community cluster (C1: n=153; C2: n=76; C3: n=103) defined using Fuzzy clustering with weighted UniFrac distances. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from the figure (n=11). P values were calculated using unpaired Mann-Whitney-Wilcoxon test with multiple testing correction (MTC) using the Benjamini-Hochberg (BH) method. Significance codes: ns: p>0.05, *: p<0.05, **: p<0.01, ***: p<0.001. B) Principal Coordinate Analysis (PCoA) of all 343 samples from 151 participants using weighted UniFrac distances. Samples are coloured by community cluster generated using Fuzzy clustering with weighted UniFrac distances. Bacterial vaginosis (BV) status based on Nugent scoring is displayed as shapes. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of these clusters were excluded from downstream analyses (n=11, "no.cluster" in figure). C) Barplot depicting the relative abundance of the 30 top most abundant species in the uCHOOSE-A-Star cohort identified by 16S rRNA microbiota profiling. The samples (n=332) are grouped by microbial community cluster (C1, C2, C3) established using Fuzzy clustering with weighted UniFrac distances and ordered based on the abundance of the most dominant species in each community type (C1: G. vaginalis, C2: L. crispatus and C3: L. iners). Samples that did not meet the minimum probability of ≥ 60% of belonging to any of tree clusters (n=11) were excluded from the figure. The alpha diversity for each sample measured using the Shannon diversity Index is depicted below the barplot.

Table 4.2. Top 30 taxa in community clusters C1-C3.

Fuzzy cluster C1			Fuzzy cluster C2			Fuzzy cluster C3		
Genus	**Species**	**mRA**	**Genus**	**Species**	**mRA**	**Genus**	**Species**	**mRA**
Gardnerella	*vaginalis*	20.2%	*Lactobacillus*	*crispatus_acidophilus*	82.1%	*Lactobacillus*	*iners*	84.0%
Shuttleworthia	BVAB1	16.6%	*Lactobacillus*	*iners*	9.3%	*Lactobacillus*	*crispatus_acidophilus*	4.0%
Megasphaera		11.4%	*Prevotella*	*bivia*	1.2%	*Gardnerella*	*vaginalis*	2.6%
Lactobacillus	*iners*	8.3.%	*Lactobacillus*	*jensenii*	1.1%	*Lactobacillus*	*jensenii*	1.8%
*Leptotrichiaceae**		7.9%	*Atopobium*	*vaginae*	0.8%	*Megasphaera*		1.7%
Prevotella	*amnii*	7.3%	*Prevotella*	*amnii*	0.4%	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*	0.7%
Prevotella		3.6%	*Gardnerella*	*vaginalis*	0.4%	*Prevotella*	*timonensis*	0.5%
Atopobium	*vaginae*	3.5%	*Streptococcus*	*infantis_mitis_oralis*	0.4%	*Prevotella*	*bivia*	0.4%
Prevotella	*timonensis*	3.5%	*Prevotella*	*timonensis*	0.4%	*Streptococcus*	*infantis_mitis_oralis*	0.4%
Sneathia		3.3%	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*	0.4%	*Shuttleworthia*	BVAB1	0.4%
Prevotella	*bivia*	1.7%	*Leptotrichiaceae**		0.3%	*Lactobacillus*	*reuteri_cluster*	0.3%
Aerococcus	*christensenii*	1.3%	*WAL_1855D*		0.3%	*Anaerococcus*	*prevotii_tetradius*	0.3%
*Mycoplasmataceae**		1.0%	*Prevotella*	*corporis*	0.3%	*Atopobium*	*vaginae*	0.3%
Lactobacillus	*crispatus_acidophilus*	0.9%	*Gemella*	*morbillorum_cluster*	0.3%	*WAL_1855D*		0.3%
Clostridium	*BVAB2*	0.9%	*Finegoldia*	*magna*	0.2%	*Staphylococcus*		0.3%
Mycoplasma	*hominis*	0.9%	*Mycoplasma*	*hominis*	0.2%	*Finegoldia*	*magna*	0.2%
Streptococcus	*infantis_mitis_oralis*	0.8%	*Lactobacillus*	*reuteri_cluster*	0.2%	*Leptotrichiaceae**		0.2%
Gardnerella		0.7%	*Megasphaera*		0.2%	*Aerococcus*	*christensenii*	0.2%
Anaerococcus	*prevotii_tetradius*	0.7%	*Prevotella*		0.2%	*Ureaplasma*	*parvum_urealyticum*	0.2%
Veillonella	*montpellieriensis*	0.7%	*Anaerococcus*	*prevotii_tetradius*	0.2%	*Prevotella*		0.2%
Prevotella	*melaninogenica*	0.7%	*Clostridium*	*perfringens*	0.2%	*Peptoniphilus*	*harei_asaccharolyticus_grossensis*	0.1%
Dialister	*succinatiphilus_propionicifaciens*	0.6%	*Staphylococcus*		0.2%	*Prevotella*	*disiens*	0.1%
Parvimonas	*micra*	0.6%	*Peptoniphilus*	*harei_asaccharolyticus_grossensis*	0.1%	*Streptococcus*	*agalactiae*	0.1%
Lactobacillus	*johnsonii_gasseri_taiwanensis*	0.5%	*Peptostreptococcus*	*anaerobius*	0.1%	*Veillonella*	*montpellieriensis*	0.1%
Fusobacterium	*nucleatum*	0.5%	*Shuttleworthia*	BVAB1	0.1%	*Haemophilus*	*parainfluenzae*	0.1%
Prevotella	*disiens*	0.4%	*Dialister*	*succinatiphilus_propionicifaciens*	0.1%	*Prevotella*	*corporis*	0.1%
Gemella	*asaccharolytica*	0.4%	*Sneathia*		0.1%	*Gemella*	*asaccharolytica*	0.1%
Porphyromonas	*eunonis*	0.4%	*Corynebacterium*	*amycolatum_lactis*	0.1%	*Fusobacterium*	*nucleatum*	0.1%
Dialister	*micraerophilus*	0.4%	*Bacteroides*	*coagulans*	0.1%	*Dialister*	*succinatiphilus_propionicifaciens*	0.1%
Mobiluncus	*mulieris*	0.3%	*Haemophilus*	*parainfluenzae*	0.1%	*Prevotella*	*amnii*	0.1%

Operational taxonomic units (OTUs) merged at lowest taxonomic level. mRA: mean relative abundance.
*Note: these taxa only annotated down to family level.

The relative abundance of several of the taxa known to be associated with BV, e.g. BVAB1-2, *Sneathia*, *Prevotella* spp., *Dialister* spp., *A. vaginae*, *Megasphaera* and *G. vaginalis*, were positively correlated with alpha diversity, Nugent scoring and vaginal pH (**Figure 4.6**). Conversely, *L. crispatus* and *L. iners* were negatively correlated with these measures.

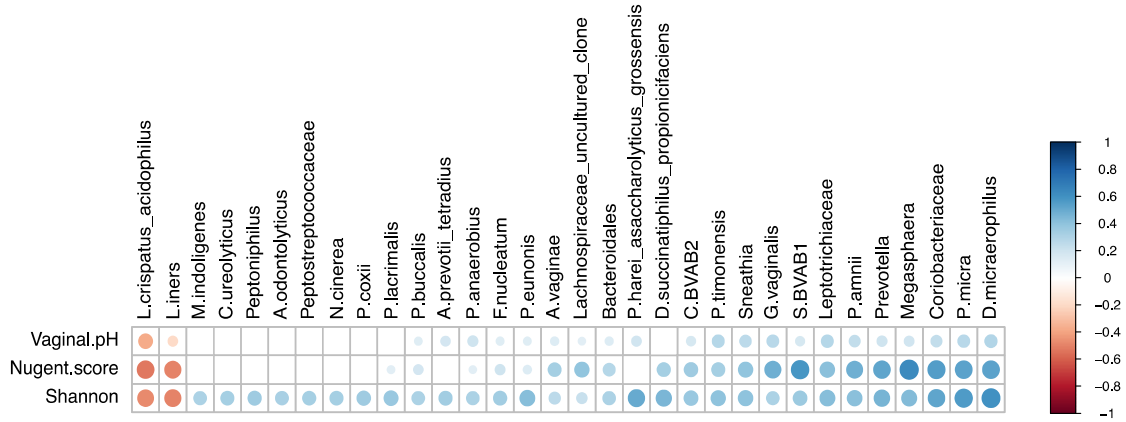


Figure 4.6. Correlation between vaginal pH, Nugent score and alpha diversity with the standardized read counts of individual bacteria. Significant positive (blue) or negative (red) correlations between standardized read counts of bacteria (merged at lowest taxonomic level) and vaginal pH, Nugent scoring assessed by Gram staining and alpha diversity measured with Shannon Index using Spearman's rank correlation. Adjusted p value of 0.05 set as cut-off. Only R values>0.3 displayed. Each OTU has ≥10 counts in ≥30% of samples.

Of the 147 samples available for analysis at baseline (screening visit), 73 were assigned to the C1 cluster, 30 to C2 and 38 to C3. Six samples were not assigned to any cluster. As was the case with all samples, at baseline the alpha diversity measured was significantly higher in the C1 communities compared to the C2 and C3 communities (adj. p=3.7e-14 and p=3.5e-14, respectively) while no difference in diversity was observed between the C2 and C3 (adj. p=0.276) (**Table 4.3**). Vaginal pH was significantly higher in the diverse C1 community (median 5.12, IQR 4.66-5.58) compared to the lactobacilli-dominant clusters (C2: median 4.62, IQR 3.97-5.27; C3: median 4.76, IQR 4.20-5.32; p=3.3e-7). We observed less STI prevalence among participants with a vaginal microbiota belonging the *L. crispatus* dominant community type (C1: 33.3%) compared to the *L. iners* dominant (C2: 46.6%) and BV-like communities (C3: 44.7%) however this was not statistically significant (**Table 4.3**). A higher prevalence of *C. trachomatis* was detected in C3 (40.7%) versus C1 (34.2%) and C2 (33.3%) but not significantly so (**Table 4.3**). There were also no differences in *C. trachomatis*

infection according to BV status (p=0.966). HSV-serology was more common in participants with a C3 vaginal microbiota (35.1%) compared to C1 (28.2%) and C2 (13.8%), yet this was not significant. Information on HSV-2 shedding is not available for this study, but in a prior study conducted in the same community (WISH cohort described in Chapter 2.1.3 and Chapter 3), shedding was detected in 5% of women (Barnabas et al., 2017). Participants with the C3 community type had a higher proportion of yeast infections compared to C1 and C2 (26.3% versus 9.59% and 16.7%) yet this difference was not significant (**Table 4.3**). However, when looking at yeast infection according to BV status, there were significantly more BV negative participants with yeast cells detected by microscopy (n=15, 23.4%) compared to amongst BV intermediate (n=3, 12.0%) and BV positive (n=4, 6.45%) participants (p=0.019). No significant differences were found between the three clusters according to age, BMI, educational level or intra-vaginal practices (**Table 4.3**). We also did not observe any differences between clusters in regard to self-reported age at sexual debut, transactional sex or number of sexual partners over the past year (new or old). Fewer participants in the C2 group reported having had intergenerational sex with a partner ≥5 years older than participants in the C1 and C3 group (11.1% versus 26.6% and 32.3%, p value not significant), but a high percentage of C2 participants replied "not sure" to this particular question (55.6%). Participants with a vaginal microbiota assigned to C1 were significantly less likely to report condom use at their last penile-vaginal intercourse while participants in the C3 cluster reported a significantly higher level of sex acts per week (**Table 4.3**). Although not statistically significant, participants in the C1 cluster also reported less condom use in general than participants in the other two clusters (always or almost always (C1: n=34, 56.7% versus C2: n=22, 81.4% and C3: n=23, 74.2%).

Table 4.3. Cohort characteristics at baseline according to Fuzzy cluster.

	C1 - diverse (n=73)	**C2 – *L. crispatus*** (n=30)	**C3 – *L. iners*** (n=38)	**P value**
Age at screening, median years (IQR)	17 (16-18)	17 (16-18)	17 (16-18)	0.205
BMI, median (IQR)	24.8 (21.5-29.2)	25.0 (22.3-27.1)	25.1 (22.3-29.1)	0.897
STI prevalence				
Any STI(s)	34 (46.6%)	10 (33.3%)	17 (44.7%)	0.457
Ct	25 (34.2%)	10 (33.3%)	11 (40.7%)	0.849
Ng	10 (13.7%)	3 (10.0%)	5 (13.2%)	0.945
Mg	2 (2.74%)	0 (0.00%)	2 (5.26%)	0.542
Tv	9 (12.3%)	1 (3.33%)	3 (7.89%)	0.399
BV prevalence				**<2.2e-16**
BV positive	61 (83.5%)	0 (0.00%)	2 (5.26%)	
BV intermediate	10 (13.7%)	1 (3.33%)	3 (7.89%)	
BV negative	2 (2.74%)	29 (96.7%)	33 (86.8%)	
HSV-2 serology[1]	20 (28.2%)	4 (13.8%)	13 (35.1%)	0.145
Yeast cells present	7 (9.59%)	5 (16.7%)	10 (26.3%)	0.067
Shannon Index, median (IQR)	2.12 (1.84-2.42)	0.41 (0.26-0.82)	0.63 (0.43-1.06)	**<2.2e-16**
Vaginal pH, mean (sd)	5.12 (4.66-5.58)	4.62 (3.97-5.27)	4.76 (4.20-5.32)	**3.3e-7**
Endocrinology, median (IQR)[2]				
E2 (pmol/L)	108 (82-157)	93 (75-133.5)	85 (63.5-127.3)	**0.041**
LH (U/L)	4.8 (2.0-6.7)	4.6 (3.5-5.95)	2.95 (0.68-4.93)	**0.010**
FSH (U/L)	4.9 (3.6-6.1)	4.9 (3.7-5.85)	4.9 (2.33-5.98)	0.511
Use of hormonal contraception[3]				0.752
None	21 (28.8%)	6 (20.0%)	7 (18.4%)	
Net-En	35 (47.9%)	17 (56.7%)	20 (52.6%)	
COCP	3 (4.11%)	1 (3.33%)	4 (10.5%)	
DMPA	10 (13.7%)	5 (16.7%)	4 (10.5%)	
Implanon	2 (2.74%)	1 (3.33%)	0 (0.00%)	
Intra-vaginal practices[4]				
Douching (y/n)	1 (1.39%)	0 (0.00%)	0 (0.00%)	1.000
Washing with water (y/n)	13 (18.1%)	2 (6.67%)	2 (5.56%)	0.327
Washing with soap (y/n)	10 (13.9%)	1 (3.33%)	2 (5.56%)	0.211
Cloth (y/n)	3 (41.7%)	0 (0.00%)	1 (2.28%)	0.809
Drying (y/n)	1 (1.39%)	0 (0.00%)	0 (0.00%)	1.000
Medication (y/n)	6 (8.33%)	0 (0.00%)	1 (2.28%)	0.227
Tampon use (y/n)	4 (5.56%)	1 (3.33%)	1 (2.28%)	0.869
Herbs (y/n)	1 (1.39%)	0 (0.00%)	0 (0.00%)	1.000
Sexual risk behaviour				
Median age of sexual debut in years (IQR)[5]	15 (14-16)	15 (14-16)	15 (14-16)	0.858
# Sexual partners, median[6] (IQR)	1 (1-2)	1 (1-2)	1 (1-2)	0.648
Multiple sexual partners, n[6]	1 (1.7%)	0 (0.0%)	0 (0.0%)	0.163
New partner, median (IQR)[6]	0 (0-1)	0 (0-1)	0 (0-0)	0.321
General condom use[7]				0.209
Never	8 (13.3%)	1 (3.7%)	3 (9.7%)	
Almost never	7 (11.7%)	2 (7.4%)	4 (12.9%)	
Not sure	11 (18.3%)	2 (7.4%)	1 (3.2%)	
Almost always	15 (25%)	11 (40.7%)	15 (48.4%)	
Always	19 (31.7%)	11 (40.7%)	8 (25.8%)	
Condom use during last PV intercourse, n[7]	31 (52.5%)	22 (81.5%)	20 (64.5%)	**0.035**
# PV sex acts per week, median (IQR)	1 (1-2)	1 (1-2)	2 (1-3)	**0.025**
Intergenerational sex with older partner (≥5 years)[6]				0.341
No	26 (43.3%)	9 (33.3%%)	11 (35.5%)	
Unsure	20 (33.3%)	15 (55.6%)	12 (38.7%)	
Yes	14 (23.3%)	3 (11.1%)	8 (25.8%)	
Transactional sex, n[6]	1 (1.67%)	0 (0.00%)	0 (0.00%)	1.000
Penile-anal intercourse, n[6]	4 (6.67%)	0 (0.00%)	0 (0.00%)	0.177

Education[6]				
School attendance, n	53 (82.8%)	23 (85.2%)	29 (90.6%)	0.626
Highest grade, median (IQR)	9 (7-12)	8 (6-12)	9 (7-11)	0.614
Tertiary attendance, n	4 (6.25%)	2 (7.41%)	0 (0.00%)	0.321

Chi-square or Fisher's for count data. Kruskal-Wallis with Dunn's post hoc test using BH method for non-parametric data. ANOVA with Tukey's post hoc test using BH. BMI, body mass index; BV; bacterial vaginosis; Ct, Chlamydia trachomatis*; E2, oestradiol; FSH, follicle-stimulating hormone, HSV-2, herpes simplex virus type-2 seropositive; IQR, interquartile range; LH, luteinizing hormone; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. One equivocal results, missing values from three participants.

2. Missing values from two participants.

3. Missing data from five participants (C1: n=2, 2.7%; C2: n=0, 0.0%; C3: n=3, 7.9%).

4. Missing data from four participants (C1: n=2, 2.7%; C2: n=0, 0.0%; C3: n=2, 5.3%).

5. Missing data from six participants (C1: n=4, 5.5%; C2: n=0, 0.0%; C3: n=2, 5.3%)

6. Missing data from 23 participants (C1: n=13, 17.8%; C2: n=3, 10.0%; C3: n=7, 18.4%).

7. Missing data from 24 participants (C1: n=14, 19.2%; C2: n=3, 10.0%; C3: n=7, 18.4%).

4.3.3 The vaginal microbiota and endogenous hormones

To investigate the association of female sex hormones and vaginal microbial communities, we measured the levels of naturally occurring oestradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the blood of each participant and related these to the vaginal microbiota. Hormone concentrations were only measured at the screening and exit visits. Hormone levels from two participant visits were unavailable (one from screening and one from exit). The hormone assays only measure naturally occurring endogenous hormones and not the synthetic steroids delivered by contraceptives. We therefore decided to first limit the analysis to samples taken at screening from participants who were not on HC when initiating the study (n=36) to evaluate the interplay between naturally occurring hormones during adolescence and the vaginal microbiota without interference of exogenous hormones. For these participants, the median E2 concentration was 129 pmol/l (95% range: 69.4-523 pmol/l) and the median number of days since reported last menstrual period start date was 28.5 days (IQR: 14-126 days). The reference ranges of E2 vary in women by menstrual cycle, maturity stage (puberty, menopause) and pregnancy (**Table 4.4**). Furthermore, reference ranges for individual hormones can vary from one clinical lab to another depending on the method of evaluation. **Table 4.4** show the predefined normal references ranges for E2, LH and FSH during the menstrual cycle in adult women as reported by our contract laboratory BARC (kit from Abbott Laboratories).

Table 4.4. Reference ranges of endogenous hormones in adult women.

E2 normal ranges		
Menstrual phase	**Median**	**95% range**
Follicular	198	76.7 – 921 pmol/l
Mid-cycle	719	139 – 2,382 pmol/l
Luteal	363	77 – 1,145 pmol/l
Menopausal (Untreated)	< 37	< 37* – 102 pmol/l
FSH normal ranges		
Menstrual phase	**Reference range (95% range)**	
Follicular	3.0 - 8.1 U/L	
Mid-cycle	2.6 - 16.7 U/L	
Luteal	1.4 - 5.5 U/L	
Menopausal (Untreated)	26.7 - 133.4 U/L	
LH normal ranges (95% range)		
Menstrual phase	**Reference range**	
Follicular	2.9 - 21.7 IU/L	
Mid-cycle	18.1 - 90.2 IU/L	
Luteal	0.8 - 16.2 IU/L	
Menopausal (Untreated)	14.2 - 52.3 IU/L	

Note: Reference ranges apply to adults. Predefined normal reference ranges provided by BARC (kit from Abbott Laboratories). *37.0 pmol/l is the analytical sensitivity of the test (lower detection level).

The E2 results in this study are in the lower end of the reference ranges provided by the contract laboratory for adult women. A recent study reported the E2 reference range during adolescence to be 20–300 pg/ml or 73.5-1,103 pmol/l (conversion factor: pg/ml x 3.676 = pmol/l (molecular weight = 272)) (Stanczyk & Clarke, 2014). Puberty is a time of rapid and unstable hormonal fluctuations and major physical changes. In girls these physical changes include changes in body shape, development of mammary glands, growth of pubic hair and an increase in fat tissue. Tanner Staging is an objective classification system used to assess the development of secondary sex characteristics of children during puberty. At screening, five participants were described as Tanner stage 3, 108 as Tanner stage 4 and thirty as Tanner stage 5. The median E2 level for the participants with Tanner stage of 3 was 78 pmol/l (95% range: 53.0-281.3 pmol/l), for participants with Tanner stage 4 the median E2 level was 98 pmol/l (37.0-531.5 pmol/l) and for participants with Tanner stage 5 the median level

was 103 pmol/l (95% range: 40.5-636.9 pmol/l) (**Figure 4.7**). There is thus a slight increase in E2 levels with increase in Tanner stage.

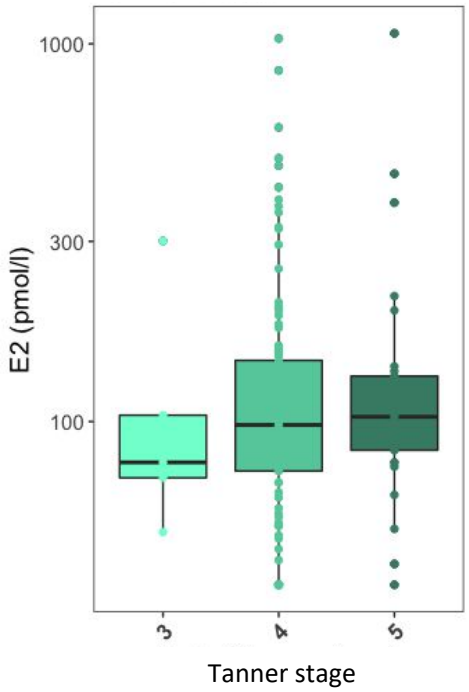


Figure 4.7. Oestradiol levels according to Tanner staging. Boxplot depicting the serum oestradiol (E2) levels according to Tanner staging at baseline (n=142). Y-axis is log10 transformed.

The median concentration of FSH in participants not on HC was 4.6 U/L (range 0.7-10.8 U/L) and for LH the median concentration was 3.6 IU/L (range 0.5-14 IU/L). These results are also in the lower end of the reference ranges provided by BARC for adult women. We did not observe any statistically significant differences between the three vaginal community clusters in relation to the concentration of any of the three endogenous hormones tested (**Figure 4.8** and **Table 4.5**) even after adjusting for last menstrual period and there was no significant correlation between the alpha diversity and the hormone levels for either E2, FSH or LH (**Figure 4.9**).

Table 4.5. Endogenous hormones and fuzzy clusters at baseline in participants not on hormonal contraception.

	C1 **Diverse** (n=21)	**C2** ***L. crispatus*** (n=7)	**C3** ***L. iners*** (n=7)	**P**	**C1 vs. C2**		**C1 vs. C3**		**C2 vs. C3**	
					P	P adj.	P	P adj.	P adj.	P adj.
E2 (pmol/l)	134 (103-215)	105 (84-136)	86 (85-365)	0.733	0.234	0.703	0.308	0.462	0.427	0.428
FSH (U/L)	4.1 (3.6-5.4)	5.0 (3.4-6.3)	5.2 (4.5-5.6)	0.714	0.426	0.426	0.205	0.614	0.301	0.451
LH (IU/L)	3.7 (2.0-5.9)	4.0 (2.5-4.2)	2.9 (1.5-4.6)	0.647	0.266	0.399	0.207	0.622	0.438	0.438

P values generated with Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests using Benjamini-Hochberg (BH) method for multiple testing correction (MTC). E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone. n=35.

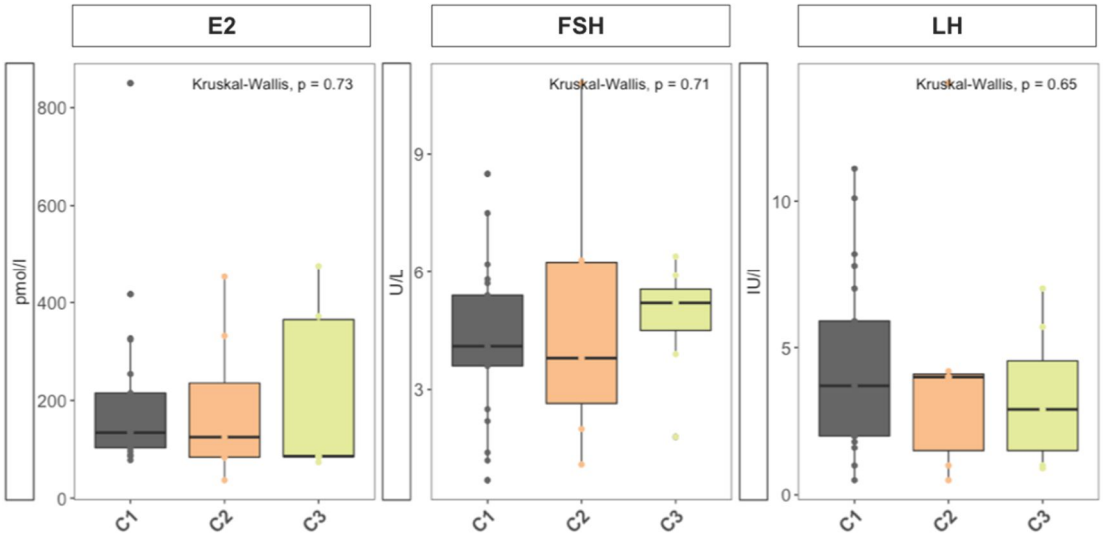


Figure 4.8. Endogenous hormones and vaginal community clusters at baseline in participants not on hormonal contraception. Boxplots of endogenous oestradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels in baseline samples from 35 participants not on hormonal contraception (including hormonal contraceptive naïve) at the screening visit stratified by community cluster generated using Fuzzy clustering with weighted UniFrac distances (C1, n=21; C2, n=7; C3, n=7). One sample that did not meet the minimum probability of ≥60% of belonging to any of the clusters and were excluded from the analysis. P values were generated using the Kruskal-Wallis test.

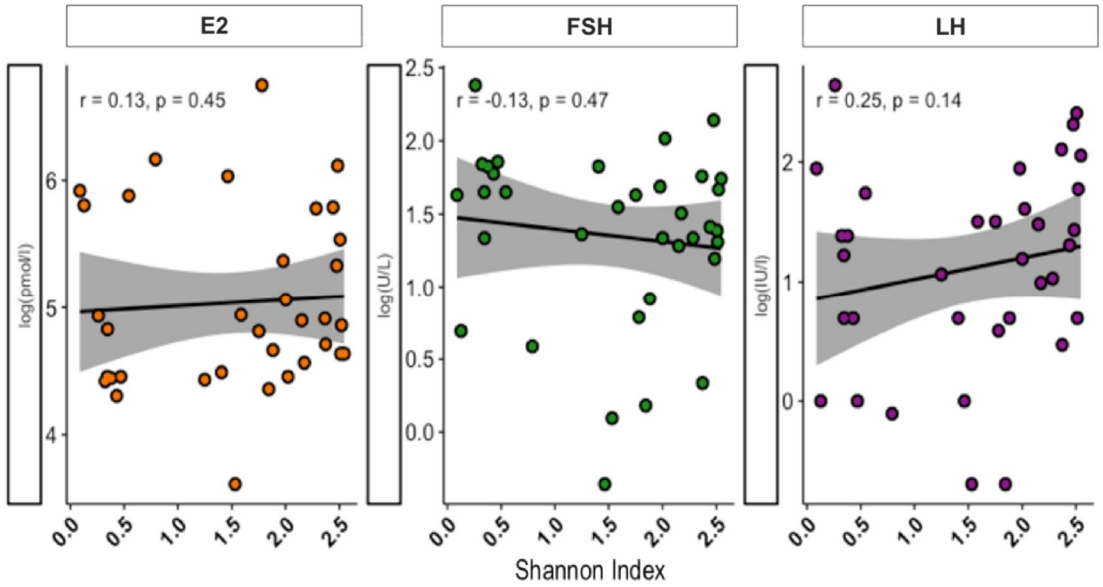


Figure 4.9. Endogenous hormones and the vaginal microbiota in participants not on hormonal contraception. Correlation plots of endogenous oestradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels according to alpha diversity measured with Shannon Index in baseline samples from 36 participants not on hormonal contraception (including hormonal contraceptive naïve) at the screening visit. The Spearman correlation coefficient is depicted on the figure for each individual hormone.

When including all samples from screening visit with hormone measurement and information on HC method (n=141), the median E2 concentration was 102 pmol/l (95% range: 37.0-487 pmol/l). The median concentration of E2 in participants using any hormonal contraceptive at screening was 94 pmol/l (95%

range: 37-431 pmol/l). This difference was significantly lower (p=0.0007) than in the participants not using HC indicating that HC use may suppress endogenous E2 production (**Table 4.6**). In a linear regression model adjusting for last menstrual period, there was a trend for lower E2 in participants not using HC compared to any HC use (OR: 1.6e-23, 95% CI 1.5e-50-1.8e+04, 0.098). No significant differences in LH and FSH concentrations were observed between HC and non-HC users also after adjustment for menstrual cycle (**Table 4.6**). We binarized the E2 levels based on this median (102 pmol/l) to assess whether any specific HC method or other factors could impact systemic E2 concentrations.

Table 4.6. Endogenous hormone levels at screening in non-hormonal contraceptive users and hormonal contraceptive users.

	All	No HC	Any HC	P value
E2 (pmol/l), median (95% range)	102 (37-487)	129 (69.4-523)	94 (37-431)	**0.0007**
FSH (U/L), median (range)	5.0 (0.1-10.8)	4.6 (0.7-10.8)	5.0 (0.1-9.0)	0.434
LH (IU/L), median (range)	4.2 (0.5-19.8)	3.6 (0.5-14.0)	4.6 (0.5-19.8)	0.505

P values generated using unpaired Mann-Whitney-Wilcoxon test between hormonal contraception users (Any HC) and non-users (No HC). E2, oestradiol; FSH, follicle-stimulating hormone, HC, hormonal contraception; LH, luteinizing hormone.

Participants in the high E2 group had a higher alpha diversity (1.87 vs. 1.25, p=0.006) and were more likely to have a diverse C1 vaginal microbiota than the low E2 group (63.6% vs. 40.6%, p=0.028) (**Table 4.7**). Yet, there were no significant correlation between E2 levels and alpha diversity (**Figure 4.10**). COCP, injectable DMPA and contraceptive implant (Implanon) use were equally distributed between the two E2 groups. Of the non-HC users, however, 69.4% fell into the high E2 category, while this was only the case for 37.3% of Net-En users (p=0.017), suggesting that Net-En may have more of a suppressive effect on systemic E2. Using a linear regression model, Net-En use was significantly associated with reduced E2 levels (p=0.017, OR: 6.1e-35, 95% CI: 7.4e-63-5.0e-07) compared to no HC use while COCP and injectable DMPA use was not. Net-En use remained significantly associated with lower E2 levels after adjusting for age, Tanner stage, BMI and last menstrual cycle (p<0.01). We did not find any significant differences in age or BMI between the two groups (**Table 4.7**) and neither age nor BMI were significantly correlated with E2 levels (**Figure 4.11A-B**).

Table 4.7. Participant characteristics according to oestradiol binarization.

	High E2 (n=70)	Low E2 (n=71)	P value
Age in years, median (IQR)	17 (16-18)	17 (16-18)	0.123
BMI, median (IQR)	25.1 (21.9-29.7)	24.6 (21.6-28.5)	0.830
Tanner stage, median (IQR)	4 (4-4)	4 (4-4)	0.659
Age in years at menarche, median (IQR)	13 (13-14)	13 (12-14)	0.344
Last mestruation[2]			0.603
<2 months before sampling	43 (62.3%)	39 (56.5%)	
>2 months before sampling	26 (37.7%)	30 (43.5%)	
Fuzzycluster[1]			**0.028**
C1	42 (63.6%)	28 (40.6%)	
C2	11 (16.7%)	19 (27.5%)	
C3	13 (19.7%)	22 (31.8%)	
BV prevalence			0.082
BV positive	36 (51.4%)	24 (33.8%)	
BV intermediate	8 (11.4%)	8 (11.3%)	
BV negative	26 (37.1%)	39 (54.9%)	
Vaginal pH, mean (sd)	4.95 (4.31-5.60)	4.90 (4.36-5.45)	0.630
Shannon Index, median (IQR)	1.87 (0.73-2.30)	1.25 (0.45-1.89)	**0.006**
STI prevalence			
Any STI(s)	27 (38.6%)	31 (43.7%)	0.658
Ct	21 (30.0%)	23 (32.4%)	0.900
Ng	8 (11.4%)	9 (12.7%)	1.000
Tv	7 (10.0%)	3 (4.2%)	0.208
Mg	3 (4.3%)	1 (1.4%)	0.366
HSV-2 serology[3]	18 (25.7%)	20 (28.2%)	0.849
Yeast cells present	11 (15.7%)	11 (15.5%)	1.000
Use of hormonal contraception			**0.017**
None	25 (35.7%)	11 (15.5%)	
Net-En	28 (40.0%)	47 (66.2%)	
COCP	5 (7.1%)	3 (4.2%)	
DMPA	10 (14.3%)	9 (12.7%)	
Implanon	2 (2.9%)	1 (1.4%)	
Intra-vaginal practices			
Douching	1 (1.4%)	0 (0.0%)	0.497
Washing with water	7 (10.0%)	12 (16.9%)	0.340
Washing with soap	6 (8.6%)	7 (9.9%)	1.000
Cloth	1 (1.4%)	0 (0.0%)	0.497
Drying	3 (4.3%)	1 (1.4%)	0.366
Medication	3 (4.3%)	4 (5.6%)	1.000
Tampon use	5 (7.1%)	3 (4.2%)	0.493
Herbs	1 (1.4%)	0 (0.0%)	0.497
Sexual risk behaviour[4]			
# Sexual partners, median (IQR)	1 (1-1)	1 (1-1)	0.481
Multiple sexual partners, n (%)	0 (0.0%)	1 (1.7%)	1.000
New partner(s), n (%)	20 (33.3%)	14 (23.3%)	0.311
General condom use			0.595
Never	6 (10.0%)	5 (8.3%)	
Almost never	7 (11.7%)	6 (10.0%)	
Not sure	10 (16.7%)	5 (8.3%)	
Almost always	18 (30.0%)	24 (40.0%)	
Always	18 (30.0%)	21 (35.0%)	
Condom use during last PV intercourse			
Yes	32 (53.3%)	42 (70.0%)	0.091
PV sex acts per week, median (IQR)	1 (1-1)	1 (1-1)	0.152
Intergenerational sex with older partner (≥5 years)			0.430
No	22 (36.7%)	26 (43.3%)	
I don't think so	2 (3.3%)	6 (10.0%)	
Not sure	19 (31.7%)	17 (28.3%)	
I think so	2 (3.3%)	2 (3.3%)	

Yes	15 (25.0%)	9 (15.0%)	
Transactional sex	0 (0.0%)	1 (1.7%)	1.000
Penile-anal intercourse	1 (1.7%)	3 (5.0%)	1.000

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency between groups, unpaired Mann-Whitney-Wilcoxon test for comparison of medians and unpaired Student's t test for comparison of means. BV, bacterial vaginosis; Ct, Chlamydia trachomatis*; E2, oestradiol; HSV-2, herpes simplex virus type 2; IQR, interquartile range; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. No Fuzzy cluster assignment for six samples (four in "High E2" and two in "Low E2").

2. Missing data from three participants (one in "High E2" and two in "Low E2").

3. One equivocal result.

4. Missing data from 21 participants (ten from "High E2" and eleven from "Low E2")

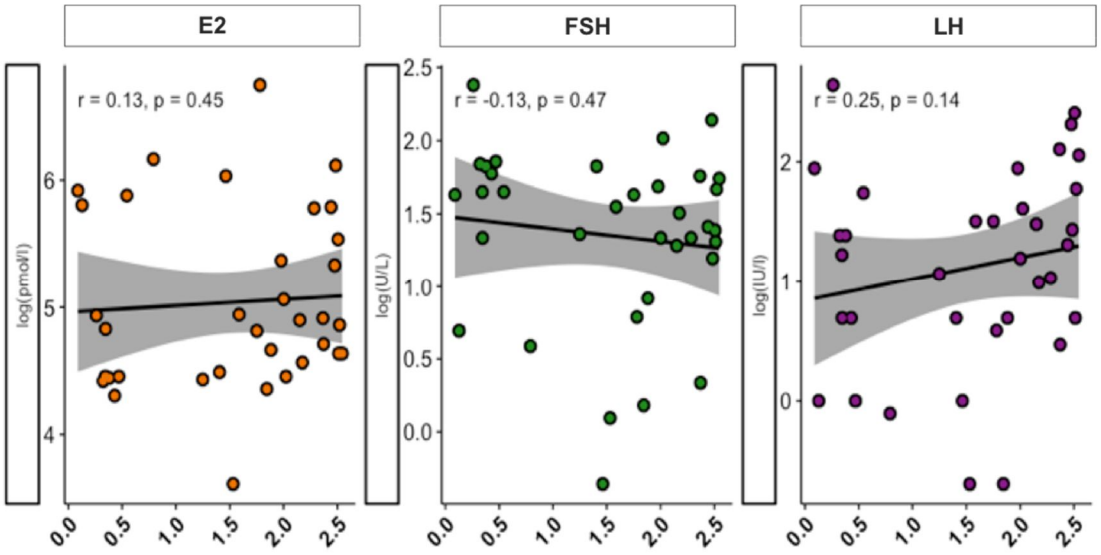


Figure 4.10. Endogenous hormones and the alpha diversity in non-hormonal contraceptive users at screening. Correlation plots of endogenous oestradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels according to alpha diversity measured with Shannon Index in baseline samples from 37 participants not on hormonal contraception (including hormonal contraceptive naïve) at the screening visit. The Spearman correlation coefficient is depicted on the figure for each individual hormone.

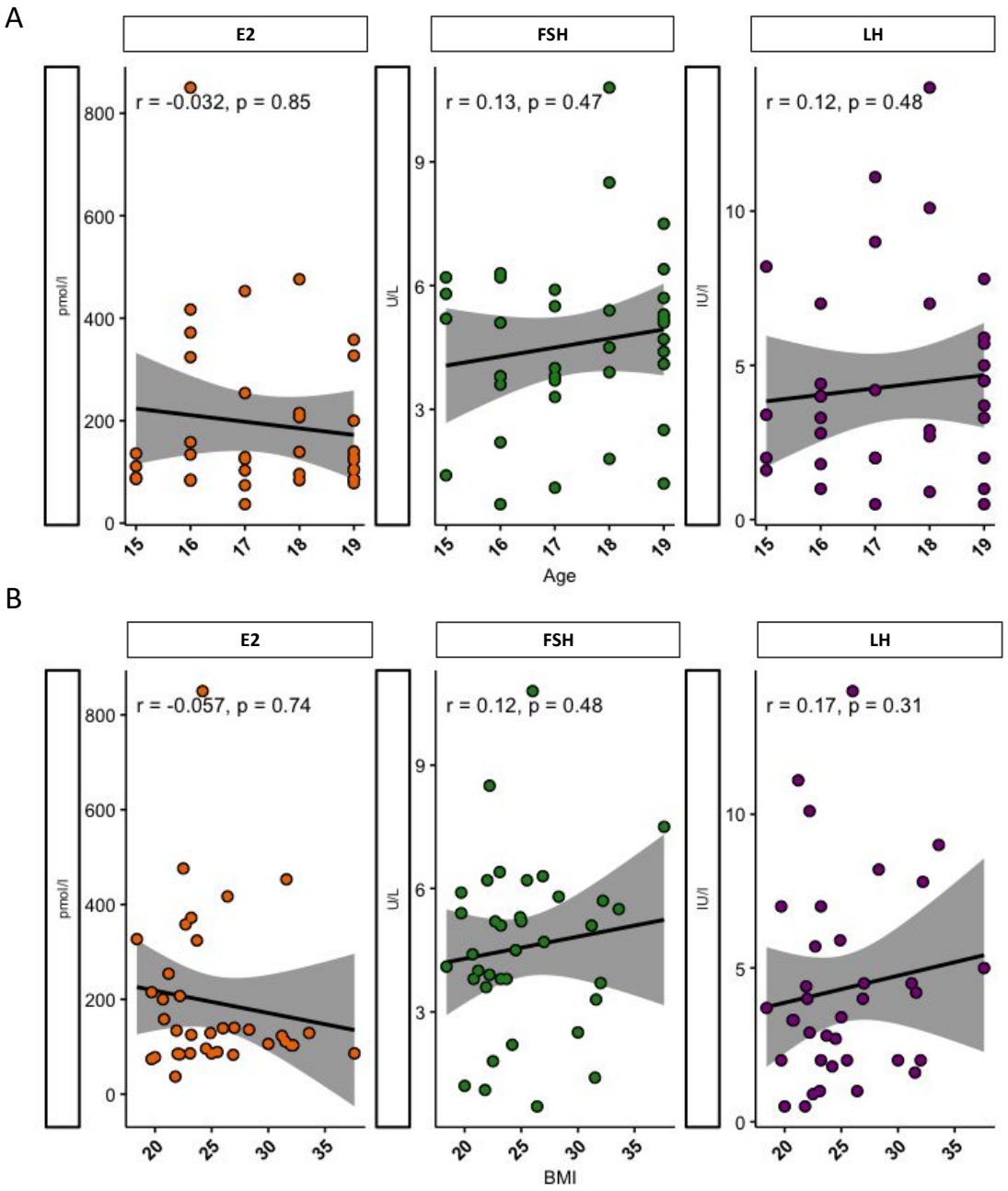


Figure 4.11. Endogenous hormones, age and BMI in non-hormonal contraceptive users at screening
Correlation plots of endogenous oestradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (measured in pmol/l, U/L and IU/L, respectively) according to A) the age and B) the body mass index (BMI) of 36 participants not using any hormonal contraception at screening. The Spearman correlation coefficient (rho, r) and the p values for each individual hormone depicted on the figure.

When looking at the level of endogenous hormones in the participants at baseline with information on HC method (n=139, excluding contraceptive implant (Implanon) users due to low numbers), we did observe a significant lower E2 concentration in participants using Net-En compared to non-HC users at baseline (**Figure 4.12**, **Table 4.8** and **Table 4.9**). This remained significant after adjusting for last menstrual cycle (p=0.003). In regard to FSH concentrations, these were significantly lower in participants using COCP compared to progestin-only injectable users and non-HC users before adjusting for multiple comparisons and after adjusting for last menstrual cycle (p=0.003) (**Figure 4.12**, **Table 4.8** and **Table 4.9**). Only the difference between COCP and Net-En stayed significant after adjusting for multiple comparisons (adj. p=0.034) (**Table 4.9**). A similar pattern was observed regarding LH concentrations with COCP users having lower LH concentrations, albeit these differences were not statistically significant (**Table 4.9**).

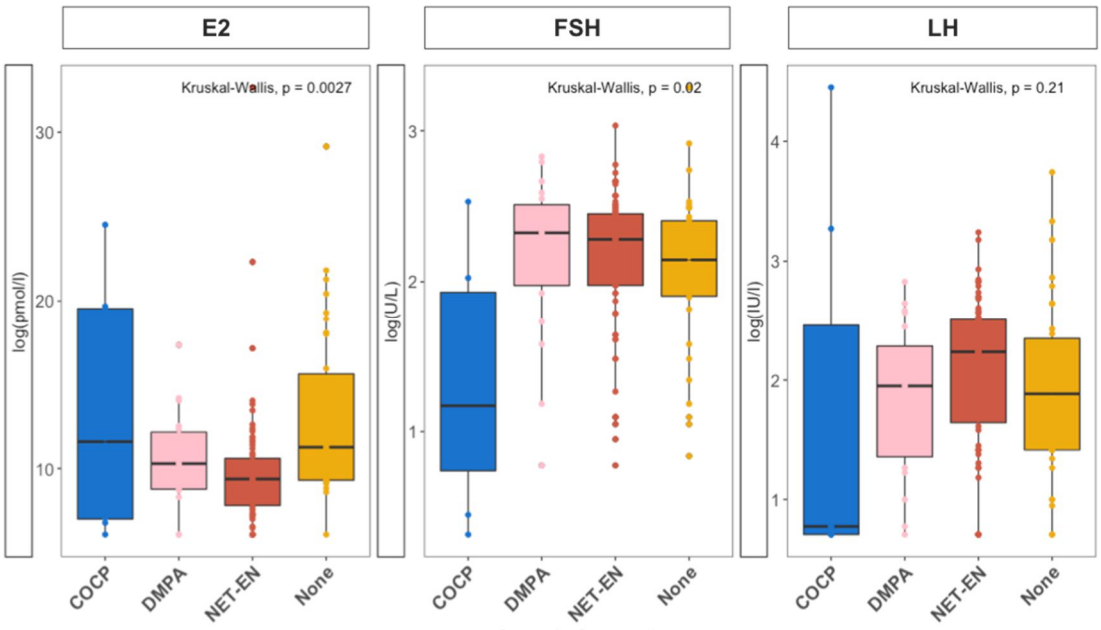


Figure 4.12. Endogenous hormones according to hormonal contraceptive method at screening. Boxplots of endogenous oestradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (log transformed concentrations) in baseline samples from 139 participants at the screening visit stratified by hormonal contraceptive method (COCP: n=8; DMPA: n=19; Net-En: n=76; None: n=36). Information on contraceptive method was missing from five participants and participants on the implant Implanon were excluded due to low numbers (n=3). P values were generated using the Kruskal-Wallis test.

Table 4.8. Endogenous hormones at baseline according to hormonal contraceptive method.

	COCP (n=8)	DMPA (n=19)	Net-En (n=76)	None (n=36)	P value
Endocrinology, median (IQR)					
E2 (pmol/l)	135 (49-382)	106 (77-148)	88 (64-117)	129 (88-225)	**0.002**
FSH (U/L)	1.4 (0.6-3.7)	5.4 (3.9-6.3)	5.2 (3.9-6.0)	4.6 (3.7-5.7)	**0.020**
LH (IU/L)	0.6 (0.5-6.3)	3.8 (1.9-5.3)	5.0 (2.7-6.3)	3.6 (2.0-5.8)	0.213

P values generated with Kruskal-Wallis test using Benjamini-Hochberg (BH) method for multiple testing correction (MTC). E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Table 4.9. Differences in endogenous hormone levels between hormonal contraceptive method at screening.

	E2		FSH		LH	
	P	P adj.	P	P adj.	P	P adj.
COCP vs. DMPA	0.614	0.736	**0.011**	0.065	0.312	0.401
COCP vs. Net-En	0.302	0.401	**0.004**	**0.034**	0.169	0.372
COCP vs. None	0.974	0.974	**0.016**	0.073	0.217	0.390
DMPA vs. Net-En	0.100	0.358	0.740	0.827	0.172	0.372
DMPA vs. None	0.140	0.372	0.299	0.401	0.781	0.827
Net-En vs. None	**0.0002**	**0.004**	0.260	0.401	0.186	0.372

P values calculated using unpaired Mann-Whitney-Wilcoxon test using Benjamini-Hochberg method for multiple testing correction (MTC). E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone. Participants using the contraceptive implant, Implanon, were excluded due to low numbers (n=3). COCP: n=8; DMPA: n=19; Net-En: n=76; None: n=36.

For 146 participants (regardless of HC use) both hormone concentrations and microbiota data from the screening visit were available for analysis and for 140 of these, the vaginal microbiota was assigned to one of the three community clusters. The E2 concentrations were significantly higher in the diverse C1 cluster compared to both the *L. crispatus* and *L. iners*-dominant communities (adj. p=0.048 and p=0.039, respectively) (**Figure 4.13** and **Table 4.9**) while the E2 levels were similar between women with the latter two communities (adj. p=0.427). In contrast, LH levels were significantly lower in the *L. iners* dominated community (C3) compared to the both diverse BV-like community (C1) and the *L. crispatus* dominated community (C2) (adj. p=0.007 and adj. p=0.011, respectively (**Figure 4.13** and **Table 4.9**). LH concentrations did not differ between C1 and C2 (adj. p=0.442). There were no differences in FSH levels between the three Fuzzy clusters at baseline (adj. p=0.511) (**Figure 4.13** and **Table 4.9**).

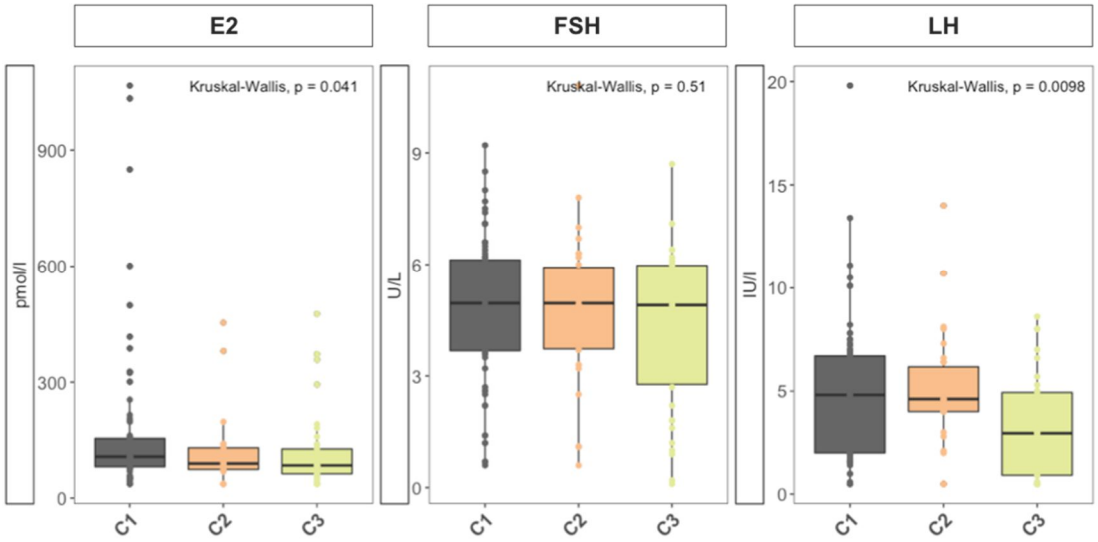


Figure 4.13. Endogenous hormones and the vaginal microbiota at screening. Boxplots of endogenous oestradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (measured in pmol/l, U/L and IU/L, respectively) in baseline samples from 140 participants at the screening visit stratified by community cluster (C1: n=72; C2: n=30; C3: n=38) generated using Fuzzy clustering with weighted UniFrac distances. P values were generated using the Kruskal-Wallis test.

Table 4.10. Endogenous hormones and fuzzy clusters at screening.

	C1 **Diverse** (n=72)	**C2** ***L. crispatus*** (n=30)	**C3** ***L. iners*** (n=35)	**P**	**C1 vs. C2**		**C1 vs. C3**		**C2 vs. C3**	
					P	P adj.	P	P adj.	P adj.	P adj.
E2 (pmol/l)	108 (81-155)	90 (74.5-130)	85 (63.5-127)	**0.041**	**0.052**	0.078	**0.032**	0.078	0.729	0.729
FSH (U/L)	5.0 (3.7-6.1)	5.0 (3.7-5.9)	4.9 (2.8-6.0)	0.511	0.869	0.869	0.262	0.627	0.418	0.627
LH (IU/L)	4.8 (2.0-6.7)	4.6 (4.0-6.2)	2.9 (0.93-4.9)	**0.010**	0.933	0.933	**0.005**	**0.016**	**0.011**	**0.017**

P values generated with Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests using Benjamini-Hochberg (BH) method for multiple testing correction (MTC). E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Furthermore, E2 and LH concentrations showed a weak, but significant positive correlation with alpha diversity (**Figure 4.14A**), which is in line with the Fuzzy cluster results, while no correlation was observed between the hormone concentrations and vaginal pH (**Figure 4.14B**), age or BMI (**Figure 4.15**). Using Spearman correlation, no specific taxa (merged at the lowest taxonomic level) were significantly correlated with either E2, FSH or LH levels (adj. p=0.1).

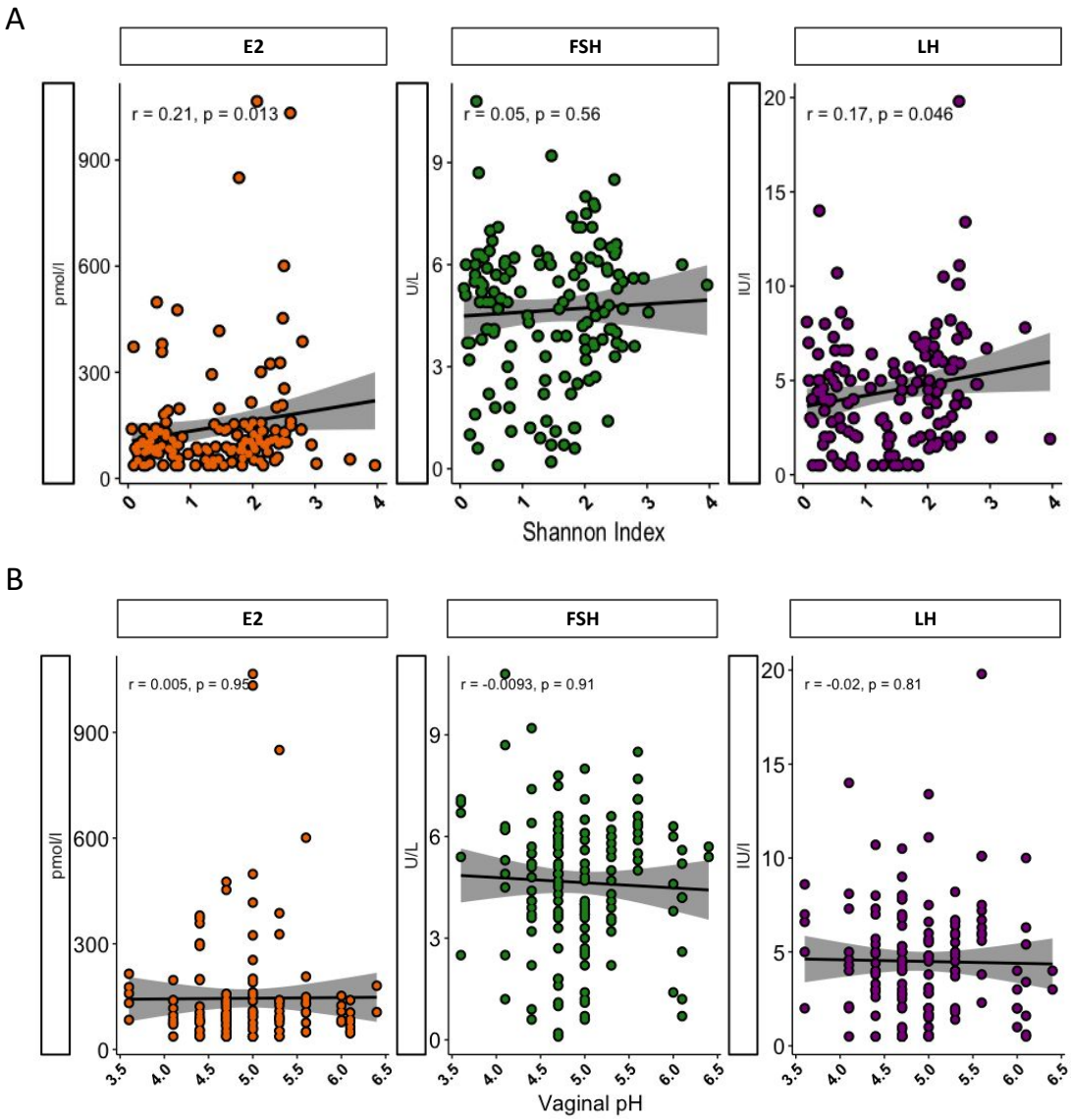


Figure 4.14. Endogenous hormones, alpha diversity and vaginal pH at screening. Correlation plots of endogenous oestradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (measured in pmol/l, U/L and IU/L, respectively) according to A) alpha diversity measured with Shannon Index and B) vaginal pH in baseline samples from 146 participants at the screening visit. The Spearman correlation coefficient (rho, r) and the p values for each individual hormone depicted on the figure.

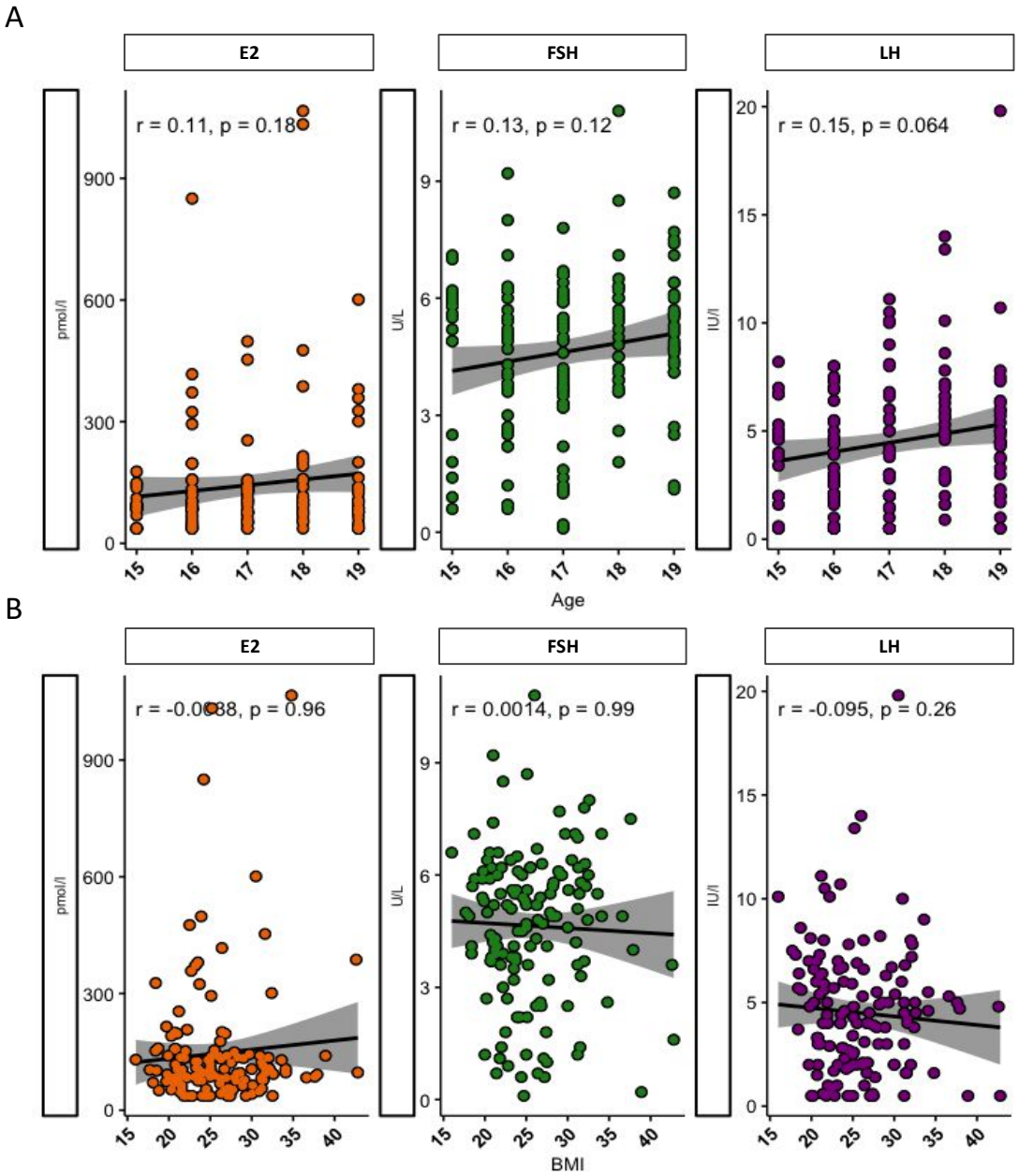


Figure 4.15. Endogenous hormones, age and BMI at screening. Correlation plots of endogenous oestradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (measured in pmol/l, U/L and IU/L, respectively) according to A) the age and B) the body mass index (BMI) of participants at screening for 146 participants. The Spearman correlation coefficient (rho, r) and the p values for each individual hormone depicted on the figure.

When comparing the alpha diversity of the vaginal microbiota of the participants not on hormonal contraception at screening to those using hormonal contraception at baseline, no differences in alpha diversity (p=0.729) or in community composition based on beta diversity (adonis, p=0.972) were found (**Figure 4.16** and **Table 4.11**).

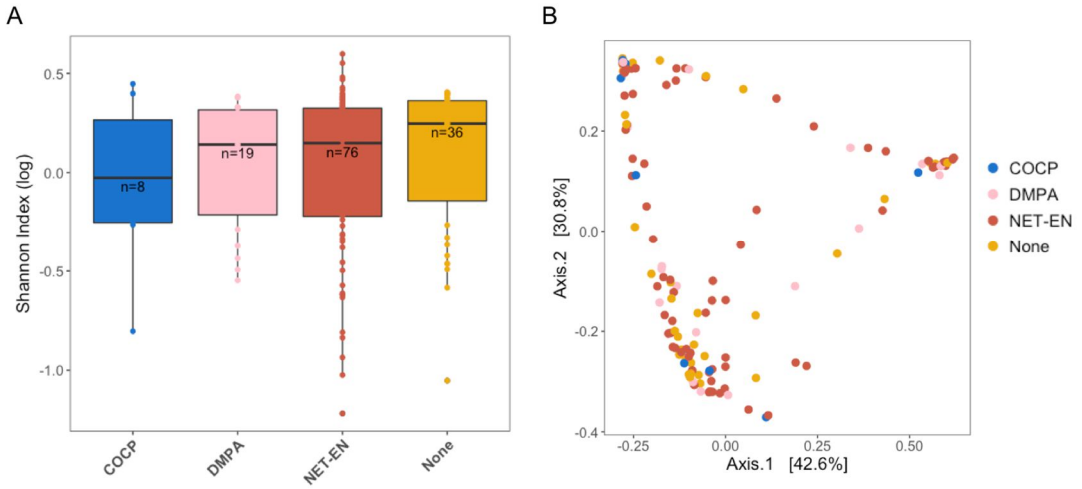


Figure 4.16. Alpha and beta diversity according to hormonal contraception at screening. A) Boxplot depicting the alpha diversity measured using Shannon Index (log transformed values) of the microbiota from 139 participants at baseline according to the hormonal contraceptive used at time of sampling. P values were calculated using Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests and adjusted for multiple comparisons using Benjamini-Hochberg (BH) method. B) Principal Coordinate Analysis (PCoA) of the vaginal microbiota from 139 participants at baseline using weighted UniFrac distances. Samples are coloured by the hormonal contraceptive method at the time of sampling (COCP: n=8; DMPA: n=19; Net-En: n=76; NuvaRing: n=36). Information on contraceptive method was missing from five participants and participants using the contraceptive implant Implanon were excluded due to low numbers (n=3).

Table 4.11. Alpha diversity according to hormonal contraceptive method.

	Shannon Index	
	P	P adj.
All hormonal contraceptive methods	0.729	0.729
COCP vs. DMPA	0.937	0.937
COCP vs. Net-EN	0.743	0.937
COCP vs. None	0.616	0.937
DMPA vs. Net-En	0.907	0.937
DMPA vs. None	0.326	0.937
Net-En vs. None	0.330	0.937

P values calculated using Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon test using Benjamini-Hochberg method for multiple testing correction (MTC). E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone. Five participants did not have any information on contraceptive method at baseline. For six participants, information on hormonal contraceptive use was missing. Participants using the contraceptive implant Implanon were excluded due to low numbers (n=3). COCP: n=8; DMPA: n=19; Net-En: n=76; NuvaRing: n=36.

For 228 samples from screening (n=140) and exit (n=88) visits taken from a total of 144 participants, we had information on HC method, endogenous hormone levels and high-quality microbiota data (participants using the contraceptive implant, Implanon, at baseline were excluded due to low numbers, n=3). We completed a similar analysis on the impact of HC method on the endogenous hormone levels as performed on the samples at baseline to increase the sample size and to include the impact of NuvaRing use, since NuvaRing was not used by any participants at baseline (**Figure 4.17**). As seen with the screening samples alone, participants on Net-En had significantly lower oestrogen levels compared to participants not using any HC method, which remained significant after adjusting for last menstrual period (p=0.023) (**Figure 4.17, Table 4.12** and **Table 4.13**). Using linear mixed-effects (lme) model, including visit (screening and exit) and HC use as fixed effects and participant ID as random effect, E2 levels were significantly lower in Net-En users compared to participants not using any HC (p=0.009). The oestrogen levels were significantly lower in participants on NuvaRing compared to participants on Net-En and participants not using any HC method (adj. p=0.044 and 0.002, respectively). This stayed significant after adjusting for last menstrual period (adj. p<0.01). However, these differences were not significant in the lme model. Participants on COCP and NuvaRing had significantly lower levels of FSH compared to participants on the DMPA and Net-En injections or no HC method also after adjusting for condom use and menstrual cycle (adj. p<0.001). This was also significant in the lme model (p<0.05). Participants on NuvaRing also had a significantly lower level of LH compared to participants on Net-En or not using HC (adj. p<0.01, lme model, p<0.05) and participants on COCP had a lower concentration of LH compared to Net-En (adj. p=0.022, lme model, p=0.015) (**Figure 4.17, Table 4.12** and **Table 4.13**). This remained significant after adjusting for last menstrual cycle.

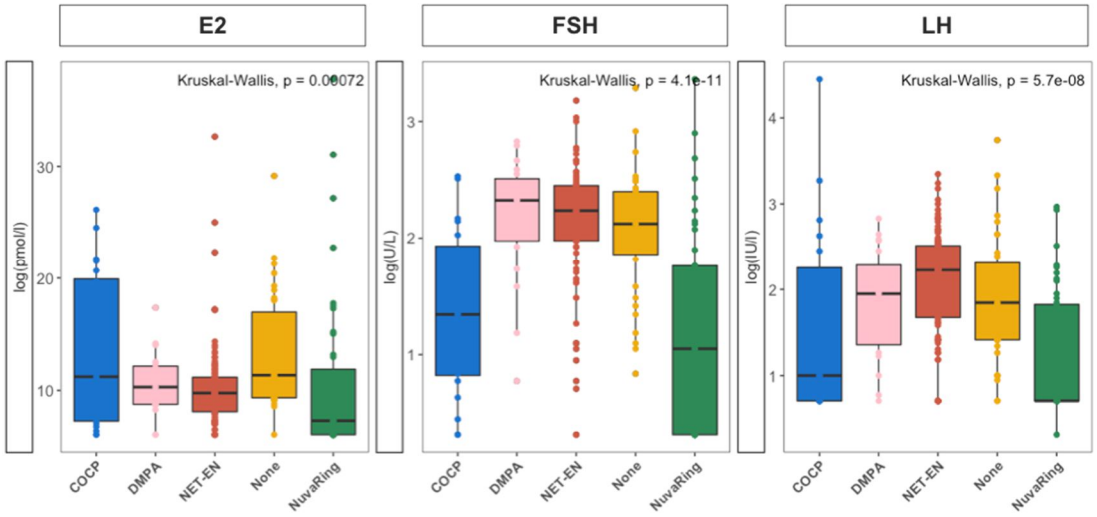


Figure 4.17. Endogenous hormones and hormonal contraceptive method across all visits. Boxplots of endogenous oestradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels in 228 samples from screening and exit visits stratified by hormonal contraceptive method at time of sampling (COCP: n=20; DMPA: n=19; Net-En: n=107; None: n=37; NuvaRing: n=45). Information on contraceptive method was missing from five participants and participants on the contraceptive implant, Implanon, at screening were excluded due to low numbers (n=3). P values were generated using the Kruskal-Wallis test.

Table 4.12. Endogenous hormones according to hormonal contraceptive method at screening and exit visits.

	COCP	DMPA	Net-En	None	NuvaRing	
E2 (pmol/l)	126 (53-397)	106 (77-148)	95 (67-128)	129 (89-254)	53 (37-133)	**0.0072**
FSH (U/L)	1.8 (0.7-3.7)	5.4 (3.9-6.3)	5.0 (3.9-6.0)	4.5 (3.6-5.7)	1.1 (0.1-3.1)	**4.1e-11**
LH (IU/L)	1.0 (0.5-5.1)	3.8 (1.9-5.3)	4.9 (2.8-6.3)	3.4 (2.0-5.7)	0.5 (0.5-3.3)	**5.7e-08**

P values generated with Kruskal-Wallis test using Benjamini-Hochberg (BH) method for multiple testing correction (MTC). E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Table 4.13. Endogenous hormones according to hormonal contraceptive method at screening and exit visits.

	E2		FSH		LH	
	P	P adj.	P	P adj.	P	P adj.
COCP vs. DMPA	0.584	0.648	**0.0006**	**0.002**	0.105	0.169
COCP vs. Net-En	0.271	0.313	**1.1e-05**	**8.39e-05**	**0.009**	**0.022**
COCP vs. None	0.668	0.716	**0.001**	**0.003**	**0.044**	0.087
COCP vs. NuvaRing	**0.026**	0.056	0.255	0.313	0.159	0.228
DMPA vs. Net-En	0.266	0.313	0.711	0.735	0.120	0.180
DMPA vs. None	0.107	0.169	0.250	0.313	0.885	0.885
DMPA vs. NuvaRing	0.064	0.121	**3.7e-05**	**0.0002**	**0.001**	**0.003**
Net-En vs. None	**0.0007**	**0.002**	0.184	0.251	0.077	0.135
Net-En vs. NuvaRing	**0.019**	**0.044**	**8.5e-10**	**2.5e-8**	**7.4e-09**	**1.1e-07**
None vs. NuvaRing	**0.0005**	**0.002**	**5.8e-06**	**5.8e-5**	**8.3e-05**	**0.0004**

P values calculated using unpaired Mann-Whitney-Wilcoxon test using Benjamini-Hochberg method for multiple testing correction (MTC). E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone. Participants on the contraceptive implant, Implanon, were excluded due to low numbers (n=3). COCP: n=20; DMPA: n=19; Net-En: n=107; None: n=37, NuvaRing: n=45.

We also looked at any association between endogenous hormones and BV status. No differences in endogenous hormone levels were found according to BV status at baseline, although the E2 level in BV positive participants was significantly higher than in the BV negative participants before adjusting for multiple comparisons (**Appendix VI**). When looking at samples from both screening and exit, the difference in E2 between BV positive and BV negative participants was significant (adj. p=0.043) (**Figure 4.18** and **Table 4.14**). We also observed a significantly higher level of LH in BV positive and BV negative participants (adj. p=0.043) (**Figure 4.18** and **Table 4.14**).

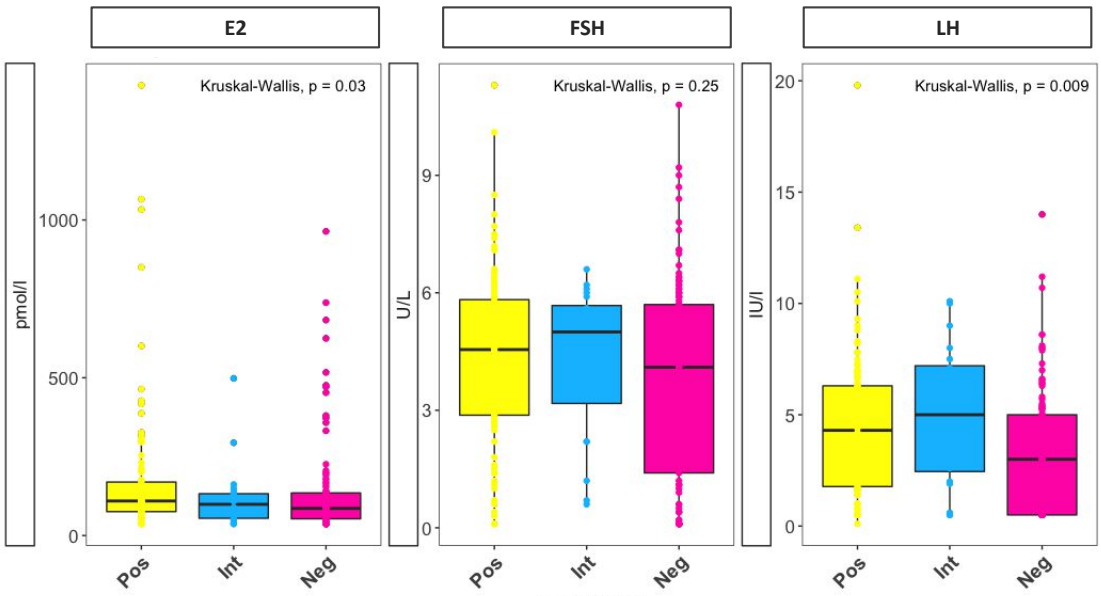


Figure 4.18. Endogenous hormones and BV status at screening and exit. Boxplots of endogenous oestradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels (measured in pmol/l, U/L and U/L, respectively) in 237 samples from 151 participants at either the screening or exit visits stratified by BV status according to Nugent score (BV+: n=100, BV int: n=20, BV-: n=117). P values were generated using the Kruskal-Wallis test.

Table 4.14. Endogenous hormones according to BV status at screening and exit.

	BV positive (n=100)	Intermediate (n=20)	BV negative (n=117)	P	BV- vs. BV+		BV- vs. Int		BV+ vs. Int	
					P	P adj.	P	P adj.	P	P adj.
E2 (pmol/l)	110 (75.8-170)	99.0 (54.8-133)	86.0 (53.0-135)	**0.030**	**0.009**	**0.043**	0.821	0.916	0.211	0.380
FSH (U/L)	4.55 (2.88-5.83)	5.00 (3.18-5.68)	4.10 (1.40-5.70)	0.250	0.130	0.292	0.300	0.449	0.916	0.916
LH (IU/L)	4.30 (1.78-6.30)	5.00 (2.45-7.20)	3.00 (0.50-5.00)	**0.009**	**0.008**	**0.043**	**0.031**	0.094	0.440	0.566

P values generated with Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests using Benjamini-Hochberg (BH) method for multiple testing correction (MTC). BV, bacterial vaginosis; E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone. BV+, BV positive; Int, BV intermediate, BV-, BV negative. BV status according to Nugent scoring.

4.3.4 Temporal variations in the vaginal microbiota

In order to evaluate the temporal stability of the FGT microbiota in adolescence, we looked at the shifts (or lack thereof) in community cluster over time within each participant who completed all three visits (n=85), excluding 10 participants whose vaginal microbiota was not assigned to a cluster at one of the three visits (thus leaving n=75). The overall distribution of clusters did not change over time in the study cohort (**Table 4.15**). The vaginal microbiota of forty percent of the participants (n=30) did not shift between the three community clusters over the course of the three visits (**Figure 4.22**). The community cluster that showed the highest stability (i.e. the vaginal community least likely to shift to another cluster throughout the study) was the diverse C1 cluster (n=17, 51.1%) followed by the *L. crispatus* (n=8, 47.1%) dominant C2 cluster with the least stable being the *L. iners* dominant C3 cluster (n=5, 20.0%) (**Figure 4.19**).

Table 4.15. Distribution of Fuzzy clusters over time in matched participants.

	Screening (n=75)	**Crossover** (n=75)	**Exit** (n=75)	**P value**
C1 – Diverse	34 (45.3%)	33 (44.0%)	33 (44.0%)	0.985
C2 – *L. crispatus*	19 (25.3%)	19 (25.3%)	17 (22.7%)	
C3 – *L. iners*	22 (29.3%)	23 (30.7%)	25 (33.3%)	

P value generated using Chi-squared test for the assessment of association of frequency among groups.

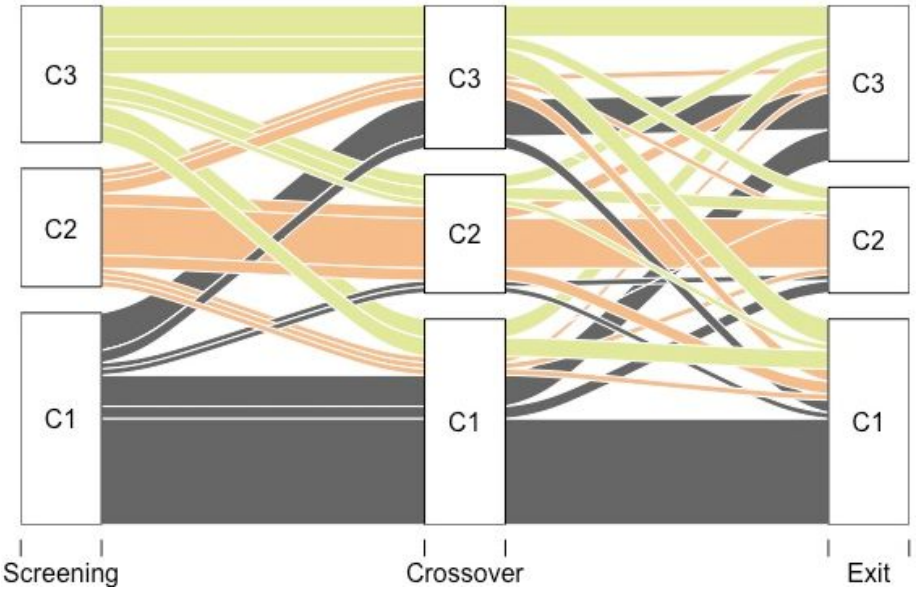


Figure 4.19. Changes in community cluster assignment over time independently of hormonal contraceptive method. Alluvial diagram showing the distribution of community clusters (white boxes) identified by fuzzy clustering at each sample visit for 75 participants with all three sample visits available and no "no cluster" assignment to samples at any visit. Each line represents a participant and shows the

changes in cluster assignment of the vaginal community over time. Each line is coloured according to the cluster assignment at screening.

Paired data from at least two consecutive visits were available for 104 participants for which the vaginal microbiota was assigned to one of the three clusters. For some of these participants, data was available for all three visits providing us with a total of 179 combinations of two consecutive, paired samples (**Table 4.16**). When looking at the community cluster transitions from one visit to the next, the diverse C1 cluster was the most stable community with 66.7% of C1 assigned communities being assigned to C1 again at the following visit. In cases where a transition from C1 to another community type was observed, C1 communities were much more likely to shift to the *L. iners* dominant C3 community (25.9%) over a *L. crispatus* dominant C2 community (7.4%). The *L. crispatus* dominant C2 community was the second most stable community type with no changes in cluster observed in 57.8% of cases. Yet, for 24.4% of vaginal communities assigned to C2, a transition to the *L. iners* dominant C3 community type was observed. For 17.8% the observed shift was to the diverse C1 community type. The least stable cluster was the *L. iners* dominant C3 cluster, however just over half of C3 communities were also assigned to C3 (50.9%) at the following visit. Most transitions from C3 were to the diverse C1 community type (30.2%) with the remainder shifted to the *L. crispatus* dominant C2 community type (18.9%).

Table 4.16. Distribution of shifts from one community cluster to another.

To / From	C1	C2	C3
C1	54	6	21
C2	8	26	11
C3	16	10	27

We also looked at BV status over time (**Figure 4.20**, **Table 4.17** and **Table 4.18**). The overall prevalence of BV remained stable with 43.0% at screening, 41.3% at crossover and 42.7% at exit. Participants that were BV positive at one visit would remain BV positive in 74.7% of cases while 21.3% would change to BV negative. In 27.3% of cases, participants with an intermediate Nugent score

would change to BV positive Nugent score while the remaining 72.7% changed to BV negative. The vast majority of BV negative participants would stay BV negative at the subsequent visit (76.3%) while 21.5% would become BV positive. Among the 85 participants completing all three visits, nearly a fourth of the participants were BV positive throughout the study (n=19, 22.4%) and approximately a third stayed BV negative (n=28, 32.9%) at all three visit. Taken together, these data suggest a high degree of stability of the vaginal microbiota over time. Of note, there was lower baseline prevalence in BV among the participants completing all three visits compared to the overall cohort (35.3% versus 43.0%), suggesting that this subsample may not be representative of the cohort. In this study, treatment for BV was not provided if the participant was asymptomatic. However, participants were treated with antibiotics for laboratory diagnosed STIs, which could have impacted the BV status over time. Using logistic regression, we found no association with the presence of an STI or antibiotic treatment with BV at crossover or exit ($p>0.5$).

Table 4.17. BV prevalence over time in participants completing all three visits.

	Screening (n=85)	**Crossover** (n=85)	**Exit** (n=85)	**P value**
BV positive	30 (35.3%)	34 (40.0%)	38 (44.7%)	0.985
Intermediate	9 (10.6%)	3 (3.5%)	2 (2.4%)	
BV negative	46 (54.1%)	48 (56.5%)	45 (52.9%)	

P value generated using Chi-squared test for the assessment of association of frequency among groups.

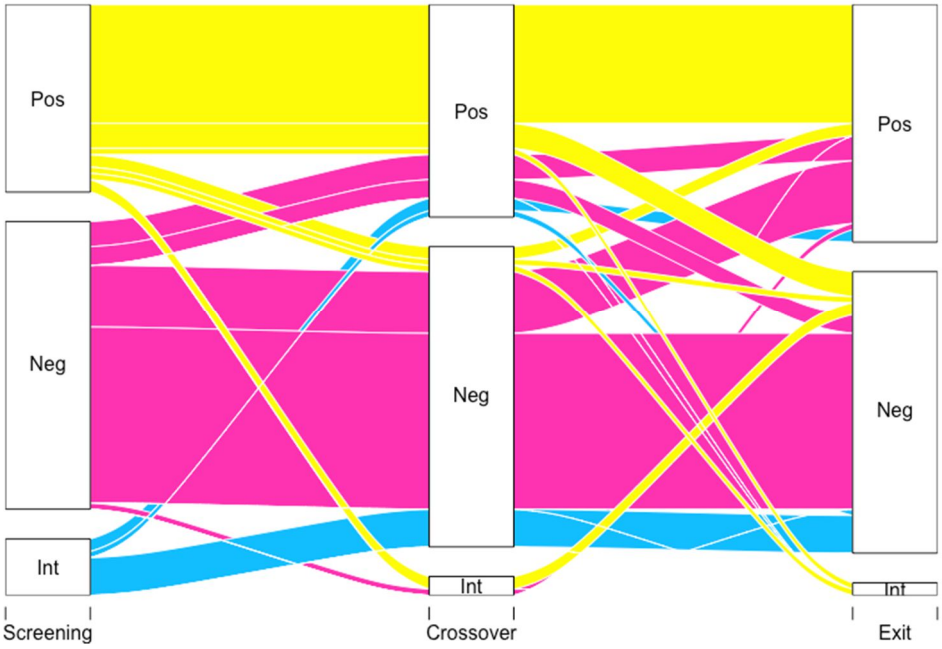


Figure 4.20. Changes in BV status over time. Alluvial diagram showing the distribution of bacterial vaginosis (BV) (white boxes) by Nugent score at each sample visit for 85 participants completing all three sample visits. Each line represents a participant and shows the changes in BV status of the vaginal community over time. Each line is coloured according to the BV status at screening.

Table 4.18. Number of shifts from one BV status to another.

From \ To	BV+	BV Int	BV-
BV+	56	3	16
BV Int	3	0	8
BV-	20	2	71

4.4 Discussion

4.4.1 The vaginal microbiota in South African adolescents

For this study, a total of 151 adolescent girls from a high HIV-risk community were screened and followed longitudinally for up to three visits for analysis of the vaginal microbiota. In concordance with other studies on African women, three major FGT bacterial community types were identified of which two were dominated by *Lactobacillus* species (*L. crispatus* and *L. iners*, respectively) and one consisted of a diverse group of anaerobic bacteria associated with BV (Anahtar et al., 2015; Gosmann et al., 2017; Lennard et al., 2017). As seen in other studies on African women, the prevalence of BV in this adolescent cohort was very high and the majority of cases were asymptomatic. According to

Nugent scoring, 43.0% of the screened participants were BV positive and this prevalence remained stable throughout the study despite HC intervention (discussed in Chapter 5) and antibiotic treatment for STIs. Of note, in line with national South African management guidelines, treatment for BV was not provided if a participant did not present with clinical symptoms. Since BV is a risk factor for several STIs, including HIV regardless of whether symptomatic (Mlisana et al., 2012; Atashili et al., 2008), decreasing the BV prevalence among young African women is of crucial importance. However, the cause of BV is still poorly understood and no primary aetiological agent or organism has yet been identified. Symbiotic relationships appear to play an important role in the pathogenesis of BV, thereby implicating several species in its aetiology. Of major concern is the growing resistance to current treatment methods, including the antibiotic metronidazole, and high recurrence rates of BV (Bradshaw et al., 2006; Sobel et al., 1993; Francis et al., 2016). A better understanding of the key bacteria involved and how they interact with one another is needed for the development of new and successful therapeutic approaches.

In this cohort, we also observed a very high prevalence of bacterial STIs, particularly *C. trachomatis*, which has also been associated with increased HIV risk (Galvin & Cohen, 2004; Chesson & Pinkerton, 2000; Fleming et al., 1999; Martin et al., 1998). Most women, including the participants in this cohort, do not present with symptoms when infected with an STI. Yet, asymptomatic infections have been associated with similar levels of genital inflammatory markers as symptomatic disease, and represent an equivalent risk of HIV (Masson et al., 2014, 2018). It is a huge concern that the prevalence of STIs remains so high in regions of high HIV prevalence and there is an urgent need to develop strategies to help identify and treat STIs and BV within resource poor settings (Mlisana et al., 2012; Masson et al., 2015a; Barnabas et al., 2017).

4.4.2 Temporal stability of the vaginal microbiota

Of the three identified community clusters, C3, the *L. iners* dominant community type, was the most unstable and thus, potentially the most easily disturbed community. In this study, any shifts from the *L. iners* dominated community type to another was most commonly to the diverse, BV-like community type and vice

versa. Any shifts from the *L. crispatus* dominated community was typically to *L. iners.* Certain participants did not change from an *L. iners* community types over time potentially indicating strain differences in terms of resilience. This data was generated in the context of an intervention (hormonal contraceptive use), which could impact the observed pattern. Chapter 5 will explore this further.

4.4.3 Endogenous hormones, hormonal contraception and the vaginal microbiota

In this study, we investigated the association of the naturally occurring female sex hormones: oestradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the circulation, and the FGT microbiota. To our knowledge, this is one of the first studies to describe the relationship between genital tract microbial communities and endogenous hormones in adolescents. We did not observe any differences in E2, FSH or LH levels between the three vaginal Fuzzy clusters or between BV positive and negative participants when analysing the data from participants not using hormonal contraception, although the sample size was modest. In a study on vaginal bacterial communities in relation to changing oestrogen levels in adults, the mean serum oestrogen level at baseline was found to be significantly lower in participants with BV compared to participants without BV (Wilson et al., 2007). Following hormonal treatment with recombinant FSH, oestradiol levels rose accompanied by a significant reduction of abnormal microbiota (Wilson et al., 2007). Similarly, in a study on postmenopausal women with atrophic vaginitis, low-dose systemic oestrogen therapy resulted in increases in the relative abundance of *Lactobacillus* spp. and a decreased relative abundance of *Gardnerella* (Shen et al., 2016). Our results also differ from results from the WISH study based in the same setting as the uCHOOSE cohort as described in Chapter 2.1.3. In the WISH cohort, oestradiol was significantly lower in participants with a C1 vaginal microbiota relative to C2 ($p=0.001$) and was also lower than in women with C3 vaginal microbiota ($p=0.07$) and LH was significantly lower in women with C1 communities relative to C2 ($p=0.02$) and C3 ($p=0.02$) in the 47 participants not using hormonal contraception (Lennard et al., 2017). The participants enrolled in the WISH study were slightly older (16-22 years) compared to the participants in the uCHOOSE study (15-19 years). Menstrual cycles are often irregular during adolescence and

during the first years after menarche anovulation and long menstrual cycles may occur (Flug et al., 1984; Widholm & Kantero, 1971; World Health Organization, 1986). Despite a lack of association between age and E2 levels in this study, the older age of the WISH participants could have impacted the stability of the menstrual cycle among the non-HC users and the E2 levels leading to a stronger association of endogenous hormone levels and the composition of the vaginal microbiota. Other factors such as sexual risk behaviour could also play a role. A major limitation to the endogenous hormone analysis at baseline is the low number of participants who were either HC naïve or not using HC (n=36) when enrolling in the study. A larger sample size would have provided more power to evaluate the impact of endogenous hormones in the absence of exogenous hormones on the vaginal microbiota during adolescence.

Appropriate physiological oestradiol reference ranges derived from well-characterized, adequately sized adolescent populations are lacking (Stanczyk & Clarke, 2014). Furthermore, endogenous hormone reference ranges may differ between various populations because of differences in their physiology and/or behaviour for instance dietary differences (Dorgan et al., 2003; van Gemert et al., 2015; de Roon et al., 2018). In this study, we did not observe any impact of BMI on oestradiol levels but a food questionnaire was introduced at the exit visit for potential future analysis in relation to oestradiol levels. More research on the endogenous hormone patterns in adolescence and particularly in sub-Saharan African women and girls is needed.

When we analysed the correlation between naturally occurring hormones and the vaginal community in all participants at screening and exit, we found that there was a higher concentration of oestrogen in the circulation of participants whose vaginal microbiota was assigned to the lactobacillus-deficient C1 community type compared to both lactobacillus dominant communities (C2 and C3) as well as in BV positive compared to BV negative participants. These results were surprising, as oestrogen has been proposed to induce glycogen production within the FGT epithelium and *Lactobacillus* levels and acidic vaginal pH have been shown to correlate positively with free glycogen (Mirmonsef et al., 2014, 2015; Mitchell et al., 2018). Of interest however, in recent studies vaginal glycogen levels did not appear to be associated with serum oestrogen

concentrations (Mitchell et al., 2017; Mirmonsef et al., 2016). It would therefore be of interest to analyse the glycogen levels or mucosal oestrogen levels instead of serum oestrogen levels to evaluate this association further.

Additionally, the level of endogenous hormones measured in our study was strongly affected by the use of HC. We therefore compared the endogenous hormone levels according to HC method and found that participants not using any HC method also had a significantly higher oestrogen level compared to participants on Net-En. This is in line with data showing that use of progestin-only injectables cause profound suppression of endogenous oestrogen (Miller et al., 2000b). Interestingly, the oestrogen levels of participants on NuvaRing were also significantly lower than in participants not using HC while this was not the case for those using COCP, suggesting that despite both COCP and NuvaRing being combination contraceptives, they exert different effects on endogenous oestrogen, potentially due to dose and delivery route differences. The wide confidence intervals for levels of E2 measured in COCP participants suggests there were varying degrees of compliance as daily adherence is acquired for COCP use. Some of the variation observed could also be due to time of sampling in relation to the time COCP tablets were taken (e.g. morning versus evening). At screening, all participants who reported using COCP had been on Nordette. In this study, two different COCPs, Triphasil and Nordette, both containing ethinyl oestradiol and levonorgestrel, could be offered to the participants. While Triphasil is triphasic, meaning that the pills contains three different doses of hormones to be taken at three different phases throughout the menstrual cycle (six tablets containing 30 μg ethinyloestradiol and 50 μg levonorgestrel, followed by five tablets containing 40 μg of ethinyloestradiol and 75 μg levonorgestrel, followed by ten tablets containing 30 μg ethinyloestradiol and 125 μg levonorgestrel), Nordette is monophasic, thus the hormone doses stay the same (30 μg ethinyloestradiol and 150 μg levonorgestrel) during the menstrual cycle. Based on our documentation, the vast majority of participants on COCPs throughout the study received Nordette. Only one participant at the exit was taking Triphasil and it is thus unlikely that the wide confidence intervals for the hormone results are due to differences between the COCP used. Future studies on the impact of dose and type of oestrogen would be interest. Participants on

COCP and NuvaRing had similar suppressive effects on FSH and LH levels when compared to participants on Net-En and participants not using HC in line with a negative feedback mechanism suggested by oestrogen present in combined hormonal contraceptives (D'Arpe et al., 2016). Using Kruskal-Wallis test for this analysis is somewhat flawed due to violation of the assumptions of the test (as it includes multiple samples from the same adolescents. A linear mixed-effects model was thus generated with visit and participant ID included in the model. The results were in line with the Kruskal-Wallis data.

In the WISH cohort described in Chapter 2.1.3, endogenous oestrogen concentrations was significantly lower in participants using injectable progestin-only HCs compared to those not using HCs as observed in this study (Dabee et al., unpublished data). In contrast to this study, however, where LH levels were slightly higher in Net-En users versus non-HC users (median 4.9 versus 3.4), the LH levels in the WISH study were two-fold lower in Net-En users compared to participants not using HC (p=0.017). The differences observed between the cohorts could be due to the timing of mucosal sampling. At all three visits in the uCHOOSE cohort, sampling was conducted just prior to initiation of a new HC method and therefore at the end of a Net-En injection period associated with decreasing levels of progestin and close to or shortly after menstruation in the case of NuvaRing and COCP users. For the WISH cohort, study visits were scheduled two weeks after injection for participants on injectable contraceptives, a time of peak progestin levels, or otherwise during the luteal phase of their menstrual cycles (between day 14–28) if they were on COCPs or not using hormonal contraceptives.

4.4.4 Sexual risk behaviour and intravaginal practices and their influence on vaginal microbiota

The menstrual cycle has been suggested as an important factor influencing the vaginal microbiota. In addition to fluctuations in endogenous sex hormones, a wide range of other factors could affect the composition of the vaginal microbiota including intravaginal practices, sexual behaviour and antibiotic use (Fashemi et al., 2013; Hickey et al., 2013; Mayer et al., 2015; Schwebke et al., 1999). In this study, we did not see any effect of douching or other vaginal

cleansing practices on microbial diversity, however the number of participants reporting engaging in intravaginal practices was very low, potentially masking any influence on the vaginal microbiota. In this study, participants with a diverse C1 cluster were less likely to report condom usage at their last intercourse before screening than participants in the lactobacilli dominated clusters and participants in the C1 cluster also reported less condom use in general. These results are in line with studies showing that the presence of prostate-specific antigen (proxy for recent sex) is significantly associated with increased microbial diversity (Jespers et al., 2017) and that recent sexual exposure had a negative effect on the presence of *L. crispatus* and *Lactobacillus* species in general (Jespers et al., 2015). Furthermore, consistent condom use has been associated with increased vaginal colonization of *L. crispatus* (Ma et al., 2013). In a recent cross-sectional study of 426 Kenyan, South African and Rwandan women at risk for HIV, sexual risk behaviour was related to the presence and quantity of different *Lactobacillus* species and BV-related bacteria. The authors found that having had multiple sexual partners in the last three months was associated with an increased prevalence of *G. vaginalis* and *L. iners* (Jespers et al., 2015). Furthermore, BV has been shown to be associated with exposure to a new sexual partner (Schwebke & Desmond, 2005). We did not observe any associations between these risk behaviours and the vaginal microbiota potentially due to a low number of participants reporting multiple and/or new sexual partners. Of note, the only four participants reporting anal sex all had vaginal microbiotas consisting of diverse bacterial communities not dominated by lactobacilli.

4.4.5 Conclusions

Three major FGT bacterial community types were identified in this high risk adolescent cohort; two dominated by *Lactobacillus* species (*L. crispatus* and *L. iners*, respectively) and one consisting of a diverse group of anaerobic bacteria. *L. iners* was the most common bacterial species but also associated with the least stable community. The prevalence of BV and STIs were very high in this cohort placing these adolescent girls at high risk of HIV. Elevated levels of naturally

occurring luteinizing hormone and oestrogen, which was altered by hormonal contraceptive use, were associated with increased bacterial diversity and BV

Chapter 5: Impact of hormonal contraception on the adolescent female genital tract microbiota in a randomized trial

Parts of this Chapter (**Table 5.1**, **Figure 5.2A**, **Figure 5.3A**, **Figure 5.20A,B**, **Figure 5.21**, **Figure 5.22**, **Figure 5.23A** and **Figure 5.24A**) have been presented at the HIVR4P Conference in Madrid, Spain, on Oct 23rd, 2018.

5.1 Introduction

Adolescent girls and young women (AGYW) are at extremely high risk of HIV acquisition, particularly in sub-Saharan Africa. Almost half of all new HIV infections occur in women under 25 years of age, and young women (aged 15-24 years) are more than four times as likely to become HIV-infected as young males in the same age group (Cowan & Pettifor, 2009; UNAIDS, 2017). AGYW in sub-Saharan Africa are also faced with a high risk of unintended pregnancy, which is associated with high maternal and infant mortality and morbidity particular in developing countries (Aitken et al., 2008). Hormonal contraceptive methods play a crucial role in preventing unplanned pregnancy. However, a number of observational studies have suggested that the use of hormonal contraception, particularly progestin-only injectables, may influence women's susceptibility to HIV-1 (Baeten et al., 2007; Morrison et al., 2010, 2012; Heffron et al., 2012; Crook et al., 2014; McCoy et al., 2013; Wand & Ramjee, 2012). Young women (<24 years) on depot medroxyprogesterone acetate (DMPA) injections appear to be at especially increased risk of HIV acquisition (Morrison et al., 2010, 2012). Different biological mechanisms by which hormonal contraception could alter the female genital tract (FGT) mucosal environment and influence HIV-1 acquisition have been suggested including alterations in the vaginal microbiota (Chandra et al., 2013; Ghanem et al., 2005; Ildgruben et al., 2003; Prakash et al., 2002, 2005; Zang et al., 2002; Miller et al., 2000a; Borgdorff et al., 2015). Indeed, it is becoming increasingly more accepted that microorganisms in the FGT may have an impact on HIV susceptibility (Atashili et al., 2008; Mitchell et al., 2014; Myer et al., 2005a). Molecular-based studies of the vaginal microbiota of healthy, reproductive-age women have described a distinct number of common bacterial community types consisting of a group of low-diversity communities dominated by a single *Lactobacillus* species (*Lactobacillus crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii*) and one to two high-diversity communities, comprised of a mixture of anaerobic bacteria associated with bacterial vaginosis (BV)(Gajer et al., 2012; Ravel et al., 2011; Anahtar et al., 2015; Gautam et al., 2015). BV is a condition characterized by the displacement of protective *Lactobacillus* species by a diverse group of facultative anaerobic bacteria including *Gardnerella vaginalis, Atopobium vaginae, Megasphaera, Prevotella, Sneathia* and BV-associated

bacteria (BVAB) 1-3. *Lactobacillus* species are recognized as an important component of the vaginal mucosal defence against pathogens, including HIV-1, due to their production of antimicrobial compounds, hydrogen peroxide (H_2O_2) and lactic acid, which maintains a low vaginal pH. High-diversity communities, like those characteristic of BV, have been shown correlate with high levels of genital pro-inflammatory cytokine concentrations and an increased number of CCR5+ CD4+ T cells in the genital mucosa both of which are associated with increased HIV susceptibility (Anahtar et al., 2015; Rebbapragada et al., 2008). Progestin-only injectables may impair vaginal colonization by decreasing *Lactobacillus* species due to the absence of oestrogen (Miller et al., 2000a; van de Wijgert et al., 2013). Oestrogen is associated with glycogen deposition in epithelial cells of the FGT, which aid lactic acid metabolism, hence promoting colonization of *Lactobacillus* species (Farage & Maibach, 2006; Hillier, 1998). Progesterone has the opposite effect on glycogen deposition thus hindering lactobacilli colonization (Hillier, 1998; Hickey et al., 2015). Hence, perturbations to the mucosal environment by hormonal contraception might translate into compositional and functionally distinct microbial communities, which in turn could alter HIV risk. The aim of this study was to assess whether use of different hormonal contraception methods could drive changes in diversity and composition of the FGT microbiota using a unique randomized cohort of South African adolescents.

5.2 Materials and Methods

For this analysis, we included 131 adolescent girls enrolled in the uCHOOSE-A-Star study described in Chapter 2.1.1 (p. 46-48) who were randomized to one of the three study arms. The study procedures were approved by the Human Research Ethics Committee of the University of Cape Town, and informed consent or assent (if <18 years) was obtained from all participants before initiation of the study. Informed consent was obtained from a parent or legal guardian of participants younger than 18 years. Information on demographics, medical and reproductive history and sexual behaviours were collected. As described in Chapter 2.1.2 (p. 49-50), vulvovaginal and lateral wall swabs were collected for STI, BV and genital candidiasis screening and vaginal pH

measurement and an additional vaginal swab was obtained for microbiota analyses. DNA extraction from vaginal lateral wall swabs, 16S rRNA amplicon sequencing library generation and pre-processing of sequencing reads, including generation of OTU table and taxonomic annotation, were performed as described in Chapters 2.2.1, 2.2.3 and 2.2.4 (p. 51-55), respectively. All downstream statistical analyses were performed in RStudio as described in Chapter 2.3 (p. 55-56).

5.3 Results

5.3.1 Intention-to-treat analysis (randomization)

Of the 151 participants successfully screened for the uCHOOSE-A-Star study, 131 were randomized to one of the three study arms and received either combined oral contraceptive pills (COCPs, n=41), the progestin-only norethisterone enanthate injection (Net-En, n=45) or a combined contraceptive vaginal ring (NuvaRing, n=45) for four months. Samples were collected at baseline and at the four month visit. To identify potential factors, which may confound the analyses of the relationship between hormonal contraception and the composition of the vaginal microbiota, we examined the baseline demographics and reported behaviour between participants assigned to the three study arms. The participants assigned to each study arm were similar at baseline in terms of their demographics, medical and reproductive history and sexual risk behaviour including age, body mass index (BMI), intravaginal practices and prior hormonal contraceptive use as well as BV, yeast and STI prevalence (**Table 5.1**). The only notisable difference observed was in regard to penile-anal intercourse with four participants in the NuvaRing arm reporting penile-anal sex compared to none in both of the other study arms.

Table *5*.1. Randomization characteristics at screening.

	COCP (n=41)(31.3%)	**Net-En** (n=45)(34.3%)	**NuvaRing** (n=45)(34.3%)
Age at screening, median years (IQR)	17 (16-18)	17 (16-18)	17 (16-18)
BMI, median (IQR)	25.7 (21.5-28.3)	25.0 (22.5-29.1)	25.0 (22.1-27.8)
STI prevalence			
Any STI(s)	16 (39.0%)	22 (48.9%)	18 (40.0%)
Ct	12 (29.3%)	17 (37.8%)	15 (33.3%)
Ng	4 (9.8%)	5 (11.1%)	5 (11.1%)
Tv	4 (9.8%)	5 (11.1%)	3 (6.7%)
Mg	1 (2.4%)	2 (4.4%)	0 (0.0%)
BV prevalence			
BV positive	17 (41.5%)	21 (46.7%)	20 (44.4%)
BV intermediate	4 (9.8%)	6 (13.3%)	2 (4.4%)
BV negative	20 (48.8%)	18 (40.0%)	23 (51.1%)
Fuzzy cluster distribution[1,2]			
C1	17 (44.7%)	23 (57.5%)	22 (51.2%)
C2	8 (21.1%)	8 (20.0%)	11 (25.6%)
C3	13 (34.2%)	9 (22.5%)	10 (23.2%)
Vaginal pH, mean (sd)[1]	4.80 (4.26-5.32)	5.05 (4.43-5.67)	4.82 (4.30-5.34)
Shannon Index, median (IQR)[1]	0.92 (0.43-2.03)	1.78 (0.64-2.27)	1.47 (0.61-2.15)
HSV-2 serology[3]	15 (36.6%)	13 (28.9%)	11 (25.0%)
Yeast cells present	7 (17.0%)	4 (8.9%)	10 (22.2%)
Clue cells	17 (41.5%)	21 (46.7%)	20 (44.4%)
Antibiotics	0 (0.0%)	4 (8.9%)	4 (8.9%)
Age menarche, median (IQR)[4]	13 (12-14)	13 (12-14)	13 (12-14)
Tanner, median (IQR)[5]	4.0 (4.0-4.0)	4.0 (4.0-4.0)	4 .0 (4.0-4.3)
Allergies, n[6]	6 (15%)	4 (9.3%)	6 (13.6%)
Leucocyte count (x10^9/L)[7]	8.3 (6.9-8.3)	7.7 (5.9-8.6)	7.1 (5.8-8.3)
Haemoglobin (g/dL)[7]	13.1 (12.3-13.7)	12.9 (12.4-13.6)	13.3 (12.5-13.9)
Biochemistry[7]			
Na (mmol/L)	139 (137-140)	138 (137-140)	139 (138-140)
K (mmol/L)	4.2 (4.0-4.5)	4.2 (4.0-4.6)	4.4 (4.1-4.6)
Creatinine (umol/L)	53.0 (48.0-60.0)	54.0 (49.5-60.0)	55.5 (51.0-59.0)
Glucose (mmol/L)	4.8 (4.5-5.0)	4.4 (4.0-4.8)	4.5 (4.1-4.8)
Endocrinology[8]			
E2 (pmol/l)	89 (76-136)	106 (89.8-157.3)	104 (71-141)
FSH (U/l)	4.7 (3.0-5.6)	4.7 (3.6-5.8)	5.5 (4.3-6.2)
LH (IU/l)	4.0 (2.1-5.3)	4.6 (2.0-6.5)	4.4 (2.0-5.9)
Parity[7]			
Previously pregnant	4 (10%)	7 (15.6%)	6 (13.3%)
Vaginal delievery (VD)	3 (7.5%)	5 (11.5%)	2 (4.4%)
Caesarean section (CS)	1 (2.5%)	1 (2.2%)	3 (6.7%)
Abortion (AB)	0 (0.0%)	1 (2.2%)	1 (2.2%)
Use of hormonal contraception[7]			
Naive	2 (5.0%)	1 (2.3%)	2 (4.5%)
Not currently	10 (25.0%)	10 (22.7%)	6 (13.6%)
Net-En	20 (50.0%)	22 (50.0%)	27 (61.4%)
COCP	1 (2.5%)	2 (4.5%)	3 (6.8%)
DMPA	7 (17.5%)	7 (15.9%)	5 (11.4%)
Implanon	0 (0.0%)	2 (4.5%)	1 (2.2%)

Intra-vaginal practices[9]			
Douching	0 (0.0%)	1 (2.2%)	0 (0.0%)
Washing with water	6 (15.0%)	7 (15.6%)	3 (6.8%)
Washing with soap	2 (5.3%)	7 (15.6%)	3 (6.8%)
Cloth	1 (2.5%)	2 (4.7%)	1 (2.3%)
Drying	0 (0.0%)	1 (2.2%)	0 (0.0%)
Medication	0 (0.0%)	4 (8.9%)	1 (2.3%)
Tampon use	1 (2.5%)	5 (11.1%)	2 (4.5%)
Herbs	0 (0.0%)	1 (2.2%)	0 (0.0%)
Sexual risk behaviour[10]			
Age of sexual debut, median (IQR)	15 (14-16)	15 (14-16)	15 (14-16)
Sexual partners past year, median (IQR)	1 (1-1)	1 (1-2)	1 (1-1)
Multiple sexual partners past year, n (%)	0 (0.0%)	1 (2.2%)	0 (0.0%)
New partner past year, median (IQR)	0 (0-0)	0 (0-1)	0 (0-1)
General condom use			
Never	3 (7.3%)	6 (14.0%)	3 (6.8%)
Almost never	4 (9.8%)	2 (4.7%)	7 (15.9%)
Not sure	3 (7.3%)	7 (16.3%)	5 (11.4%)
Almost always	20 (48.8%)	13 (30.2%)	13 (22.7%)
Always	11 (26.8%)	15 (34.9%)	16 (36.4%)
Condom use during last PV intercourse			
Yes	25 (61.0%)	24 (61.9%)	17 (61.4%)
PV sex acts per week, median (IQR)	1 (1-2)	1 (1-2)	1 (1-2)
Intergenerational sex with older partner (≥5 years)			
No	19 (46.3%)	16 (37.2%)	15 (34.1%)
Unsure	14 (34.2%)	17 (39.5%)	21 (47.7%)
Yes	8 (19.5)	10 (23.3%)	8 (18.2%)
Transactional sex	0 (0.0%)	1 (2.3%)	0 (0.0%)
Penile-anal intercourse	0 (0.0%)	0 (0.0%)	4 (9.1%)
Education[11]			
School attendance	36 (87.8%)	39 (86.7%)	37 (86.0%)
Highest grade, median (IQR)	10 (8-11)	10 (9-11)	9 (8-10)
Tertiary attendance	3 (7.9%)	5 (11.4%)	4 (9.3%)

BV, bacterial vaginosis; Ct, Chlamydia trachomatis*; HSV-2, herpes simplex virus type 2; IQR, interquartile range; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. Based on samples with available microbiota data. (COCP: n=40; Net-En: n=42; NuvaRing: n=45)

2. Six samples not assigned any cluster (COCP: n=2; Net-En: n=2; NuvaRing: n=2)

3. One equivocal result (NuvaRing: n=1).

4. Missing data from six participants (COCP: n=4; Net-En: n=1; NuvaRing: n=1)

5. Missing values for two participants (COCP: n=0; Net-En: n=1; NuvaRing: n=1).

6. Missing values for four participants (COCP: n=1; Net-En: n=2; NuvaRing: n=1).

7. Missing data from three participants (COCP: n=0; Net-En: n=2; NuvaRing: n=1).

8. Missing value for one participant (Net-En: n=1).

9. Missing values for two participants (COCP: n=1; Net-En: n=0; NuvaRing: n=1).

10. Missing data from three participants (COCP: n=1; Net-En: n=1; NuvaRing: n=1).

11. Missing samples from two participants (NuvaRing: n=2).

Among the randomized participants with adequate sequencing data from the screening visit (n=127; COCP: n=40; Net-En: n=42 and NuvaRing: n=45), there were an equal baseline distribution of community types generated using Fuzzy

clustering and weighted UniFrac distances (as described in Chapter 4, section 4.3.3), vaginal pH or within sample diversity (i.e. alpha diversity, measured by Shannon Index (SI)) between the three study arms (**Table 5.1**). However, we did observe a slight skewing of vaginal pH and alpha diversity between the study arms with a higher vaginal pH (5.05 versus 4.80 and 4.82) and increased alpha diversity (1.78 versus 0.92 and 1.47) observed in the participants assigned to the Net-En arm compared to participants assigned to the COCP and NuvaRing arms (**Table 5.2**).

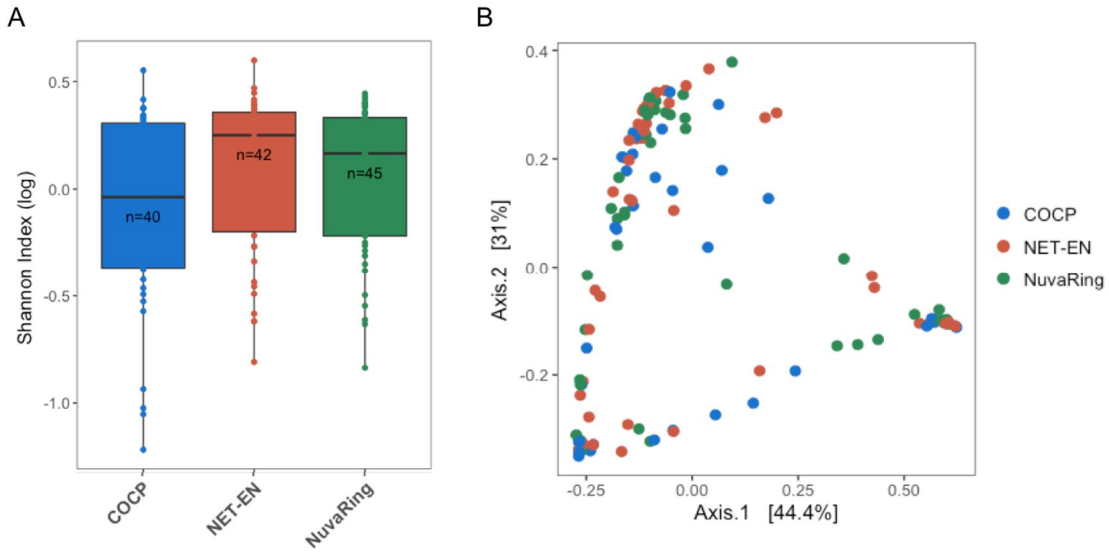


Figure 6.1. Alpha and beta diversity at baseline according to study arm. A) Boxplot depicting the alpha diversity measured using Shannon Index (log transformed values) of the vaginal microbiota from 104 participants at baseline according to their assigned study arm (COCP arm: n=40; Net-En arm: n=42; NuvaRing arm: n=45). P values were calculated using Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests and adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. B) Principal Coordinate Analysis (PCoA) of samples from 104 participants at baseline using weighted UniFrac distances. Samples are coloured by the hormonal contraceptive method that the participant was assigned to at the randomization visit (COCP arm: n=40; Net-En arm: n=42; NuvaRing arm: n=45).

Table 6.2. Alpha diversity at baseline according to assigned study arm.

	Shannon Index	
	P	P adj.
All study arms	0.410	0.410
COCP arm vs. Net-En arm	0.094	0.283
COCP arm vs. NuvaRing arm	0.188	0.283
Net-En arm vs. NuvaRing arm	0.322	0.322

P values generated using Kruskal-Wallis test with Dunn's post hoc test using the Benjamini-Hochberg (BH) method for multiple comparisons testing (MTC). COCP arm, n=40; Net-En arm, n=42; NuvaRing arm, n=45

Of the 131 randomized participants, 24 did not complete the crossover visit (18.3% loss-to-follow-up). Of these, four where initially assigned to the COCP arm (retention rate 96.7%), eleven to the Net-En arm (retention rate 75.6%) and ten were assigned to the NuvaRing arm (retention rate 77.8%) (p=0.087). One participant seroconverted between the screening and the crossover visit and mucosal sampling was therefore not performed. Sequencing data was available for 104 of the participants who completed the crossover visit. In an intention-to-treat analysis at crossover, we observed a significant difference in the distribution of vaginal community types between participants assigned to the three study arms at baseline (p=0.044) (**Table 5.3**). COCP recipients were least likely to have diverse community type C1 (32%) than either Net-En or NuvaRing (both 50%). The *L. iners* dominant C3 community type was the most prevalent in the COCP arm and significantly more prevalent in this arm compared to the Net-En study arm (adj. p= 0.028). In both the Net-En and the NuvaRing arms the diverse C1 community type was most common followed by *L. crispatus* dominant C2 community type in the Net-En arm and *L. iners* dominant C3 community type in the NuvaRing arm (**Table 5.3**). Furthermore, the vaginal microbiota of participants randomly assigned to COCPs had a significantly lower alpha diversity (median SI: 0.90) at crossover compared to the microbiota of participants assigned to both the Net-En and NuvaRing arms (median SI: 1.62, adj. p=0.004 and median SI: 1.67, adj. p=0.007, respectively) while no difference in diversity was observed between the NuvaRing and Net-En arms (adj. p=0.49) (**Figure 5.2A** and **Table 5.3**). Using a linear regression model, we adjusted for alpha diversity at screening due to the skewing observed between the study arms at baseline despite of randomization. The alpha diversity of the vaginal microbiota of participants on COCP remained significantly lower than that of the vaginal microbiota of participants assigned to the NuvaRing arm after adjusting for baseline alpha diversity (ANCOVA, p=0.012) while the difference between the alpha diversity of participants assigned to the COCP and the Net-En arm was close to, but no longer significant (p=0.054). There were no significant changes in the alpha diversity of the vaginal microbiota of adolescents within each study arm from baseline to crossover although a slight decrease in diversity in the COC (median SI (IQR): 1.02 (0.41-2.05) versus 0.90 (0.35-1.54)) and Net-En (median

SI (IQR): 1.85 (1.01-2.32) versus 1.62 (0.74-2.22)) arms and a slight increase in NuvaRing arm (median SI (IQR): 1.34 (0.55-2.13) versus 1.67 (0.80-2.02)) were observed (p>0.05, Wilcoxon Signed Rank test).

Table 6.3. Characteristics of participants at crossover according to assigned study arm (intention-to-treat).

	COCP (n=37)	**Net-En** (n=32)	**NuvaRing** (n=35)	**P value**
Fuzzy cluster distribution[1]				**0.044**
C1	12 (32.4%)	15 (50.0%)	17 (50.0%)	
C2	8 (21.6%)	11 (36.7%)	6 (17.6%	
C3	17 (45.9%)	4 (13.3%)	11 (32.4%)	
Vaginal pH, mean (sd)	4.69 (4.22-5.15)	4.82 (4.17-5.47)	4.97 (4.46-5.47)	0.096
Shannon Index, median (IQR)	0.90 (0.35-1.54)	1.62 (0.74-2.22)	1.67 (0.80-2.02)	**0.005**
HSV-2 serology	13 (35.1%)	9 (28.1%)	14 (40.0%)	0.592
Yeast cells present	4 (10.8%)	6 (18.8%)	6 (17.1%)	0.622
BV prevalence				0.565
BV positive	13 (35.1%)	15 (46.9%)	15 (42.9%)	
BV intermediate	3 (8.1%)	0 (0.0%)	1 (2.9%)	
BV negative	21 (56.8%)	17 (53.1%)	19 (54.3%)	
STI prevalence				
Any STI(s)	7 (18.9%)	9 (28.1%)	13 (37.1%)	0.226
Ct	4 (10.8%)	4 (12.5%)	8 (22.9%)	0.361
Ng	2 (5.41%)	1 (3.1%)	7 (20.0%)	0.062
Tv	1 (2.7%)	1 (3.1%)	1 (2.9%)	1.000
Mg	1 (2.7%)	3 (9.4%)	0 (0.0%)	0.111
Sexual risk behaviour since last visit[2]				
Sexual partners, median (IQR)	1 (1-1)	1 (1-1)	1 (1-1)	0.941
Multiple sexual partners, n	1 (2.9%)	1 (3.7%)	0 (0.0%)	0.741
New partner, n	2 (6.1%)	1 (3.7%)	0 (0.0%)	0.414
Condom use since last visit				0.575
Never	6 (17.1%)	7 (25.9%)	8 (24.2%)	
Less than half the time	6 (17.1%)	1 (3.70%)	3 (9.09%)	
Half the time	16 (45.7%)	11 (40.7%)	8 (24.2%)	
More than half the time	3 (8.6%)	2 (7.41%)	4 (12.1%)	
Always	4 (11.4%)	6 (22.2%)	10 (30.3%)	
Condom use during last PV intercourse				
Yes	21 (60.0%)	13 (48.1%)	20 (60.6%)	0.458
PV sex acts per week, median (IQR)	2 (1-2)	2 (1-2)	1 (1-2)	0.828
Intergenerational sex with older partner (≥5 years)				
No	1 (2.9%)	3 (11.1%)	0 (0.0%)	0.097
Unsure	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Yes	34 (97.1%)	24 (88.9%)	33 (100%)	
Transactional sex	0 (0.0%)	0 (0.0%)	0 (0.0%)	NA
Penile-anal intercourse	0 (0.0%)	0 (0.0%)	0 (0.0%)	NA

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups, Kruskal-Wallis test for comparison of medians and ANOVA test for comparison of means. BV; bacterial vaginosis; Ct, Chlamydia trachomatis*; HSV-2, herpes simplex virus type 2; IQR, interquartile range; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. Three samples not assigned any cluster (COCP: n=0; Net-En: n=2; NuvaRing: n=1).

2. Missing values for nine participants (COCP: n=2; Net-En: n=5; NuvaRing: n=2).

The vaginal pH at crossover of participants randomized to COCP was lower than the vaginal pH of participants on the Net-En and NuvaRing arms (mean pH: 4.69 versus 4.82 and 4.97), but this was not statistically significant (**Table 5.3**). There was a markedly lower prevalence of bacterial STIs amongst participants in the COCP arm compared to the Net-En and NuvaRing arms (18.9% versus 28.1% and 37.1%, respectively) and a higher prevalence of *N. gonorrhoea* in the NuvaRing arm compared to the other two arms (20.0% versus 5.41% and 3.13%, respectively), although neither of these differences reached statistical significance. We did also not observe any statistical differences in yeast infection, BV prevalence by Nugent scoring or in HSV-2 serology between study arms (**Table 6.3**).

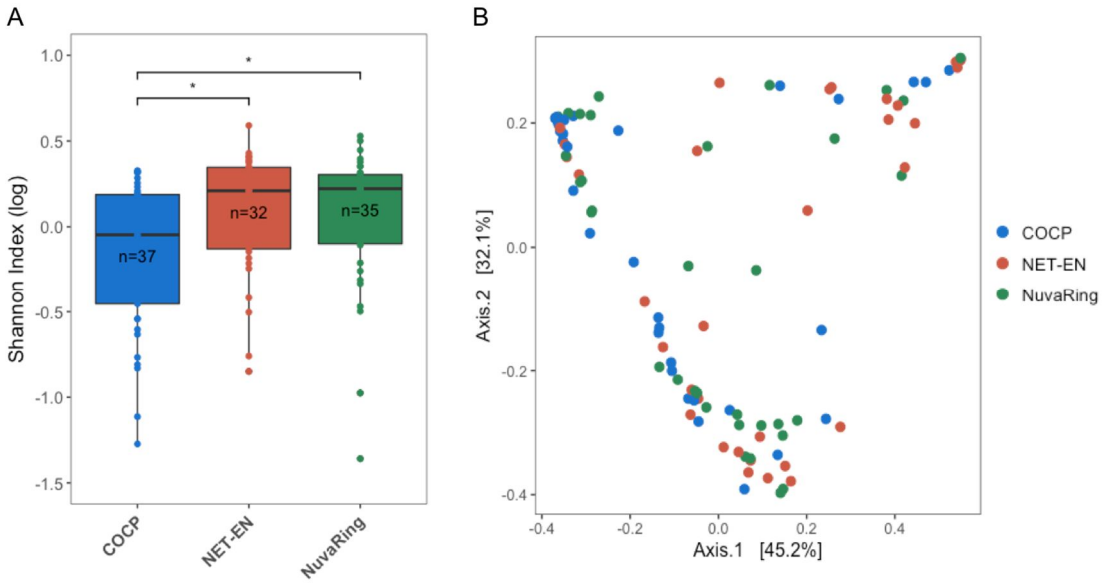


Figure 6.2. Alpha and beta diversity at crossover according to assigned study arm. A) Boxplot depicting the alpha diversity (Shannon Index) of the vaginal microbiota from 104 participants at crossover according to their assigned study arm (intention-to-treat (ITT) analysis, COCP arm: n=37; Net-En arm: n=32; NuvaRing arm: n=35). P values were calculated using Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests and adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. *=adj. p<0.05. B) Principal Coordinate Analysis (PCoA) of samples from 104 participants at crossover using weighted UniFrac distances. Samples are coloured by the hormonal contraceptive method that the participant was assigned to at the randomization visit (COCP arm: n=37; Net-En arm: n=32; NuvaRing arm: n=35).

Table 6.4. Alpha diversity according to assigned study arm at crossover.

	Shannon Index	
	P	P adj.
All study arms	**0.005**	**0.005**
COCP arm vs. Net-En arm	**0.003**	**0.004**
COCP arm vs. NuvaRing arm	**0.002**	**0.007**
Net-En arm vs. NuvaRing arm	0.490	0.490

P values generated using Kruskal-Wallis test with Dunn's post hoc test using the Benjamini-Hochberg (BH) method for multiple comparisons testing (MTC). COCP arm, n=37; Net-En arm, n=32; NuvaRing arm, n=35.

Table 6.5. Linear regression model of alpha diversity by assigned study arm at crossover adjusting for baseline diversity.

	Shannon Index	
	P	P adj.
All study arms	**0.005**	**0.010**
COCP arm vs. Net-En arm	**0.017**	0.054
COCP arm vs. NuvaRing arm	**0.012**	**0.012**
Net-En vs. NuvaRing	0.999	0.892

P values generated using analysis of covariance (ANCOVA) with Tukey's post hoc test. COCP arm, n=37; Net-En arm, n=32; NuvaRing arm, n=35.

To assess any differences in the microbial communities, as measured with beta diversity, between participants on the different study arms, we performed a permutational multivariate analysis of variance (PERMANOVA) using the adonis function in the R package 'vegan' and weighted UniFrac distance matrices. We found significant differences in beta diversity between the three assigned study arms at crossover (adonis2, p=0.012, R2=0.058, adjusted for baseline alpha diversity) suggesting a significant difference in microbiota composition between at least one of the study arms from the others. However, we did not observe any obvious clustering of the samples according to study arm using principal coordinate analysis (PCoA) (**Figure 5.2B**). To further explain the significant adonis statistic in the absence of obvious clustering, we measured the extent to which variances in the three study arms are equivalent. Dispersion of the community structures of participants in the three study arms did not significantly differ (betadisp, p=0.661, F=0.416) (**Figure 5.3**). These results suggest that that the three arms differ in overall microbial composition (difference in centroids) but not in their overall heterogeneity of species

composition (dispersion). We are thus meeting the assumptions for adonis (homogeneous dispersion) and can trust that the results from adonis statistics are not an artefact of heterogeneous dispersions.

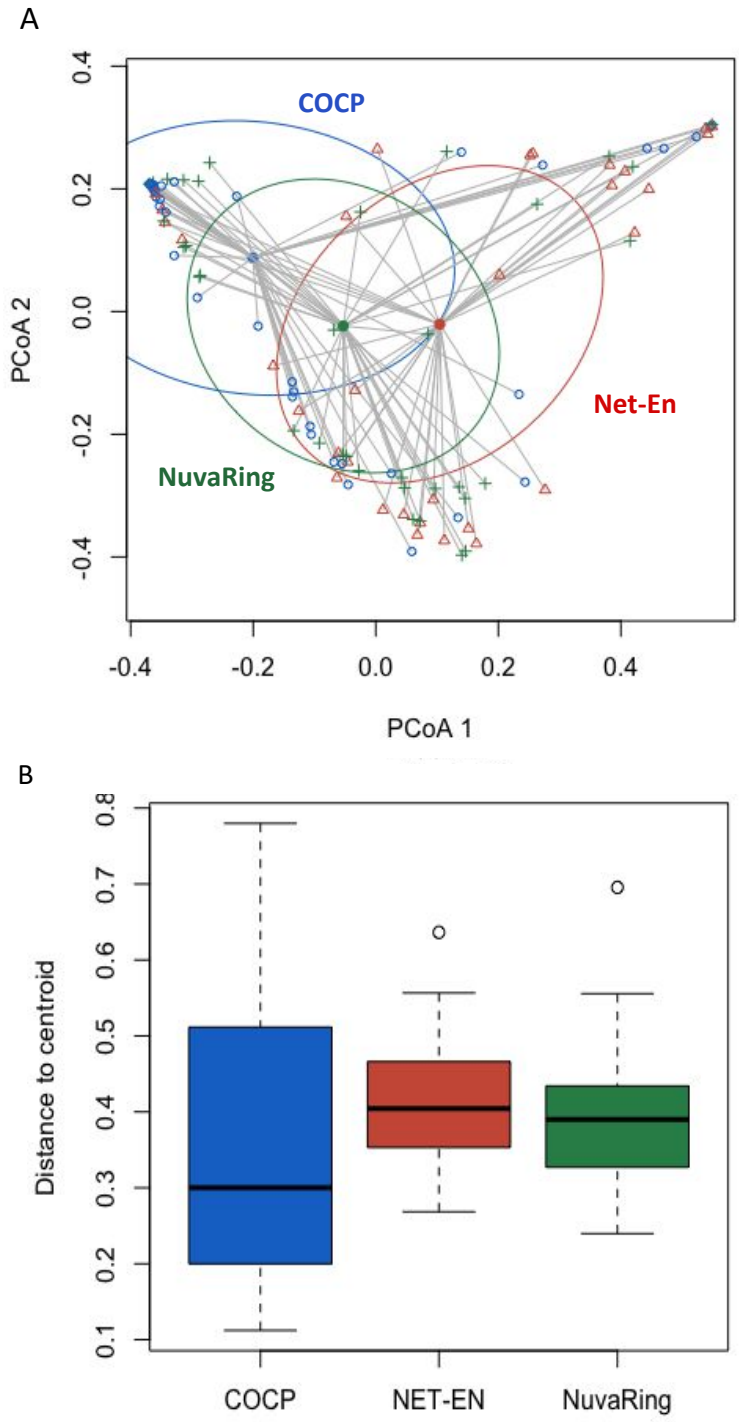


Figure 6.3. Beta diversity within assigned study arms at crossover. A) Principal Coordinates Analysis (PCoA) of beta diversity calculated using weighted UniFrac according to study arm with standard deviations ellipses around each study arm centroid. B) Barplot depicting the distance to the centroid for each sample in each study arm.

Women on certain types of hormonal contraceptives are likely behaviourally and demographically different from women who are using other kinds of contraceptive methods. Since this study was open-label, the participants were not blinded to the study products. We therefore looked into any differences in reported sexual risk behaviour based on study arm at crossover in order to evaluate if differences in vaginal microbiota is reflective of differences in behaviour depending on the study arm assigned to the participants (**Table 5.3**). No significant differences were identified. When adjusting the linear regression model for condom use since last visit, the differences in alpha diversity between study arms remained significant (p=0.025) with the differences between COCP and NuvaRing driving the difference (p=0.026). The same was found after adjusting for condom use at the latest intercourse (ANCOVA p=0.023, COCP versus NuvaRing, p=0.022). When adjusting the adonis statistic for condom use since screening visit (n=95, 9 participants excluded due to missing values) a significant difference in beta diversity was still observed between study arms (p=0.029, R2=0.047). The same was observed when adjusting for condom use during the latest intercourse (p=0.018, R2=0.047). Of note, use of condom at the last sexual act was also significantly associated with beta diversity (p=0.002, R2=0.054). The beta diversity stayed statistically significant different between study arms after adjusting for any STI(s) (p=0.010, R2=0.051).

Using Spearman's rank test, we looked at the correlation of alpha diversity at screening and crossover within individuals according to study arm. There was a significant positive correlation between the alpha diversity at screening and crossover for participants assigned to the COCP and NuvaRing arms (rho (r)=0.41, p=0.013 and r=0.42, p= 0.012, respectively) but not for participants assigned to the Net-En arm (**Figure 5.4**).

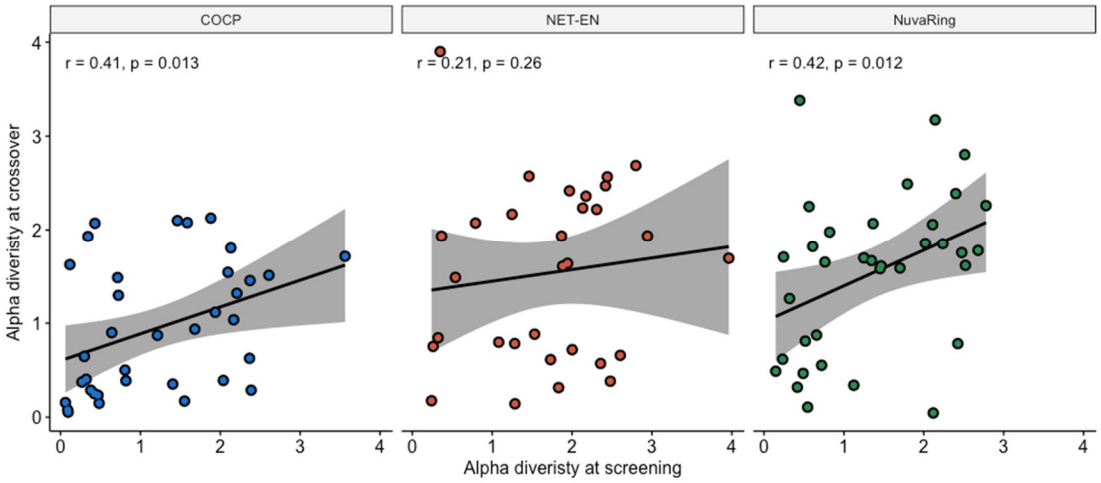


Figure 6.4. Correlation of alpha diversity at screening and crossover according to assigned study arm. The Spearman correlation coefficient (rho, r) and the p values for the different study arms are depicted in the figure.

5.3.2 Differentially abundant taxa between study arms

We looked at the mean relative abundance of the thirty most prevalent taxa according to study arm and many of the same vaginal associated genera (e.g. *Lactobacillus* spp., *Gardnerella vaginalis*, *Megaspheara*, (*Shuttleworthia*) BVAB1, *Prevotella* spp., *Sneathia, Atopobium vaginae*, *Anaerococcus prevotii_tetradius* and *Aerococcus christensenii*) were dominating in the three different arms with *L. iners* being the most prevalent in the COCP and NuvaRing arms and *L. crispatus* the most prevalent in the Net-En arm (**Table 5.6**).

Table 6.6. Most abundant taxa according to study arm.

Study arm COCP			Study arm Net-En			Study arm NuvaRing		
Genus	**Species**	**mRA**	**Genus**	**Species**	**mRA**	**Genus**	**Species**	**mRA**
Lactobacillus	*iners*	52.3%	*Lactobacillus*	*crispatus_acidophilus*	37.8%	*Lactobacillus*	*iners*	38.8%
Lactobacillus	*crispatus_acidophilus*	23.7%	*Lactobacillus*	*iners*	25.8%	*Lactobacillus*	*crispatus_acidophilus*	21.9%
Gardnerella	*vaginalis*	12.9%	*Gardnerella*	*vaginalis*	13.1%	*Gardnerella*	*vaginalis*	14.6%
Shuttleworthia	BVAB1	12.8%	*Shuttleworthia*	BVAB1	10.6%	*Megasphaera*		7.8%
Megasphaera		6.1%	*Megasphaera*		5.5%	*Leptotrichiaceae**		7.8%
Atopobium	*vaginae*	2.3%	*Prevotella*	*amnii*	5.6%	*Shuttleworthia*	BVAB1	8.0%
*Leptotrichiaceae**		2.1%	*Leptotrichiaceae**		5.5%	*Prevotella*	*amnii*	5.8%
Prevotella	*amnii*	2.1%	*Prevotella*	*timonensis*	3.5%	*Atopobium*	*vaginae*	2.9%
Prevotella	*timonensis*	1.7%	*Prevotella*		2.8%	*Lactobacillus*	*jensenii*	2.6%
Prevotella		1.3%	*Leptotrichia*		3.7%	*Prevotella*	*timonensis*	2.1%
Sneathia		1.1%	*Sneathia*		2.4%	*Prevotella*		2.1%
Prevotella	*melaninogenica*	0.9%	*Aerococcus*	*christensenii*	2.0%	*Sneathia*		2.1%
Veillonella	*montpellieriensis*	1.1%	*Atopobium*	*vaginae*	1.5%	*Prevotella*	*bivia*	1.5%
Aerococcus	*christensenii*	0.7%	*Streptococcus*	*infantis_mitis_oralis*	1.4%	*Anaerococcus*	*prevotii_tetradius*	1.3%
Lactobacillus	*johnsonii_gasseri_taiwanensis*	0.7%	*Prevotella*	*bivia*	1.1%	*Staphylococcus*		1.2%
Lactobacillus	*jensenii*	0.6%	*Anaerococcus*	*prevotii_tetradius*	0.9%	*Gardnerella*		1.0%
Streptococcus	*infantis_mitis_oralis*	0.6%	*Clostridium*	*BVAB2*	0.9%	*Prevotella*	*disiens*	0.7%
Prevotella	*bivia*	0.5%	*WAL_1855D*		0.7%	*Gemella*	*asaccharolytica*	0.8%
Streptococcus	*agalactiae*	0.5%	*Dialister*	*succinatiphilus_propionicifaciens*	0.5%	*Aerococcus*	*christensenii*	0.5%
Clostridium	*BVAB2*	0.3%	*Lactobacillus*	*jensenii*	0.7%	*Finegoldia*	*magna*	0.5%
Gemella	*asaccharolytica*	0.2%	*Fusobacterium*	*nucleatum*	0.7%	*Porphyromonas*	*eunonis*	0.5%
Dialister	*succinatiphilus_propionicifaciens*	0.2%	*Veillonella*	*montpellieriensis*	0.6%	*Streptococcus*	*infantis_mitis_oralis*	0.5%
Gardnerella		0.3%	*Mycoplasma*	*hominis*	0.6%	*Parvimonas*	*micra*	0.4%
Dialister	*micraerophilus*	0.2%	*Finegoldia*	*magna*	0.4%	*Clostridium*	BVAB2	0.4%
Parvimonas	*micra*	0.2%	*Parvimonas*	*micra*	0.4%	*Mycoplasma*	*hominis*	0.4%
Anaerococcus	*prevotii_tetradius*	0.2%	*Peptoniphilus*	*harei_asaccharolyticus_grossensis*	0.3%	*Peptoniphilus*	*harei_asaccharolyticus_grossensis*	0.3%
Finegoldia	*magna*	0.1%	*Prevotella*	*corporis*	0.5%	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*	0.4%
Ureaplasma	*parvum_urealyticum*	0.1%	*Peptostreptococcus*	*anaerobius*	0.4%	*Corynebacterium*	*tuberculostearicum*	0.4%
Gemella	*morbillorum_cluster*	0.2%	*Porphyromonas*	*eunonis*	0.4%	WAL_1855D		0.3%
WAL_1855D		0.1%	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*	0.3%	*Dialister*	*succinatiphilus_propionicifaciens*	0.3%
OTUs merged at lowest taxonomic level. mRA: mean relative abundance. *Note: this taxa only annotated down to family level								

To identify differentially abundant bacterial species between study arms, we used metagenomeSeq analysis (FDR ≤ 0.05, fold-change ≥ 1.25, taxa present in ≥ 20% of samples in at least one of the two groups being compared). The relative abundances of twelve taxa (OTUs merged at lowest taxonomic level) were significantly different between participants assigned to the COCP (n=37) and Net-En arms (n=32) (**Figure 5.5A** and **Table 5.7**). These included *L. iners*, *Staphylococcus*, *Streptococcus salivarus_thermophilus* and *Gemella morbillorum_cluster*, which were more abundant in the COCP arm and *Porphyromonadaceae*, *Chryseobacterium*, *Actinomyces hongkongensis*, *Arcanobacterium*, *Campylobacter, Proteobacteria* (note: only annotated at phylum level) and *BVAB3_M_indolicus* which were more abundant in the Net-En arm. When comparing the bacterial composition of the microbiota of participants in the COCP arm (n=37) to participants in the NuvaRing arm (n=35), eight taxa were differentially abundant between the two groups (**Figure 5.5B** and **Table 5.7**). Only *Bifidobacterium* was more abundant in the COCP arm compared to the NuvaRing arm while *Peptostreptococcus anaerobius*, *Campylobacter*, *Porphyromonadaceae*, *Prevotella bivia*, *Prevotella disiens*, *Arcanobacterium* and *Parvimonas micra* were more abundant in the NuvaRing group. When comparing the bacterial composition of the microbiota of participants in the Net-En arm (n=32) to participants in the NuvaRing arm (n=35), thirteen taxa were significantly differentially abundant between the two groups (**Figure 5.5C** and **Table 5.7**). These included *Bifidobacterium*, *Clostridium ramosum_saccharogumia*, BVAB1 and *L. crispatus* which were more abundant in the Net-En arm and *Veillonella*, *Porphyromonas bennonis*, *Enterococcus faecium_cluster*, *Gemella asaccharolytica*, *L. jensenii*, *Fusobacterium equinum*, *Corynebacterium urealyticum* and *Neisseria gonorrhoea* which were more abundant in the NuvaRing group. The increased relative abundance of *N. gonorrhoea* is consistent with the STI results (**Table 5.3**).

Table 6.7. Differentially abundant taxa between assigned study arms at crossover (intention-to-treat).

	Coeff	P adj.	Family	Genus	Species
COCP vs. Net-En	-2.481	0.0311	Lactobacillaceae	*Lactobacillus*	*iners*
	-1.583	0.0078	Staphylococcaceae	*Staphylococcus*	NA
	-1.517	0.0226	Streptococcaceae	*Streptococcus*	*salivarius_thermophilus*
	-1.498	0.0311	Gemellaceae	*Gemella*	*morbillorum_cluster*
	1.422	0.0117	Porphyromonadaceae	NA	NA
	1.547	0.0026	[Weeksellaceae]	*Chryseobacterium*	NA
	1.620	0.0028	Proteobacteria*	NA	NA
	1.760	2.34e-05	Actinomycetaceae	*Actinomyces*	*hongkongensis*
	2.059	1.13e-05	Actinomycetaceae	*Arcanobacterium*	NA
	2.219	0.0008	Campylobacteraceae	*Campylobacter*	NA
	2.468	0.0034	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
COCP vs. NuvaRing	-1.267	0.0066	Bifidobacteriaceae	*Bifidobacterium*	NA
	1.325	0.0401	Peptostreptococcaceae	*Peptostreptococcus*	*anaerobius*
	1.533	0.0219	Campylobacteraceae	*Campylobacter*	NA
	1.558	0.0034	Porphyromonadaceae	NA	NA
	1.925	0.0288	Prevotellaceae	*Prevotella*	*bivia*
	2.112	0.0006	Actinomycetaceae	*Arcanobacterium*	NA
	2.294	0.0219	[Tissierellaceae]	*Parvimonas*	*micra*
	2.317	0.0111	Prevotellaceae	*Prevotella*	*disiens*
Net-En vs. NuvaRing	-1.760	0.0421	Erysipelotrichaceae	*Clostridium*	*ramosum_saccharogumia*
	-1.591	0.0118	Bifidobacteriaceae	*Bifidobacterium*	NA
	-1.443	0.0011	Lachnospiraceae	*Shuttleworthia*	BVAB1
	-1.316	0.0095	Lactobacillaceae	*Lactobacillus*	*crispatus_acidophilus*
	1.331	0.0450	Enterococcaceae	*Enterococcus*	*faecium_cluster*
	1.339	0.0118	Gemellaceae	*Gemella*	*asaccharolytica*
	1.345	0.0118	Porphyromonadaceae	*Porphyromonas*	*bennonis*
	1.348	0.0118	Prevotellaceae	*Prevotella*	*disiens*
	1.421	0.0421	Corynebacteriaceae	*Corynebacterium*	*urealyticum*
	1.558	0.0118	Fusobacteriaceae	*Fusobacterium*	*equinum*
	2.051	0.0421	Veillonellaceae	*Veillonella*	NA
	2.490	0.0000	Lactobacillaceae	*Lactobacillus*	*jensenii*
	2.653	0.0002	Neisseriaceae	*Neisseria*	*gonorrhoea*

*Note: Proteobacteria is only annotated at phylum level.

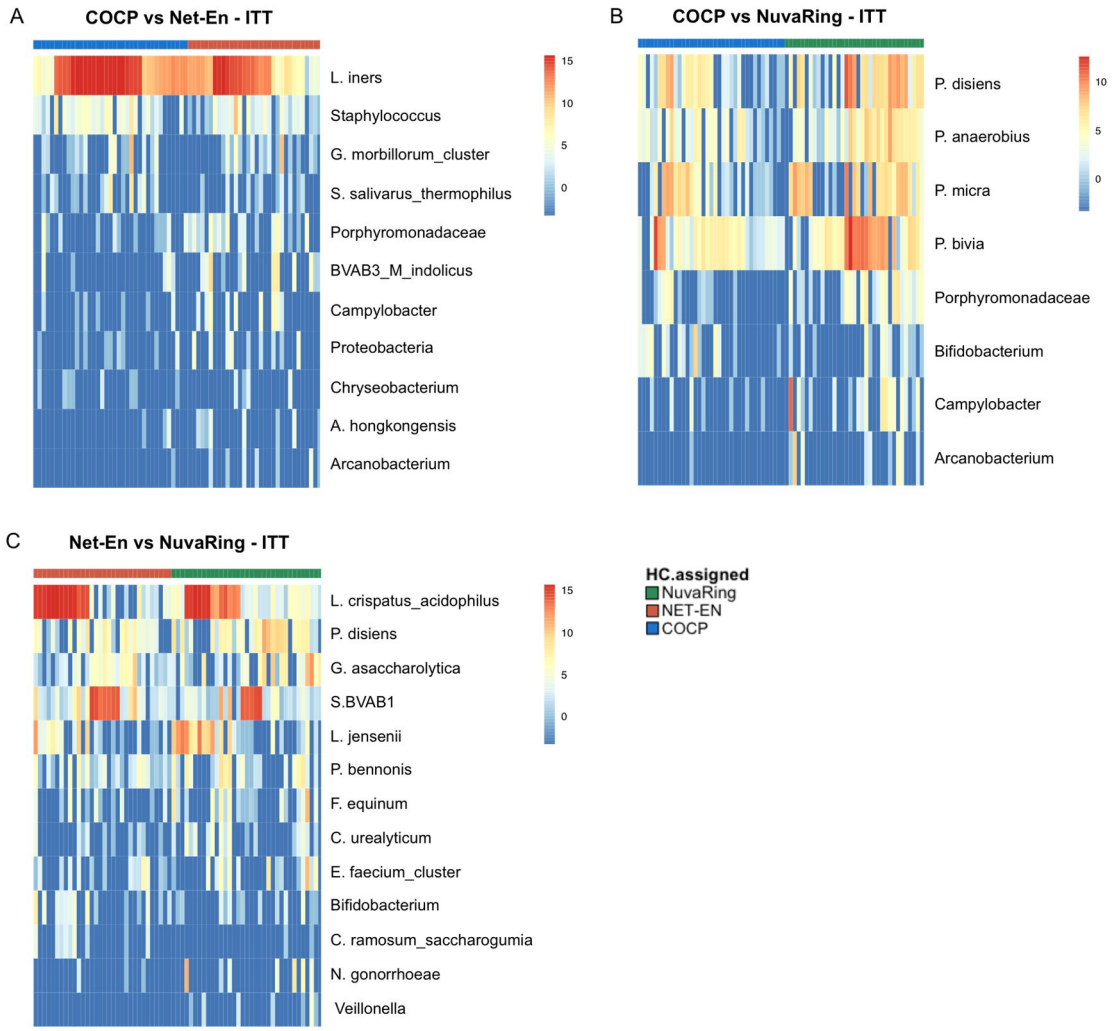


Figure 6.5. Differentially abundant taxa by study arm at crossover. Bacterial taxa significantly differentially abundant and/or frequent by study arm (A: COCP versus Net-En; B: COCP versus NuvaRing; C: Net-En versus NuvaRing) in the vaginal samples from crossover visits analysed using metagenomeSeq (FDR ≤ 0.05, fold-change ≥ 1.25, taxa present in ≥ 20% of samples in at least one of the two groups being compared). The heatmap shows unsupervised clustering of samples (columns) by Bray-Curtis distances split by contraceptive method. Log2-transformed standardized read counts are illustrated by the colour key.

Random forest analysis was additionally used to identify species predictive of study arm at crossover. The most influential taxa in differentiating COCP from Net-En according to this analysis were *Peptostreptococcus anaerobius*, *L. iners*, *Prevotella* spp. (including *P. amnii*), *Peptoniphilus*, *Peptoniphilus harei_asaccharolyticus_grossensis*, *Clostridiales*, *Acinetobacter baumanii*, *Dialister succinatiphilus_propionicifaciens*, and *Anaerococcus lactolyticus* (AUC=0.82, sensitivity=0.85 and specificity=0.68 for the training set and a validation predicted error rate of 47.83% using the five top predictive taxa) (**Figure 5.6A** and **Table 5.8**). The most influential taxa in differentiating COCP from NuvaRing were *Dialister micraerophilus*, *Prevotella disiens*, *Prevotella amnii*,

Leptotrichiaceae, *Prevotella buccalis*, *L. jensenii, L. iners*, *Atopobium vaginae*, *Peptostreptococcus anaerobius* and BVAB1 (AUC=0.69, sensitivity =0.64 and specificity=0.74 for the training set and a validation predicted error rate of 37.50% using the top five predictive taxa) (**Figure 5.6B** and **Table 5.9**). The most influential taxa in differentiating the Net-En arm from the NuvaRing arm were *Prevotella* ssp. (including *P. disiens*, *P. corporis and P. bivia*), *L. crispatus_acidophilus, Gemella asaccharolytica*, *Peptoniphilus lacrimalis*, *Parvimonas micra*, *L. iners*, *D. succinatiphilus_propionicifaciens* and *Aerococcus christensenii* (AUC=0.79, sensitivity=0.68 and specificity=0.74 for the training set and a validation predicted error rate of 54.55% using the top five predictive taxa) (**Figure 5.6C** and **Table 5.10**). In all three cases the error rates were high suggesting that the individual species are not a strong predictor of study arm.

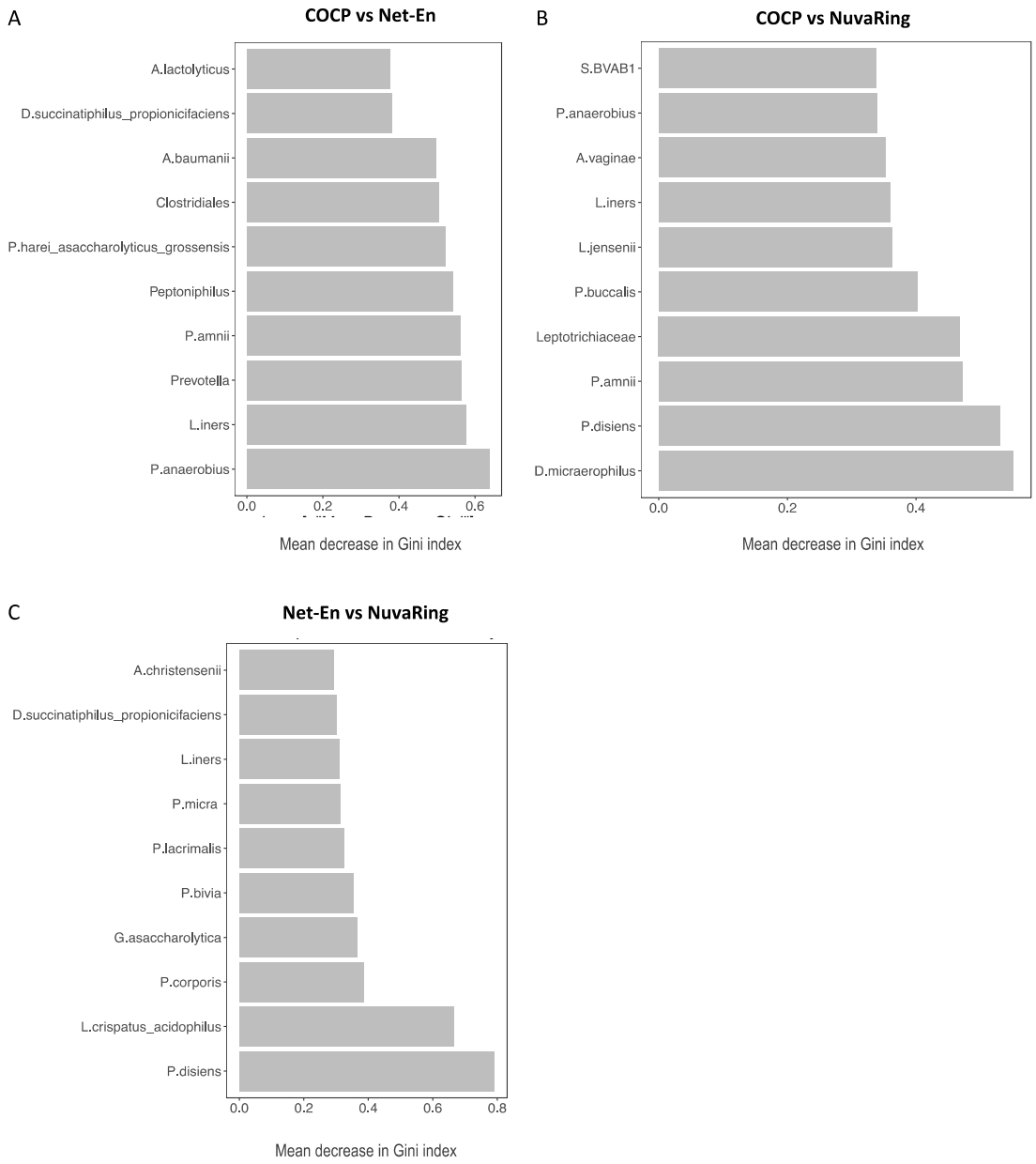


Figure 6.6. Random forest analysis of study arms at crossover (intention-to-treat). The top 10 most influential taxa by random forest analysis in predicting study arm (A: COCP versus Net-En, B: COCP versus NuvaRing, C: Net-en versus NuvaRing) at crossover. The x-axis indicates the mean decrease in Gini Index (length of bar represents predictive ability of each taxon).

Table 6.8. Random forest analysis of COCP arm versus Net-En arm at crossover.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_41	3.607	0.638	*Peptostreptococcus anaerobius*
OTU_1	2.836	0.576	*Lactobacillus iners*
OTU_9	3.279	0.563	*Prevotella*
OTU_7	4.570	0.562	*Prevotella amnii*
OTU_56	2.702	0.541	*Peptoniphilus*
OTU_35	2.726	0.521	*Peptoniphilus* harei_asaccharolyticus_grossensis
OTU_110	1.738	0.504	*Clostridiales*
OTU_57	3.532	0.497	*Acinetobacter baumanii*
OTU_14	3.683	0.383	*Dialister succinatiphilus_propionicifaciens*
OTU_1131	2.859	0.376	*Anaerococcus lactolyticus*

Training set size: 46 samples with 27 and 19 samples per arm. Test set size: 23 samples with 10 and 13 samples per arm. Validation predicted error: 47.83% (using top five predictive taxa).

Table 6.9. Random forest analysis of COCP arm versus NuvaRing arm at crossover.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_39	1.248	0.551	*Dialister micraerophilus*
OTU_25	2.161	0.531	*Prevotella disiens*
OTU_7	0.480	0.472	*Prevotella amnii*
OTU_6	-1.310	0.468	*Leptotrichiaceae*
OTU_50	0.959	0.402	*Prevotella buccalis*
OTU_90	0.293	0.363	*Lactobacillus jensenii*
OTU_1	-0.905	0.360	*Lactobacillus iners*
OTU_8	-1.869	0.352	*Atopobium vaginae*
OTU_41	-0.733	0.339	*Peptostreptococcus anaerobius*
OTU_3	-0.368	0.338	S. BVAB1

Training set size: 48 samples with 25 and 23 samples per arm. Test set size: 24 samples with 12 and 12 samples per arm. Validation predicted error: 37.50% (using top five predictive taxa).

Table 6.10. Random forest analysis of Net-En arm versus NuvaRing arm at crossover.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_25	4.986	0.791	*Prevotella disiens*
OTU_2	4.556	0.666	*Lactobacillus crispatus_acidophilus*
OTU_31	0.749	0.386	*Prevotella corporis*
OTU_37	1.622	0.364	*Gemella asaccharolytica*
OTU_11	1.734	0.354	*Prevotella bivia*
OTU_44	1.290	0.324	*Peptoniphilus lacrimalis*
OTU_24	-1.782	0.314	*Parvimonas micra*
OTU_1	1.966	0.311	*Lactobacillus iners*
OTU_14	-1.055	0.301	*Dialister succinatiphilus_propionicifaciens*
OTU_16	-0.045	0.293	*Aerococcus christensenii*

Training set size: 45 samples with 22 and 23 samples per arm. Test set size: 22 samples with 10 and 12 samples per arm. Validation predicted error: 54.55% (using top five predictive taxa).

DESeq2 can perform better differential abundance testing with small datasets (Weiss et al., 2017). Using DESeq2, three taxa (OTUs merged at lowest taxonomic level, adj. $P<0.05$, fold change >1.5 or <1/1.5) were found to be differentially abundant between the COCP and Net-En study arms at crossover (**Figure 5.7** and **Table 5.11**). *L. iners* was more abundant in the COCP study arm while *Sneathia* and *BVAB3_M_indolicus*, which were more abundant in the Net-En arm. Three taxa, *Anaerococcus prevotii_tetradius*, *Enterobacteriaceae* and *Fusobacterium equinum* were significantly more abundant in the NuvaRing arm compared to the COCP arm at crossover (**Figure 5.7** and **Table 5.11**) while none were found to be more abundant in the COCP arm. Two taxa, *Prevotella bivia* and *BVAB3_M_indolicus*, which both had a higher abundance in the NuvaRing arm, were differentially abundant between the Net-En and NuvaRing study arms at crossover (**Figure 5.7** and **Table 5.11**).

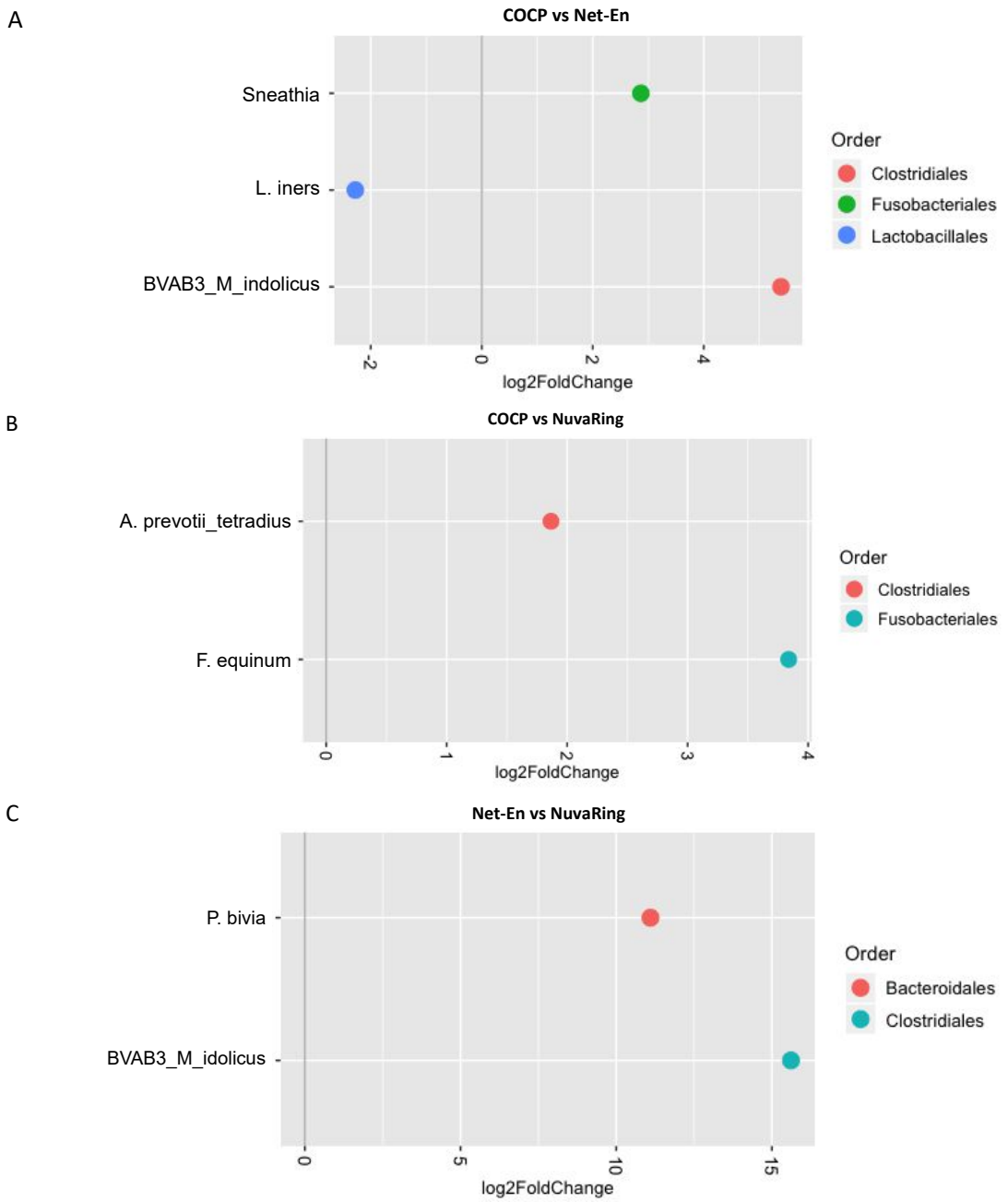


Figure 6.7. DESeq2 analysis of assigned study arms at crossover. Bacterial taxa significantly differentially abundant and/or frequent by study arm at crossover using DESeq2 (with an alpha of 0.05). A) Combined oral contraceptives (COCP) versus Net-En injection. B) COCP versus NuvaRing. C) Net-En injection versus NuvaRing. Taxa depicted at species level. Taxa without species level annotation (if >1) are grouped in the first line and order of genera corresponds to order of appearance of dots (from left to right). Figure only includes taxa annotated at the genus level.

Table 6.11. Differentially abundant taxa between assigned study arms at crossover by DESeq2.

	Log2FC	P adj.	Family	Genus	Species
COCP vs. Net-En	5.391	0.009	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
	-2.278	0.009	Lactobacillaceae	*Lactobacillus*	*iners*
	2.866	0.016	Leptotrichiaceae	*Sneathia*	NA
COCP vs. NuvaRing	3.838	0.009	Fusobacteriaceae	*Fusobacterium*	*equinum*
	3.141	0.039	Enterobacteriaceae	NA	NA
	1.865	0.044	[Tissierellaceae]	*Anaerococcus*	*prevotii_tetradius*
Net-En vs. NuvaRing	11.101	0.004	Prevotellaceae	*Prevotella*	*bivia*
	15.612	0.006	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*

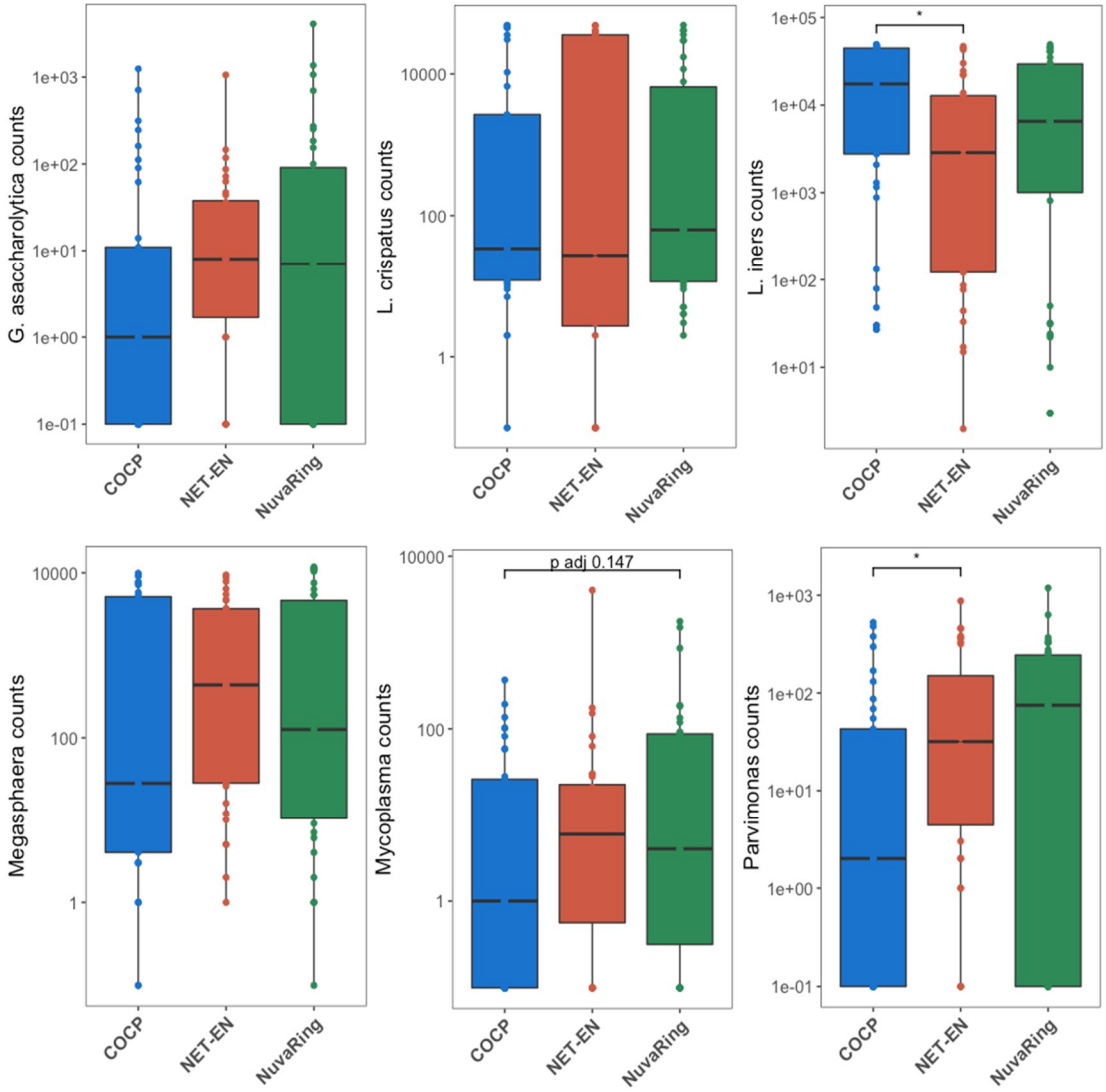
FC, fold change.

Certain bacterial species and genera have been associated with increased risk of HIV infection, including *Parvimonas*, *Prevotella* spp. (e.g. *P. amnii, P. bivia* and *P. melaninogenica*), *Mycoplasma*, *Gemella asaccharolytica*, *Sneathia*, *Veillonella montpellieriensis*, and *Megasphaera* (McClelland et al., 2018; Gosmann et al., 2017) while *Lactobacillus*, particularly non-*iners Lactobacillus*, dominated communities have been associated with protection from later HIV acquisition (Gosmann et al., 2017; Borgdorff et al., 2014). For these particular bacteria of interest, we looked at whether there were any differences in the standardized read counts between the study arms. For the read counts of *G. asaccharolytica*, *L. crispatus*, *Megasphaera*, *Sneathia* and *Veillonella montpellieriensis*, no significant differences between study arms were observed ($p<0.05$) (**Figure 5.8** and **Table 5.12**). The read counts of *L. iners* were significantly higher in the COCP study arm compared to the Net-En study arm (adj. $p=0.024$) while the opposite pattern was observed for *Parvimonas* (adj. $p=0.018$). The read counts of total lactobacilli were significantly higher in COCP arm compared to Net-En before adjusting for multiple comparisons ($p=0.033$). The read counts of *Mycoplasma* were significantly lower in COCP arm compared to NuvaRing ($p=0.049$), but not after adjusting for multiple comparisons. The same was found for *P. bivia* ($p=0.044$). When analysing *Prevotella* at the genus level, the standardised read counts were significantly higher in both the Net-En (adj. $p=0.022$) and NuvaRing (adj. $p=0.046$) arms compared to the COCP arm (**Figure 5.8** and **Table 5.12**).

Table 6.12. Standardised read counts of specific bacteria of interest according to study arm.

	COCP	Net-En	NuvaRing	P value
G. asaccharolytica	1 (0-11)	8 (2-38)	7 (0-92)	0.181
Lactobacillus	44,455 (5,957- 48,831)	29,562 (1,051-46,146)	29,577 (1,473-47,091)	0.095
L. crispatus	34 (12-2,679)	28 (3-35,674)	63 (12-6,671)	0.846
L. iners	17,449 (2,764-44,736)	2,877 (121-12,844)	6,532 (1,028-29,518)	**0.025**
Megasphaera	28 (4-5,140)	438 (28-3,677)	126 (10-4,687)	0.497
Mycoplasma	1 (0-26)	6 (1-23)	4 (1-88)	0.113
Parvimonas	2 (0-43)	32 (5-168)	75 (0-245)	**0.027**
Prevotella	223 (75-3,057)	2,025 (450-9080)	2,421 (333-5,953)	**0.016**
P. amnii	6 (1-38)	7 (1-3,784)	4 (1-1,235)	0.801
P. bivia	13 (4-55)	35 (4-202)	47 (12-490)	0.111
P. melaninogenica	1 (0-5)	0 (0-3)	1 (0-3)	0.552
Sneathia	5 (1-55)	24 (2-514)	19 (2-696)	0.270
V. montpellieriensis	1 (0-3)	1 (0-32)	1 (0-14)	0.809

P values were generated using Kruskal-Wallis test adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method.



Continued on next page.

Continued from previous page.

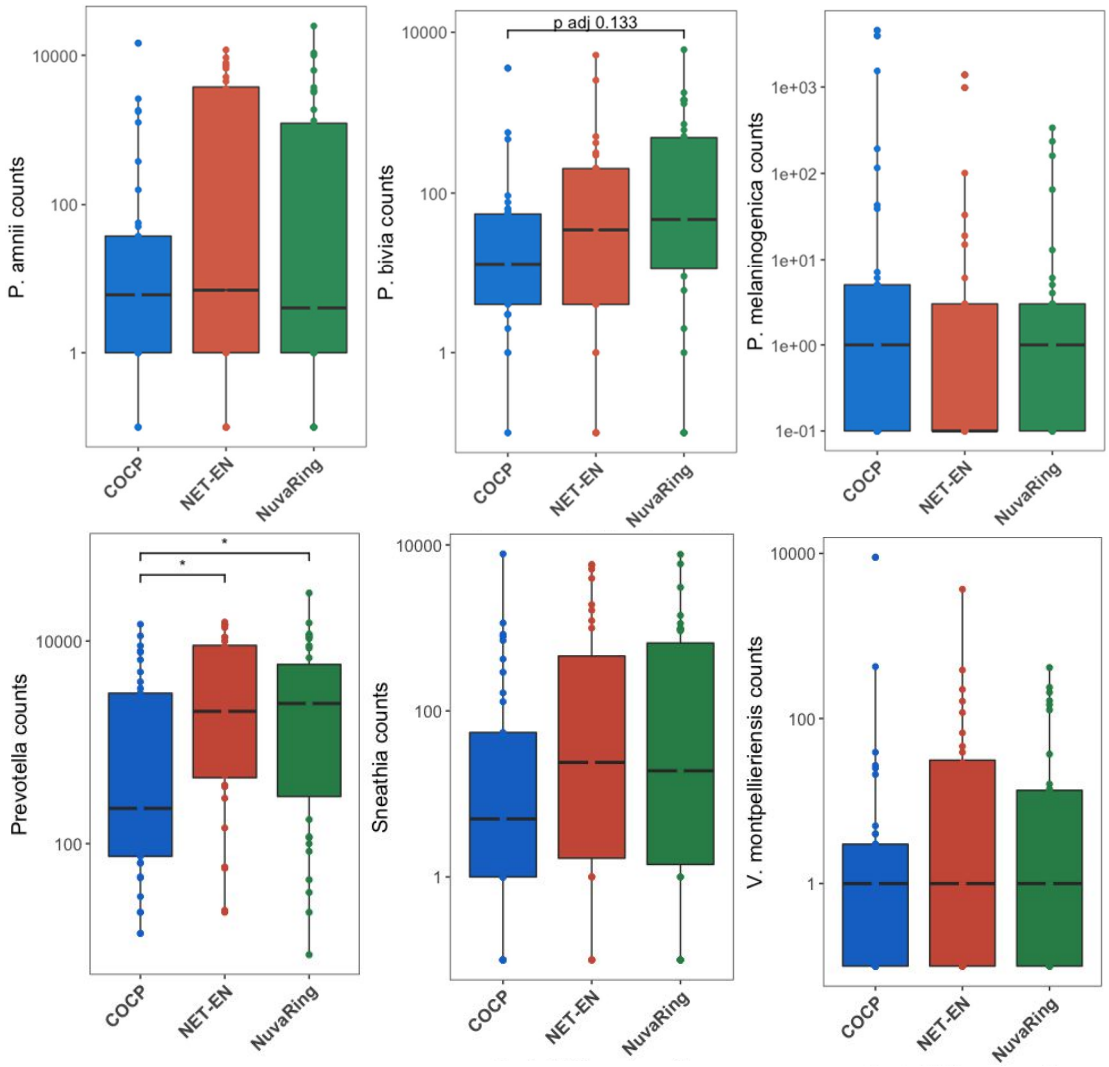


Figure 6.8. Differences in bacterial counts between study arms of bacteria of interest. Boxplot showing the standardized read counts of bacteria of interest in 104 vaginal samples crossover visits according to study arm. P values were generated using unpaired Mann-Whitney-Wilcoxon tests adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. Y-axis is log10 transformed. *=adj. p<0.05.

5.3.3 According to protocol analysis at crossover

Despite randomisation, some study participants changed hormonal contraceptive method before the crossover visit due to sideeffects (bleeding or spotting with Net-En), issues with adherence (using COCP and NuvaRing) or discomfort with study product (NuvaRing) (n=9 in all groups) (**Figure 4.1**, Chapter 4). Three participants changed from COCP to Net-En and one to NuvaRing, one participant changed from Net-En to COCP and three participants changed from NuvaRing to Net-En and one to COCP.

Table 6.13. Characteristics of participants at crossover according to hormonal contraceptive method.

	COCP (n=35)	Net-En (n=37)	NuvaRing (n=32)	P value
Fuzzy cluster distribution[1]				0.156
C1	11 (31.4%)	17 (48.6%)	16 (51.6%)	
C2	8 (22.9%)	11 (31.4%)	6 (19.4%)	
C3	16 (45.7%)	7 (20.0%)	9 (29.0%)	
Vaginal pH, mean (sd)	4.63 (4.17-5.08)	4.85 (4.24-5.47)	5.00 (4.49-5.51)	**0.017**
Shannon Index, median (IQR)	0.87 (0.32-1.54)	1.64 (0.78-2.22)	1.64 (0.74-1.90)	**0.010**
HSV-2 serology	13 (37.1%)	10 (27.0%)	13 (40.6%)	0.461
Yeast cells present	3 (8.6%)	7 (18.9%)	6 (18.8%)	0.384
BV prevalence				0.292
BV positive	11 (31.4%)	18 (48.6%)	14 (43.8%)	
BV intermediate	3 (8.6%)	0 (0.0%)	1 (3.1%)	
BV negative	21 (60.0%)	19 (51.4%)	17 (53.1%)	
STI prevalence				
Any STI(s)	4 (11.4%)	13 (35.1%)	12 (37.5%)	**0.028**
Ct	3 (8.6%)	7 (16.2%)	6 (18.8%)	0.384
Ng	1 (2.9%)	1 (2.7%)	8 (25.0%)	**0.002**
Tv	0 (0.0%)	2 (5.4%)	1 (3.1%)	0.524
Mg	1 (2.9%)	3 (8.1%)	0 (0.0%)	0.323
Sexual risk behaviour since last visit[2]				
Sexual partner(s), median (IQR)	1 (1-1)	1 (1-1)	1 (1-1)	0.673
Multiple sexual partners, n	1 (3.0%)	1 (3.1%)	1 (0.0%)	1.000
New partner(s), n	2 (6.5%)	1 (3.5%)	0 (0.0%)	0.410
Condom use				0.361
Never	5 (15.2%)	9 (28.1%)	7 (23.3%)	
Less than half the time	6 (18.2%)	1 (3.1%)	3 (10.0%)	
Half the time	15 (45.5%)	15 (46.9%)	5 (16.7%)	
More than half the time	3 (9.1%)	1 (3.1%)	5 (16.7%)	
Always	4 (12.1%)	6 (18.8%)	10 (33.3%)	
Condom use during last PV intercourse				
Yes	19 (73.7%)	15 (46.9%)	20 (66.7%)	0.289
PV sex acts per week, median (IQR)	2 (1-2)	2 (1-2)	1 (1-2)	0.624
Intergenerational sex with older partner (≥5 years)				
No	2 (6.1%)	2 (6.3%)	0 (0.0%)	0.542
Unsure	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Yes	31 (93.9%)	30 (93.8%)	30 (100%)	
Transactional sex	0 (0.0%)	0 (0.0%)	0 (0.0%)	NA
Penile-anal intercourse	0 (0.0%)	0 (0.0%)	0 (0.0%)	NA

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups, Kruskal-Wallis for comparison of medians and ANOVA test for comparison of means. BV; bacterial vaginosis; Ct, Chlamydia trachomatis*; HSV-2, herpes simplex virus type 2; IQR, interquartile range, Mg,* Mycoplasma genitalium; *Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. Three samples not assigned any cluster (COCP: n=0; Net-En: n=2; NuvaRing: n=1)

2. Missing values for nine participants (COCP: n=2; Net-En: n=5; NuvaRing: n=2)

Hence, at crossover a total of 32 (assigned and non-assigned) participants were on COCP, 37 on Net-En and the 32 on NuvaRing. Among the participants on Net-En at crossover; 27 out of 31 (87.1%, missing data from six participants) had received their last injection on time (for four participants it had been >3 months since their last injection). The main reasons for missing the injections were due

to traveling and being busy with school work. Among the participants on NuvaRing at crossover; 26 out of 31 (83.9%, data missing from one participant) reported using the ring as instructed over the two past months (if removed, the ring was removed for menstruation to occur). The main reason for not using the ring was the ring falling out and/or lost or being uncomfortable with the ring. Among the participants on COCPs at crossover; 17 reported having taken their COCPs as instructed over the past two months (48.5%), ten to have taken their COCPs inconsistently (28.6%, up to ten days without study pills) and four reported less use (11.4%, data missing from four participants).

The BV prevalence according to Nugent scoring of participants on Net-En at crossover (48.6%) was higher than for participants on COCP or NuvaRing (31.4% and 43.8%, respectively), however these differences were not statistically significant (p=0.292) (**Table 5.13**). Also, no statistical difference in distribution of Fuzzy clusters between the three groups was observed (p=0.156) (**Table 5.13**). The vaginal pH of participants on COCP was lower than the pH detected in the vaginas of participants on Net-En and NuvaRing (mean pH: 4.63 versus 4.85 and 5.00). This was significantly different between COCP and NuvaRing (adj. p=0.013) (**Table 5.13**). The vaginal microbiota of participants on COCPs at crossover had a significantly lower alpha diversity at crossover compared to the microbiota of participants on Net-En and NuvaRing (adj. p= 0.005 and 0.008, respectively), while no difference in diversity was observed between the NuvaRing and Net-En arms (adj. p=0.389) (**Figure 5.9A** and **Table 5.14**). Similar to the intention-to-treat analysis, this relationship remained significant between participants on COCP and NuvaRing in a linear regression model after adjusting for baseline alpha diversity (p=0.039), and close to, but not significantly so between participants on COCP and Net-En (p=0.051) (**Table 5.15**). As in the ITT analysis, there were no significant changes in the alpha diversity of the vaginal microbiota of adolescents within each HC group from baseline to crossover although a slight decrease in diversity in the COC (median SI (IQR): 0.87 (0.32-1.83) versus 0.81 (0.35-1.54)) and Net-En (median SI (IQR): 1.87 (0.79-2.31) versus 1.64 (0.78-2.22)) users and a slight increase in NuvaRing users (median SI (IQR): 1.41 (0.56-2.12) versus 1.64 (0.74-1.90)) were observed (p>0.05, Wilcoxon Signed Rank test).

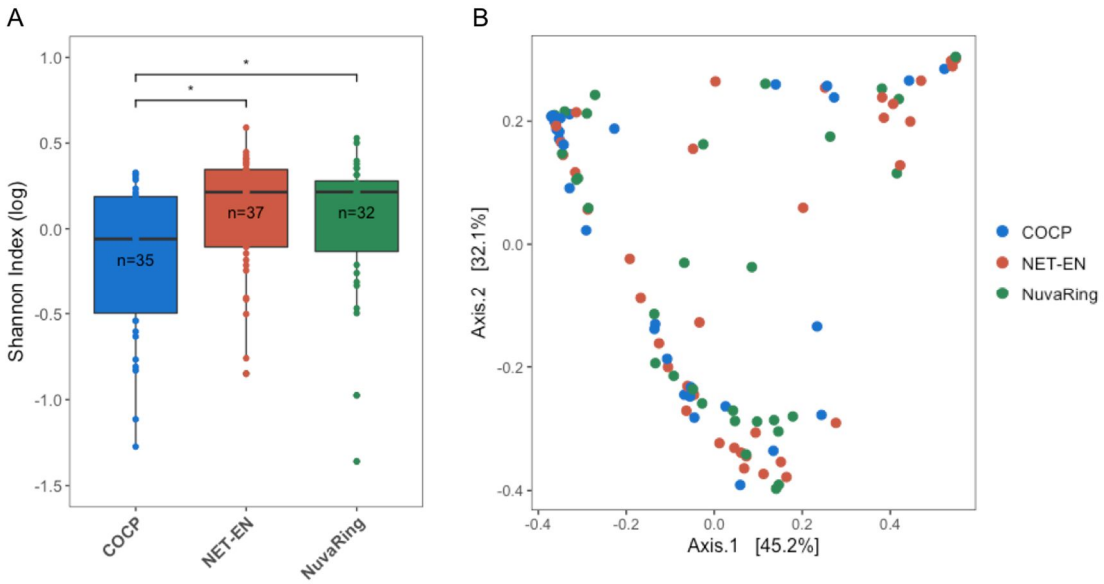


Figure 6.9. Alpha and beta diversity according to hormonal contraceptive method at crossover. A) Boxplot depicting the alpha diversity measured using Shannon Index (log transformed values) of the vaginal microbiota from 104 participants at crossover according to hormonal contraceptive method (COCP: n=35; Net-En; n=37; NuvaRing: n=32). P values were calculated using Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests and adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. *=adj. p<0.05. B) Principal Coordinate Analysis (PCoA) of samples from 104 participants at crossover using weighted UniFrac distances. Samples are coloured by the hormonal contraceptive method that the participant was on at time of sampling (COCP: n=35; Net-En; n=37; NuvaRing: n=32).

We did not observe any differences in *Candida* and BV prevalence or HSV-2 serology between HC methods (**Table 5.13**). However, there was a significant difference in overall STI prevalence between the three HC methods with participants using COCPs having a lower STI prevalence compared to participants on Net-En and NuvaRing (11.4% versus 35.1% and 37.5%, respectively). There were significantly more participants with *N. gonorrhoea* infection amongst the NuvaRing group compared to the other two methods (25% versus 2.86% and 2.30%, p=0.002).

Table 6.14. Alpha diversity according to hormonal contraceptive method at crossover.

	Shannon Index	
	P	P adj.
All hormonal contraceptive methods	**0.007**	**0.007**
COCP vs. Net-En	**0.002**	**0.005**
COCP vs. NuvaRing	**0.006**	**0.008**
Net-En vs. NuvaRing	0.389	0.389

P values generated using Kruskal-Wallis test with Dunn's post hoc test using the Benjamini-Hochberg (BH) method for multiple comparisons testing (MTC). COCP: n=35; Net-En: n=37; NuvaRing: n=32.

Table 6.15. Linear regression model of alpha diversity according to hormonal contraceptive method adjusted for baseline alpha diversity.

	Shannon Index	
	P	P adj.
All hormonal contraceptive methods	**0.006**	**0.023**
COCP vs. Net-En	**0.010**	0.051
COCP vs. NuvaRing	**0.022**	**0.039**
Net-En vs. NuvaRing	0.741	0.989

P values generated using ANCOVA with Tukey's post hoc test for multiple comparisons testing. COCP: n=35; Net-En: n=37; NuvaRing: n=32.

Overall differences in microbiota composition (beta diversity) were found between the vaginal microbiota of participants on the different HC methods at crossover (p= 0.026, R2=0.044, adjusted for baseline alpha diversity) but as in the intention-to-treat analysis no distinct clustering was observed using PCoA (**Figure 5.9B** and **Figure 5.10**). Once again, the dispersion of the bacterial vaginal community of participants on the different HC methods did not significantly differ (p=0.722, F=0.327). We also looked into any differences in sexual risk behaviour based on HC method at crossover in order to evaluate if differences in vaginal microbiota is reflective of differences in behaviour, although there were no significant differences in reported sexual behaviour (**Table 5.13**). When adjusting the linear regression model for condom use since the screening visit, the differences in alpha diversity between HC methods became less strong (p=0.056) with the differences between the COCP and NuvaRing arm being close to significant. A similar trend was seen after adjusting for condom use at the latest intercourse (ANCOVA p=0.051, COCP versus NuvaRing, p=0.066). When adjusting the adonis statistic for condom use since the screening visit (n=95, 9 participants excluded due to missing data), the difference in beta diversity between HC methods was close to, but not significant (p=0.058, R2=0.040), however, when adjusting for condom use at last intercourse it was (p=0.027, R2=0.046). Again, condom use during last sexual act was significantly associated with beta diversity (p=0.003, R2=0.055). The beta diversity stayed statistically significant different between HC methods after adjusting for any STI(s) (p=0.021, R2=0.044).

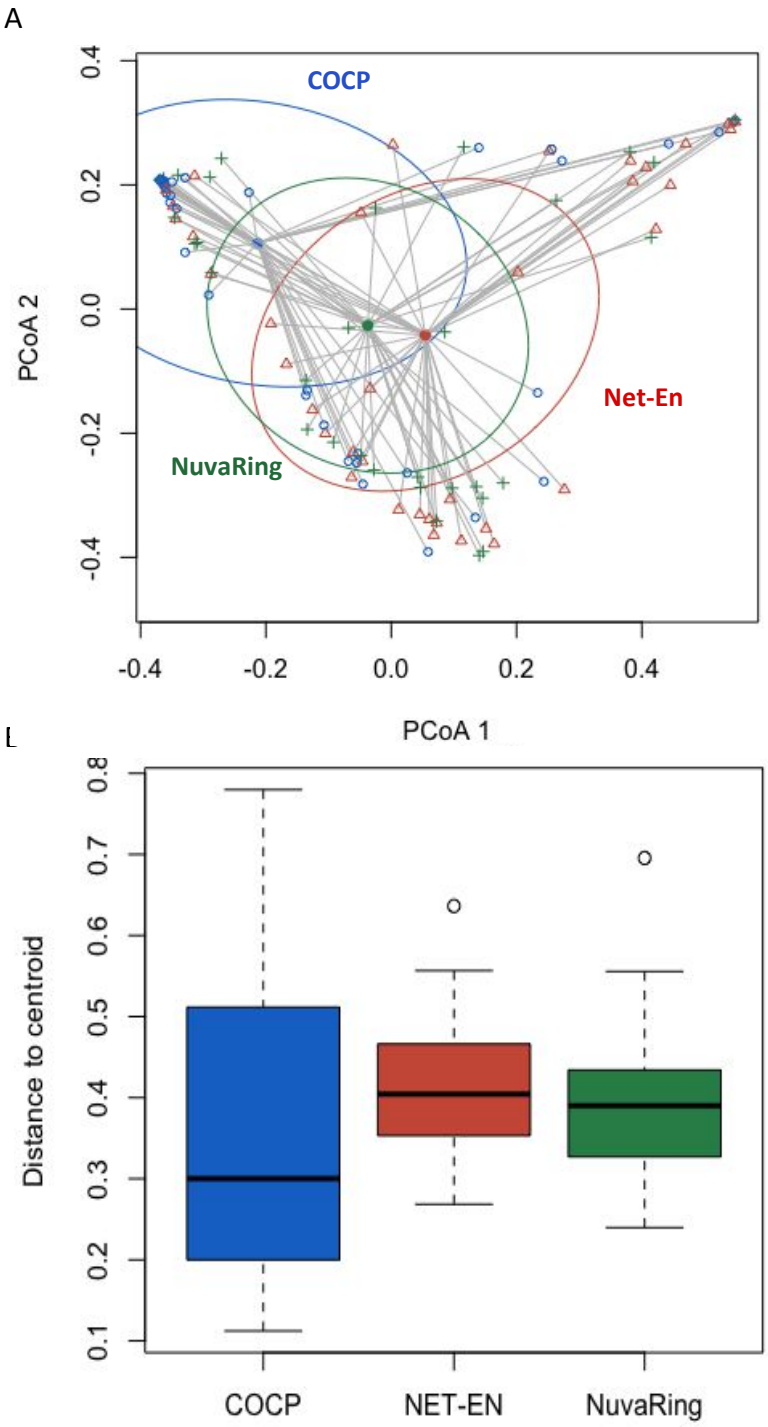


Figure 6.10. Beta diversity within hormonal contraceptive group at crossover. A) Principal Coordinates Analysis (PCoA) of beta diversity calculated using weighted UniFrac according to hormonal contraceptive group with standard deviations ellipses around each group centroid. B) Barplot depicting the distance to the centroid for each sample in each hormonal contraceptive method.

We applied Spearman's rank test to assess any correlation of alpha diversity at screening and crossover within individuals according contraceptive method. As in the intention-to-treat analysis, there was a significant positive correlation between the alpha diversity at screening and crossover for participants on COCP (r=0.42, p=0.013) and participants on NuvaRing (r=0.38, p= 0.031) but not for participants on Net-En (**Figure 5.11**).

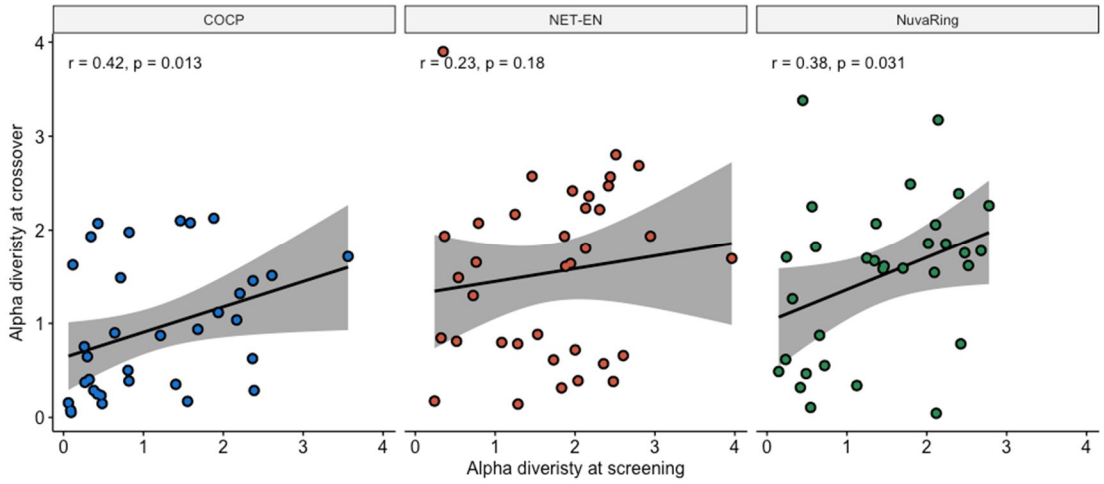


Figure 6.11. Correlation plots of alpha diversity at screening and crossover according to hormonal contraceptive method at crossover. The Spearman correlation coefficient (rho, r) and the p values for the different contraceptive methods are depicted in the figure.

5.3.4 Differentially abundant taxa between hormonal contraceptive methods

As in the intention-to-treat analysis, we looked at the mean relative abundance of the thirty most prevalent taxa according to hormonal contraceptive method. Once again, the same species previously associated with the vaginal milieu were dominating the three groups (**Table 5.16**). Combined *L. iners* and *L. crispatus* represented a mean relative abundance of 77.0% in participants using COCP with *L. iners* alone accounting for more than half (52.9%). In participants on Net-En, *L. crispatus* and *L. iners* together had a mean relative abundance of 62.3% with *L. crispatus* being more prevalent (34.05 versus 28.3%) while *L. iners* was more prevalent among participants on NuvaRing (38.5%) with *L. crispatus* and *L. iners* accounting for 62.2% overall.

Table 6.16. Top 30 most abundant taxa according to hormonal contraceptive method at crossover.

COCP			Net-En			NuvaRing		
Genus	**Species**	**mRA**	**Genus**	**Species**	**mRA**	**Genus**	**Species**	**mRA**
Lactobacillus	*iners*	52.9%	*Lactobacillus*	*crispatus_acidophilus*	34.0%	*Lactobacillus*	*iners*	38.5%
Lactobacillus	*crispatus_acidophilus*	24.1%	*Lactobacillus*	*iners*	28.3%	*Lactobacillus*	*crispatus_acidophilus*	23.7%
Shuttleworthia	BVAB1	13.7%	*Gardnerella*	*vaginalis*	13.1%	*Gardnerella*	*vaginalis*	16.5%
Gardnerella	*vaginalis*	11.3%	*Shuttleworthia*	BVAB1	11.7%	*Megasphaera*		8.4%
Megasphaera		5.8%	*Megasphaera*		5.4%	*Leptotrichiaceae**		8.0%
*Leptotrichiaceae**		2.3%	*Prevotella*	*amnii*	5.2%	*Prevotella*	*amnii*	5.8%
Atopobium	*vaginae*	2.3%	*Leptotrichiaceae**		5.1%	*Shuttleworthia*	*BVAB1*	5.3%
Prevotella	*amnii*	2.4%	*Prevotella*	*timonensis*	3.2%	*Atopobium*	*vaginae*	3.2%
Prevotella	*timonensis*	1.9%	*Prevotella*		2.9%	*Prevotella*	*timonensis*	2.1%
Prevotella		1.3%	*Sneathia*		2.4%	*Lactobacillus*	*jensenii*	2.0%
Sneathia		1.2%	*Leptotrichia*		3.2%	*Sneathia*		1.9%
Prevotella	*melaninogenica*	1.0%	*Aerococcus*	*christensenii*	1.8%	*Prevotella*		1.7%
Lactobacillus	*johnsonii_gasseri_taiwanensis*	0.8%	*Atopobium*	*vaginae*	1.3%	*Prevotella*	*bivia*	1.6%
Veillonella	*montpellieriensis*	1.1%	*Lactobacillus*	*jensenii*	1.5%	*Anaerococcus*	*prevotii_tetradius*	1.4%
Aerococcus	*christensenii*	0.7%	*Prevotella*	*bivia*	1.0%	*Staphylococcus*		1.3%
Streptococcus	*infantis_mitis_oralis*	0.6%	*Streptococcus*	*infantis_mitis_oralis*	1.2%	*Gemella*	*asaccharolytica*	0.9%
Lactobacillus	*jensenii*	0.6%	*Anaerococcus*	*prevotii_tetradius*	0.8%	*Prevotella*	*disiens*	0.8%
Prevotella	*bivia*	0.5%	*Clostridium*	BVAB2	0.8%	*Gardnerella*		1.0%
Streptococcus	*agalactiae*	0.5%	WAL_1855D		0.6%	*Finegoldia*	*magna*	0.6%
Gardnerella		0.4%	*Dialister*	*succinatiphilus_propionicifaciens*	0.5%	*Aerococcus*	*christensenii*	0.5%
Clostridium	*BVAB2*	0.4%	*Fusobacterium*	*nucleatum*	0.6%	*Streptococcus*	*infantis_mitis_oralis*	0.6%
Dialister	*succinatiphilus_propionicifaciens*	0.2%	*Veillonella*	*montpellieriensis*	0.5%	*Mycoplasma*	*hominis*	0.5%
Parvimonas	*micra*	0.2%	*Porphyromonas*	*eunonis*	0.4%	*Parvimonas*	*micra*	0.4%
Dialister	*micraerophilus*	0.2%	*Parvimonas*	*micra*	0.3%	*Clostridium*	BVAB2	0.4%
Anaerococcus	*prevotii_tetradius*	0.2%	*Finegoldia*	*magna*	0.3%	*Porphyromonas*	*eunonis*	0.4%
Finegoldia	*magna*	0.1%	*Mycoplasma*	*hominis*	0.5%	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*	0.4%
Gemella	*morbillorum_cluster*	0.2%	*Peptoniphilus*	*harei_asaccharolyticus_grossensis*	0.3%	*Corynebacterium*	*tuberculostearicum*	0.4%
Gemella	*asaccharolytica*	0.2%	*Prevotella*	*corporis*	0.4%	*Peptoniphilus*	*harei_asaccharolyticus_grossensis*	0.3%
Peptoniphilus	*harei_asaccharolyticus_grossensis*	0.1%	*Peptostreptococcus*	*anaerobius*	0.3%	WAL_1855D		0.3%
Prevotella	*disiens*	0.1%	*Dialister*	*micraerophilus*	0.2%	*Dialister*	*succinatiphilus_propionicifaciens*	0.3%

OTUs merged at lowest taxonomic level. mRA: mean relative abundance. *Note: this taxa only annotated down to family level

MetagenomeSeq analysis was once again applied to identify differentially abundant bacterial taxa according to contraceptive method at the crossover visit. The relative abundances of fourteen taxa were significantly different between participants on COCP (n=35) and Net-En (n=37) (**Figure 5.12A** and **Table 5.17**). These included the same taxa identified in the intention-to-treat analysis (i.e. *Gemella morbillorum_cluster*, *Staphylococcus*, *Streptococcus salivarius_thermophilus*) as more abundant in participants on COCP in addition to *Eikenella corrodens*, *Neisseria subflava*, *Jeotgalicoccus* and *Prevotella melaninogenica*.

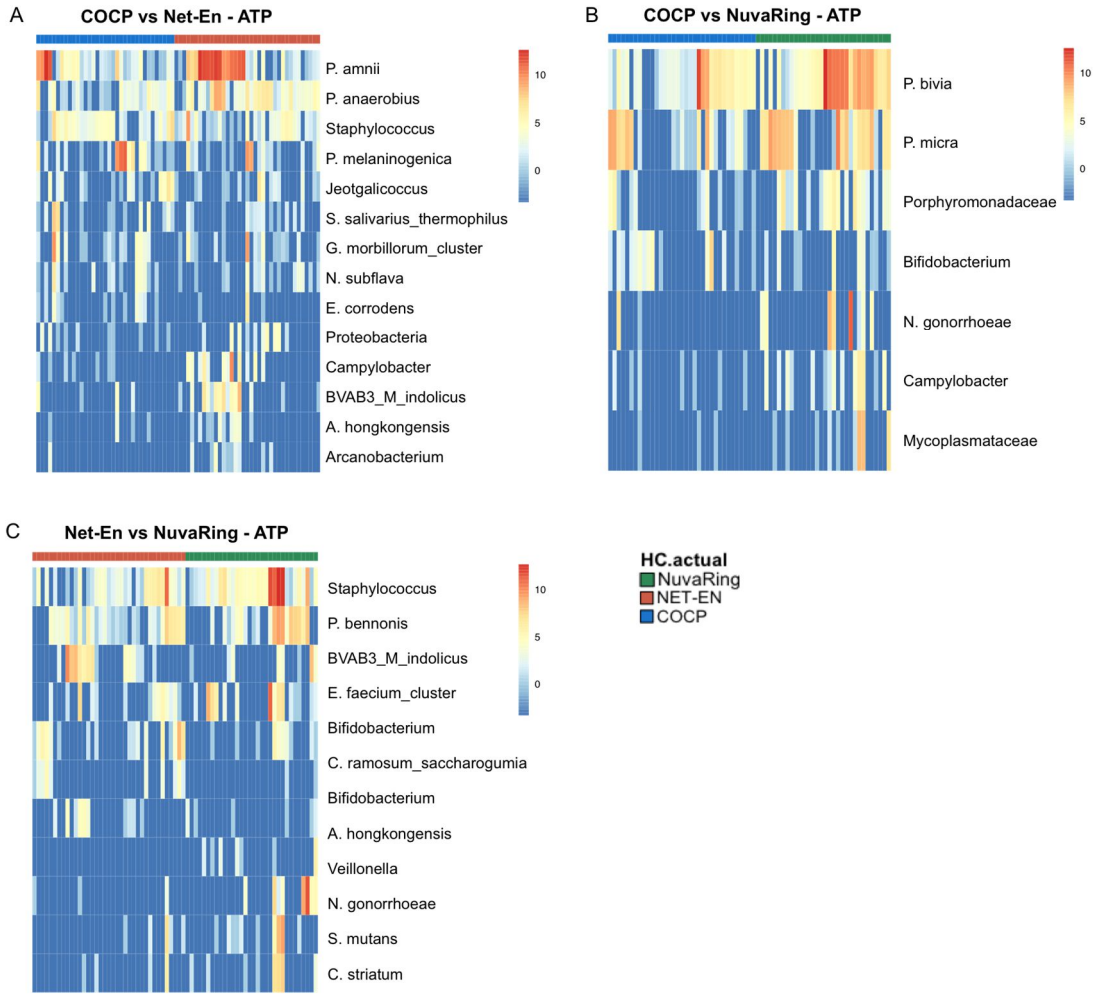


Figure 6.12. Differentially abundance taxa by hormonal contraceptive group at crossover. Bacterial taxa significantly differentially abundant and/or frequent by hormonal contraception category (A: COCP versus Net-En; B: COCP versus NuvaRing; C: Net-En versus NuvaRing) in the vaginal samples from crossover visits analysed using metagenomeSeq (FDR ≤ 0.05, fold-change ≥ 1.25, taxa present in ≥ 20% of samples in at least one of the two groups being compared). The heatmap shows unsupervised clustering of samples (columns) by Bray-Curtis distances split by contraceptive method. Log2-transformed standardized read counts are illustrated by the colour key.

Table 6.17. Differentially abundant taxa between participants on different hormonal contraceptives at crossover.

	Coeff	P adj.	Family	Genus	Species
COCP vs. Net-En	-2.388	0.0196	Prevotellaceae	*Prevotella*	*melaninogenica*
	-1.947	0.0026	Gemellaceae	*Gemella*	*morbillorum_cluster*
	-1.688	0.0058	Staphylococcaceae	*Jeotgalicoccus*	NA
	-1.662	0.0048	Staphylococcaceae	*Staphylococcus*	NA
	-1.569	0.0143	Streptococcaceae	*Streptococcus*	*salivarius_thermophilus*
	-1.567	0.0003	Neisseriaceae	*Eikenella*	*corrodens*
	-1.301	0.0062	Neisseriaceae	*Neisseria*	*subflava*
	1.317	0.0199	Proteobacteria*	*NA*	NA
	1.375	0.0337	Peptostreptococcaceae	*Peptostreptococcus*	*anaerobius*
	1.420	0.0038	Actinomycetaceae	*Arcanobacterium*	NA
	1.500	9.96e-05	Actinomycetaceae	*Actinomyces*	*hongkongensis*
	2.571	0.0019	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
	2.728	0.0399	Prevotellaceae	*Prevotella*	*amnii*
	2.968	9.96e-05	Campylobacteraceae	*Campylobacter*	NA
COCP vs. NuvaRing	-1.570	0.0020	Bifidobacteriaceae	*Bifidobacterium*	NA
	1.255	0.0029	Campylobacteraceae	*Campylobacter*	NA
	1.292	0.0270	Mycoplasmataceae	NA	NA
	1.615	0.0045	Porphyromonadaceae	NA	NA
	1.941	0.0351	Prevotellaceae	*Prevotella*	*bivia*
	2.184	0.0420	[Tissierellaceae]	*Parvimonas*	*micra*
	2.440	0.0301	Neisseriaceae	*Neisseria*	*gonorrhoeae*
Net-En vs. NuvaRing	-1.850	0.0361	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
	-1.765	9.99e-05	Actinomycetaceae	*Actinomyces*	*hongkongensis*
	-1.590	0.0005	Erysipelotrichaceae	*Clostridium*	*ramosum_saccharogumia*
	-1.369	0.0131	Bifidobacteriaceae	*Bifidobacterium*	NA
	1.381	0.0076	Porphyromonadaceae	*Porphyromonas*	*bennonis*
	1.505	0.0006	Corynebacteriaceae	*Corynebacterium*	*striatum*
	1.541	0.0002	Streptococcaceae	*Streptococcus*	*mutans*
	1.601	0.0448	Enterococcaceae	*Enterococcus*	*faecium_cluster*
	1.705	0.0131	Staphylococcaceae	*Staphylococcus*	NA
	2.281	2.88e-06	Veillonellaceae	*Veillonella*	NA
	3.198	0.0003	Neisseriaceae	*Neisseria*	*gonorrhoeae*

*Note: *Proteobacteria* only annotated at phylum level.

As in the intention-to-treat analysis, *Arcanobacterium*, *BVAB3_M_indolicus*, *Actinomyces hongkongensis* and *Campylobacter* were found to be more abundant in participants on Net-En in addition to *Peptostreptococcus anaerobius* and *Prevotella amnii*. When comparing the bacterial composition of the microbiota of participants on COCP (n=35) to participants on NuvaRing (n=32) at crossover, seven taxa were significantly differentially abundant between the two groups (**Figure 5.12B** and **Table 5.17**). *Bifidobacterium* was more abundant in participants on COCP and *Campylobacter*, *Mycoplasmataceae*, *Porphyromonadaceae*, *Prevotella bivia*, *Parvimonas micra* and *Neisseria gonorrhoea* were more abundant in the NuvaRing group. Eleven taxa were significantly differentially abundant between the vaginal microbiota of participants on Net-En (n=37) and the NuvaRing (n=32) at crossover (**Figure 5.12C** and **Table 5.17**). These included *BVAB3_M_indolicus*, *Actinomyces hongkongensis*, *Clostridium ramosum_saccharogumia,* and *Bifidobacterium* which were more abundant in participants on Net-En and *Porphyromonas bennonis, Enterococcus faecium_cluster*, *Corynebacterium striatum*, *Staphylococcus*, *Streptococcus mutans*, *Veillonella* and *N. gonorrhoea* which were more abundant in participants on NuvaRing (**Figure 5.12B** and **Table 5.17**). Once again the increased relative abundance of *N. gonorrhoea* in the NuvaRing group is in concordance with the STI results (**Table 5.13**).

To identify species predictive of hormonal contraceptive method at crossover, Random forest analysis was also applied. The top ten most influential taxa in differentiating participants on COCP from participants on Net-En were *L. iners*, *Prevotella* spp. (including *P. amnii*), *Staphylococc*us, *Dialister succinatiphilus_propionicifaciens*, *BVAB3_M_indolicus*, *Sutterella*, *Peptostreptococcus anaerobius*, *Propionibacterium eunonis* and *Leptotrichiaceae* (AUC=0.77, sensitivity=0.64 and specificity=0.70 for the training set and a validation predicted error rate of 34.72%) (**Figure 5.13A** and **Table 5.18**). Although still relatively high, the error rate for the COCP versus Net-En was markedly improved compared to the intention-to-treat analysis. The top ten most predictive taxa in differentiating vaginal microbiota from a participant on COCP from a participant on the NuvaRing were *L. crispatus*, *Prevotella spp. (including P. disiens*, *P. bivia* and P. *amnii*), *Porphyromonadaceae*,

Peptostreptococcus anaerobius, *Dialister succinatiphilus_propionicifaciens*, *L. iners*, *Peptoniphilus coxii*, *Dialister micraerophilus*, *Peptoniphilus harei_asaccharolyticus_grossensis* and *Clostridiales* (AUC=0.68, sensitivity=0.64 and specificity=0.57 for the training set and a validation predicted error rate of 36.36% using the top five predictive taxa) (**Figure 5.13B** and **Table 5.19**). The top ten most influential taxa in differentiating Net-En from NuvaRing participants were *Bacillus coagulans*, *Prevotella timonensis*, *Peptoniphilus*, *Dialister micraerophilus, N. gonorrhoea*, *Streptococcus infantis_mitis_oralis*, WAL_1855D, *Gemella asaccharolytica*, *Atopobium vaginae* and *Dialister invisus* (AUC=0.81, sensitivity=0.85 and specificity=0.68 for the training set and a validation predicted error rate of 56.52% using the top five predictive taxa) (**Figure 5.13C** and **Table 5.20**). The error rate for the Net-En and NuvaRing comparison is very high suggesting that bacterial taxa are relatively weak predictors of Net-En versus NuvaRing use.

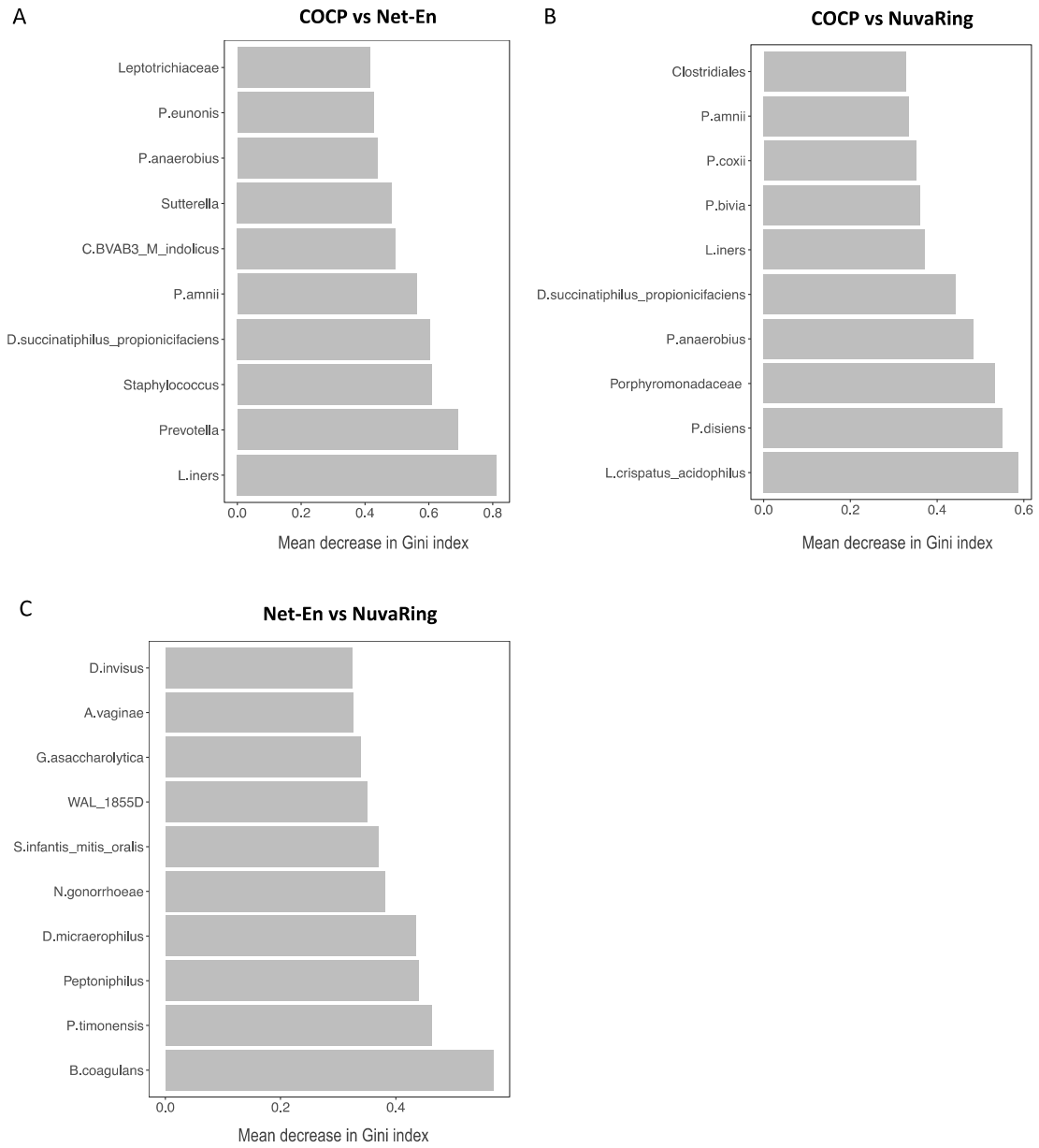


Figure 6.13. Random forest analysis of hormonal contraceptive method at crossover. The top 10 most influential taxa by random forest analysis in predicting contraceptive method (A: COCP versus Net-En, B: COCP versus NuvaRing, C: Net-En versus NuvaRing) at crossover. The x-axis indicates the mean decrease in Gini Index (length of bar represents predictive ability of each taxon).

Table 6.18. Random forest analysis of COCP versus NuvaRing at crossover.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_1	3.668	0.812	*Lactobacillus iners*
OTU_9	4.993	0.690	*Prevotella*
OTU_32	3.553	0.609	*Staphylococcus*
OTU_14	2.858	0.605	*Dialister succinatiphilus_propionicifaciens*
OTU_7	4.254	0.561	*Prevotella amnii*
OTU_51	3.972	0.495	*BVAB3_M_indolicus*
OTU_101	2.324	0.485	*Sutterella*
OTU_41	3.015	0.439	*Peptostreptococcus anaerobius*
OTU_40	1.774	0.428	*Porphyromonas eunonis*
OTU_6	2.962	0.415	*Leptotrichiaceae*

Training set size: 48 samples with 25 and 23 samples per class. Test set size: 24 samples with 10 and 14 samples per class. Validation predicted error: 54.17% using top five predictive taxa.

Table 6.19. Random forest analysis of COCP versus NuvaRing at crossover.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_2	1.462	0.588	*Lactobacillus crispatus_acidophilus*
OTU_25	2.757	0.551	*Prevotella disiens*
OTU_66	3.420	0.533	*Porphyromonadaceae*
OTU_41	-0.055	0.485	*Peptostreptococcus anaerobius*
OTU_14	-0.126	0.443	*Dialister succinatiphilus_propionicifaciens*
OTU_1	0.651	0.372	*Lactobacillus iners*
OTU_11	2.202	0.360	*Prevotella bivia*
OTU_65	2.440	0.351	*Prevotella coxii*
OTU_7	1.727	0.335	*Prevotella amnii*
OTU_110	-0.844	0.328	*Clostridiales*

Training set size: 45 samples with 22 and 23 samples per class. Test set size: 22 samples with 13 and 9 samples per class. Validation predicted error: 36.36% using top five predictive taxa.

Table 6.20. Random forest analysis of Net-En versus NuvaRing at crossover.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_52	3.532	0.569	*Bacillus coagulans*
OTU_10	0.587	0.463	*Prevotella timonensis*
OTU_56	3.843	0.440	*Peptoniphilus*
OTU_39	1.767	0.434	*Dialister micraerophilus*
OTU_1266	4.172	0.380	*Neisseria gonorrhoea*
OTU_20	3.483	0.369	*Streptococcus infantis_mitis_oralis*
OTU_29	1.269	0.351	WAL_1855D
OTU_37	0.867	0.339	*Gemella asaccharolytica*
OTU_8	-0.753	0.326	*Atopobium vaginae*
OTU_176	2.088	0.325	*Dialister invisus*

Training set size: 46 samples with 27 and 19 samples per class. Test set size: 23 samples with 10 and 13 samples per class. Validation predicted error: 56.52% using top five predictive taxa.

Using DeSeq2, ten taxa were found to be differentially abundant between the vaginal microbiota of participants on COCP and Net-En at crossover in an according to protocol analysis (**Figure 5.14A** and **Table 5.21**). The following taxa were more abundant in participants on COCP; *L. iners*, *Prevotella melaninogenica*, *Streptococcus agalactiae*, *Haemophilus parainfluenzae* and *Streptococcus infantis_mitis_oralis* while *BVAB3_M_indolicus*, *Mobiluncus mulieris*, *Campylobacter, Peptostreptococcus anaerobius* and *Sneathia* were more abundant in participants on Net-En. Six taxa were differentially abundant between participants on COCP and NuvaRing at crossover (**Figure 5.14B** and **Table 5.21**) of which two, *Enterobacteriaceae* and *Porphyromonadaceae*, were only annotated down to family level. The four remaining taxa were *L. jensenii*, *Prevotella bivia*, *A. prevotii_tetradius* and *F. equinum*, which were all more abundant in participants on NuvaRing. Only *Staphylococcus* (more abundant in NuvaRing participants) and *BVAB3_M_indolicus* (more abundant in Net-En participants) were found to be differentially abundant between the vaginal microbiota of participants on Net-En and NuvaRing at crossover (**Figure 5.14C** and **Table 5.21**).

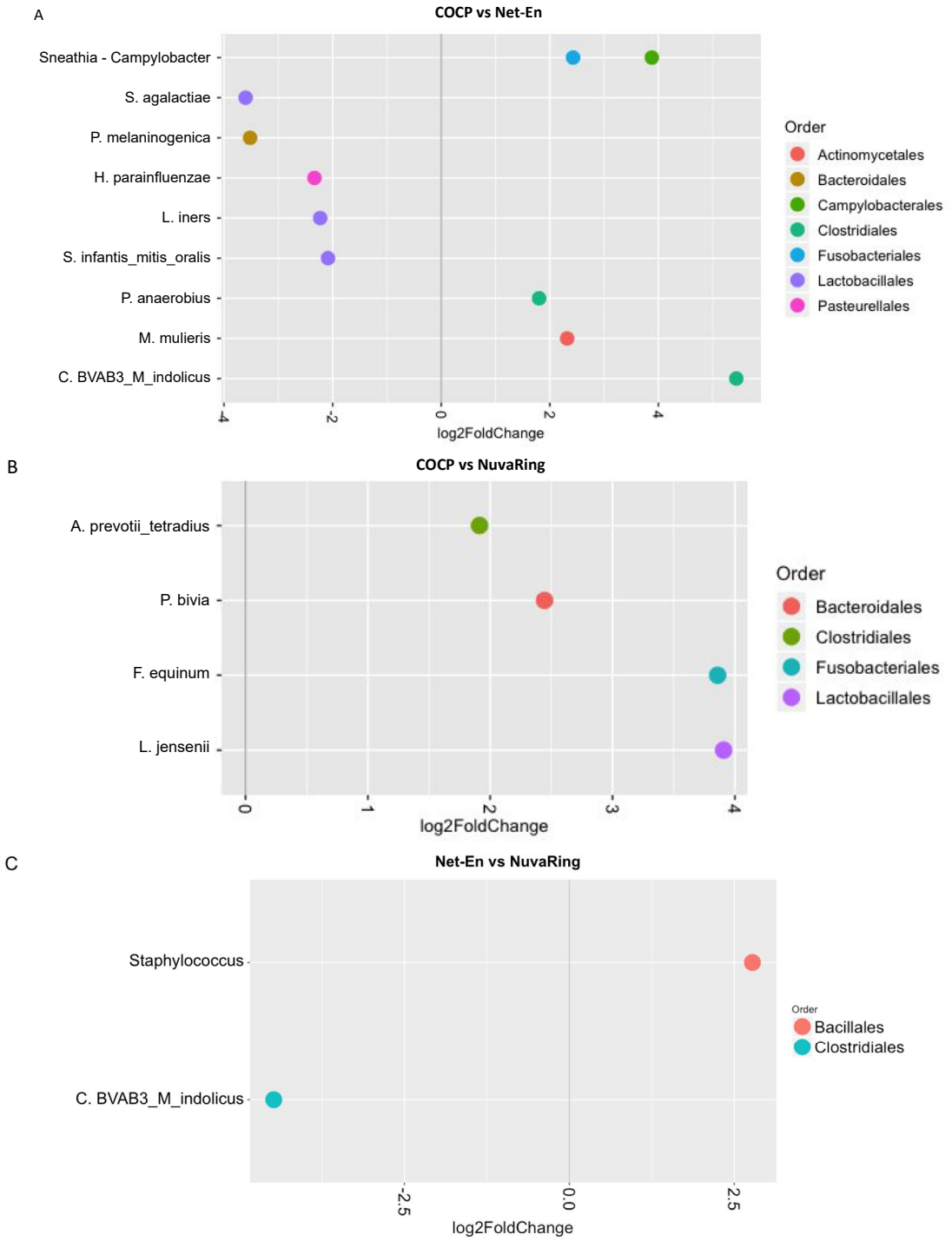


Figure 6.14. DESeq2 analysis of hormonal contraceptive methods at crossover. Bacterial taxa significantly differentially abundant and/or frequent by hormonal contraception category at crossover using DESeq2 (with an alpha of 0.05). A) Combined oral contraceptives (COCP) versus Net-En injection. B) COCP versus NuvaRing. C) Net-En injection versus NuvaRing. Taxa depicted at species level. Taxa without species level annotation are grouped in the first line (if >1) and order of genera corresponds to order of appearance of dots (from left to right). Figure only includes taxa annotated at the genus level.

Table 6.21. Differentially abundant taxa between hormonal contraceptive methods at crossover by DESeq2.

	Log2FC	P adj.	Family	Genus	Species
COCP vs. Net-En	5.434	0.003	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
	-2.229	0.004	Lactobacillaceae	*Lactobacillus*	*iners*
	-3.520	0.004	Prevotellaceae	*Prevotella*	*melaninogenica*
	2.319	0.006	Actinomycetaceae	*Mobiluncus*	*mulieris*
	3.878	0.020	Campylobacteraceae	*Campylobacter*	NA
	-3.602	0.020	Streptococcaceae	*Streptococcus*	*agalactiae*
	-2.334	0.020	Pasteurellaceae	*Haemophilus*	*parainfluenzae*
	-2.085	0.022	Streptococcaceae	*Streptococcus*	*infantis_mitis_oralis*
	2.427	0.023	Leptotrichiaceae	*Sneathia*	NA
	1.802	0.023	Peptostreptococcaceae	*Peptostreptococcus*	*anaerobius*
COCP vs. NuvaRing	3.908	0.0005	Lactobacillaceae	*Lactobacillus*	*jensenii*
	2.444	0.004	Prevotellaceae	*Prevotella*	*bivia*
	3.186	0.004	Porphyromonadaceae	NA	NA
	3.858	0.005	Fusobacteriaceae	*Fusobacterium*	*equinum*
	3.169	0.016	Enterobacteriaceae	NA	NA
	1.913	0.027	[Tissierellaceae]	*Anaerococcus*	*prevotii_tetradius*
Net-En vs. NuvaRing	2.770	0.006	Staphylococcaceae	*Staphylococcus*	NA
	-4.473	0.019	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*

FC, fold change.

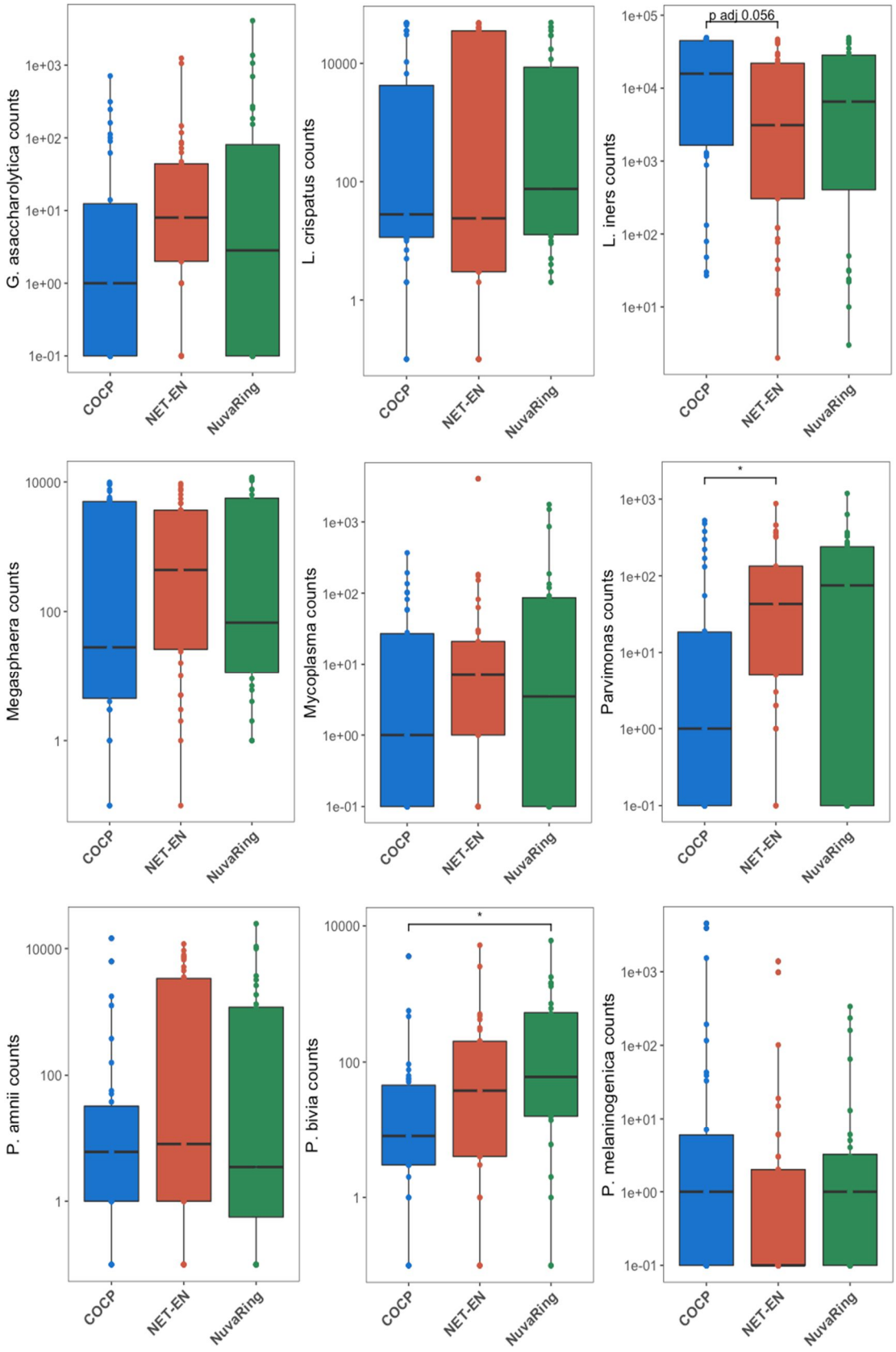
As in the intention-to-treat analysis, we looked at the standardized read counts of specific bacteria of interest for HIV acquisition in each HC group according to actual contraceptive at crossover. Similar to the intention-to-treat analyses, no significant differences in the standardised read counts of *G. asaccharolytica*, *L. crispatus*, *Megasphaera*, *Sneathia* and *Veillonella montpellieriensis*, were observed between the participants on the different HC methods ($p<0.05$) (**Figure 5.15** and **Table 5.22**). In addition, the read counts of *Mycoplasma* were no longer significantly different between the HC groups. The read counts of *L. iners* in participants using COCP were higher compared to participants on Net-En and this difference was close to significant (adj. $p=0.056$). Again the read counts of total lactobacilli were significantly higher in COCP participants compared to Net-En participants but not after adjusting for multiple comparisons ($p=0.030$) (**Figure 5.15** and **Table 5.22**). In contrast, the read counts of *Parvimonas* were significantly higher in participants using Net-En compared to participants on

COCPs (adj. p=0.005). The read counts for *P. bivia* were significantly higher in participants on NuvaRing compared to participants using COCPs (adj. p=0.023) while the read counts of *Prevotella* at the genus level were significantly higher in participants on Net-En compared to participants on COCPs (adj. p=0.013) (**Figure 5.15** and **Table 5.22**).

Table 6.22. Standardised read counts of specific bacteria of interest according to hormonal contraceptive method.

	COCP	Net-En	NuvaRing	P value
G. asaccharolytica	1 (0-13)	8 (2-44)	3 (0-88)	0.181
Lactobacillus	46,032 (4,258-48,843)	30,261 (1,460-45,891)	25,633 (1,581-469,52)	0.101
L. crispatus	28 (12-6,671)	24 (3-35,451)	77 (13-8,760)	0.103
L. iners	15,810 (1,696-44,871)	3,111 (305-21,991	6,535 (621-28,383)	0.054
Megasphaera	28 (5-4,991)	438 (26-3,668)	81 (12-5,637)	0.437
Mycoplasma	1 (0-27)	7 (1-21)	4 (0-87)	0.128
Parvimonas	1 (0-19)	43 (5-134)	75 (0-240)	**0.013**
Prevotella	223 (65-3,158)	2,059 (473 -8,791)	22,81 (159-4,657)	**0.015**
P. amnii	6 (1-33)	8 (1-3,377)	4 (1-1,190)	0.577
P. bivia	8 (3-46)	38 (4-202)	63 (16-534)	**0.021**
P. melaninogenica	1 (0-6)	0 (0-2)	1 (0-4)	0.493
Sneathia	4 (1-101)	32 (1-996)	14 (2-304)	0.274
V. montpellieriensis	1 (0-4)	1 (0-29)	1 (0-15)	0.763

P values were generated using Kruskal-Wallis test adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method.



Continued on next page,

Continued from previous page.

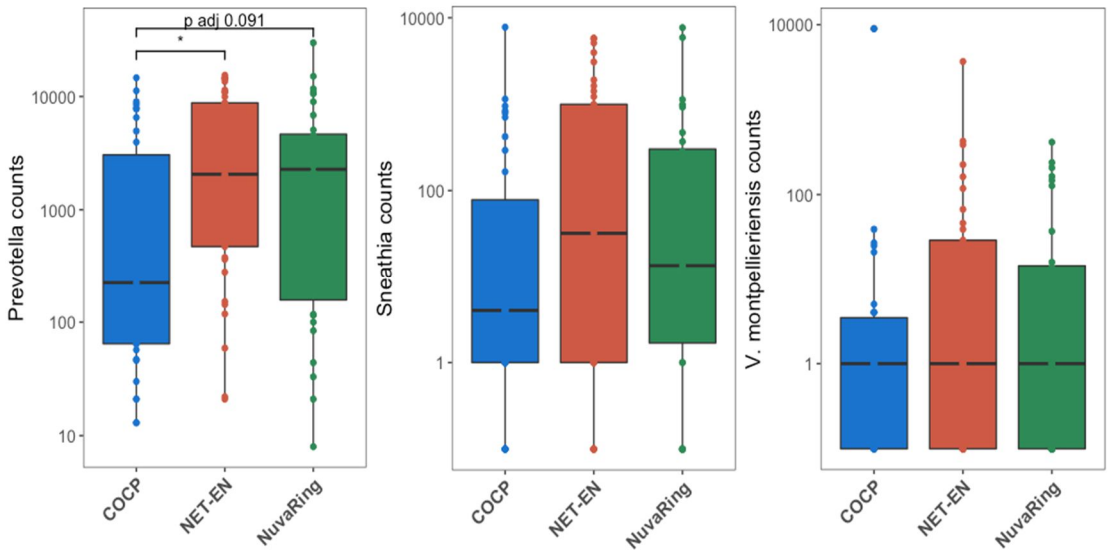


Figure 6.15. Differences in bacterial counts between hormonal contraceptive methods of bacteria of interest. Boxplot showing the standardized read counts of bacteria of interest in 104 vaginal samples from crossover visits according to hormonal contraceptive method. P values were generated using Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests and adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. Y-axis log10 transformed. *= adj. p<0.05.

5.3.5 Repeated measures cohort analysis

At crossover, all participants who were assigned to either COCP or Net-En at baseline would change their contraceptive method to NuvaRing for the final four months of the study. Participants randomized to NuvaRing at baseline were, however, given the option between the other two hormonal contraceptive options, COCPs or Net-En injection, for the remainder of the study. The majority of participants on NuvaRing at crossover chose Net-En over COCPs as their second hormonal contraceptive method (**Figure 4.1**, Chapter 4). Of the 92 participants who completed the exit visit, more than half of the participants were on the NuvaRing (n=47, 51.1%) at time of sampling as a result of the randomization and study design. Of the remaining participants, 33 participants had chosen the Net-En injection (35.9%) and only 12 had selected the COCP method (13.0%) at the crossover visit. Due to the introduction of a choice of hormonal contraceptive option amongst some of the participants we cannot include data from the exit visit as part of a randomized analysis. For inclusion of exit visit data in our analyses, we therefore applied a different analysis strategy in which the data was seen as representative of a repeated measures cohort. In the following analyses, we have combined the data from the crossover and exit

visit for all participants with available sequencing data at either or both visits. This provided a dataset consisting of samples from 193 visits taken from 104 participants. Of these samples, 47 were taken from participants on COCPs at the time of sampling, 69 from participants on Net-En and 77 from participants on the NuvaRing. Among the participants on Net-En at either crossover or exit, 55 out of 62 had received their last injection on time (88.7%, 7 participants missed their injections, missing data from 7 participants). Among the participants on NuvaRing at crossover or exit, 65 of 76 (85.5%, data missing from 1 participant) reported using the ring as instructed over the two past months. Among the participants on COCP at crossover and exit, 24 had reported taken their COCPs regularly over the past two months (54.5%), 13 reported inconsistent use (29.5%, up to ten days without study pills) and six reported less use (data missing from four participants).

Table 5.23 shows the characteristics of the cohort according to HC method. Of interest, we observed a significant difference between the three groups in terms of condom use, with participants on Net-En and NuvaRing generally reporting less condom usage compared to participants on COCP. If we look at the exit visit in isolation (Appendix VII), we observed a trend towards higher level of condom use among the participants who chose COCP and those who chose Net-En, suggesting that participants who opted for different HC methods may show slightly different behaviours. The most prevalent community cluster assigned to the vaginal microbiota of participants on COCP at time of sampling was the *L. iners* dominant C3 community type with just over 40 percent assigned to this community type while the remaining sixty percent were evenly distributed between the diverse C1 (31.9%) and the *L. crispatus* dominant (27.7%) community types. In contrast, the vaginal microbiota of participants on Net-En at the time of sampling was most commonly assigned to the diverse C1 community type (43.9%) with the remaining evenly distributed between the two lactobacilli dominant community types (C2, 27.3%; C3, 28.8%). Almost half of the vaginal communities from participants on NuvaRing at the time of sampling were assigned to the diverse C1 community type (46.7%) while just over a third were assigned to the *L. iners* dominant C3 community type (34.7%) and less than a fifth to the *L. crispatus* dominant C2 community type (18.7%). These

differences observed between the HC methods did however not reach statistical significance.

Table 6.23. Characteristics of participants at crossover and exit according to hormonal contraceptive method.

	COCP (n=47)	**Net-En** (n=69)	**NuvaRing** (n=77)	**P value**
Fuzzy cluster distribution				0.387
C1	15 (31.9%)	29 (43.9%)	35 (46.7%)	
C2	13 (27.7%)	18 (27.3%)	14 (18.7%)	
C3	19 (40.4%)	19 (28.8%)	26 (34.7%)	
Vaginal pH, mean (sd)	4.54 (4.06-5.02)	4.77 (4.17-5.37)	4.87 (4.30-5.43)	**0.008**
Shannon Index, median (IQR)	0.75 (0.30-1.60)	1.57 (0.78-2.07)	1.59 (0.62-2.06)	**0.004**
HSV-2 serology	16 (34.0)	23 (33.3%)	30 (39.0%)	0.748
Yeast cells present	4 (8.5%)	12 (17.4%)	20 (26.0%)	**0.050**
BV prevalence				0.478
BV positive	16 (34.0%)	32 (46.4%)	33 (42.9%)	
BV intermediate	3 (6.4%)	1 (1.5%)	2 (2.6%)	
BV negative	28 (59.6%)	36 (52.2%)	42 (54.5%)	
STI prevalence				
Any STI(s)	10 (21.3%)	17 (24.6%)	29 (37.7%)	0.091
Ct	7 (14.9%)	10 (14.5%)	16 (20.8%)	0.541
Ng	3 (6.4%)	3 (4.4%)	10 (13.0%)	0.158
Tv	1 (2.1%)	3 (4.4%)	2 (2.6%)	0.767
Mg	1 (2.1%)	3 (4.4%)	2 (2.6%)	0.767
Sexual risk behaviour since last visit[2]				
Sexual partners, median (IQR)	1 (1-1)	1 (1-1)	1 (1-1)	0.166
Multiple sexual partners, n	1 (2.2%)	2 (3.1%)	2 (2.7%)	0.931
New partner, n	2 (4.4%)	2 (3.1%)	1 (1.4%)	0.614
Condom use since last visit				**0.037**
Never	6 (13.3%)	22 (34.4%)	21 (28.4%)	
Less than half the time	7 (15.6%)	4 (6.3%)	14 (18.9%)	
Half the time	18 (40.0%)	22 (34.4%)	13 (17.6%)	
More than half the time	5 (11.1%)	7 (10.9%)	8 (10.8%)	
Always	9 (20.0%)	9 (14.1%)	18 (24.3%)	
Condom use during last PV intercourse				
Yes	30 (66.7%)	37 (57.8%)	47 (63.5%)	0.603
PV sex acts per week, median (IQR)	2 (1-2)	2 (1-2)	1 (1-2)	0.691
Intergenerational sex with older partner (≥5 years)				
No	2 (4.4%)	2 (3.1%)	3 (4.1%)	1.000
Unsure	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Yes	43 (95.6%)	62 (96.9%)	71 (95.9%)	
Transactional sex	1 (2.2%)	0 (0.0%)	0 (0.0%)	0.246
Penile-anal intercourse	0 (0.0%)	0 (0.0%)	0 (0.0%)	NA

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups, Kruskal-Wallis test for comparison of medians and ANOVA test for comparison of means. BV; bacterial vaginosis; Ct, Chlamydia trachomatis*; HSV-2, herpes simplex virus type 2; IQR, interquartile range; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. Five samples without any cluster assignment (COCP: n=0; Net-En: n=3; NuvaRing: n=2).

2. Missing data from nine participants (COCP: n=2; Net-En: n=5; NuvaRing: n=4).

The vaginal microbiota of participants using COCPs at either crossover or exit visits had a significantly lower alpha diversity at the time of sampling compared to the microbiota of participants who were on Net-En or NuvaRing (adj. p=0.002 and 0.004, respectively) while no difference in diversity was observed between NuvaRing and Net-En users (adj. p=0.498) (**Figure 5.16** and **Table 5.24**).

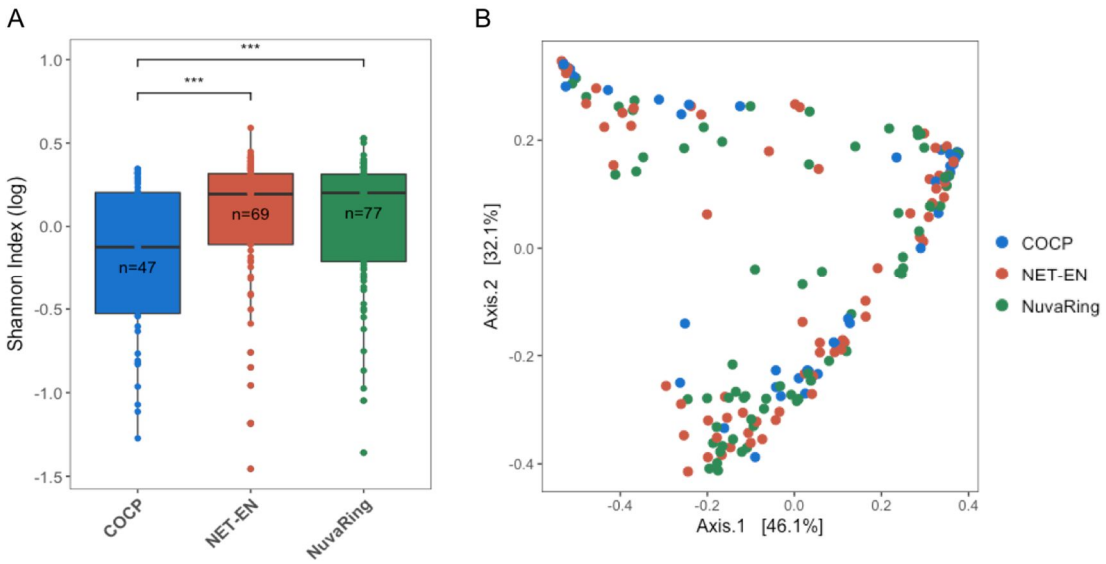


Figure 6.16. Alpha and beta diversity according to hormonal contraceptive method at crossover and exit. A) Boxplot depicting the alpha diversity measured using Shannon Index (log transformed values) of the microbiota from 193 vaginal samples from 104 participants at crossover and exit visits according to the hormonal contraceptive used at time of sampling (COCP: n=47; Net-En: n=69; NuvaRing: n=77). P values were calculated using Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests and adjusted for multiple comparisons using Benjamini-Hochberg (BH) method. Significance codes: *: adj. p<0.05, **: adj. p<0.01, ***: adj. p<0.001. B) Principal Coordinate Analysis (PCoA) of 193 samples from 104 participants at crossover and exit visits using weighted UniFrac distances. Samples are coloured by the hormonal contraceptive method that the participant was on at the time of sampling.

Using a linear regression model, the differences in alpha diversity between participants on COCP and the two other contraceptive methods stayed significantly different when adjusting for baseline alpha diversity (ANCOVA, adj. p=0.032 and p=0.008) (**Table 5.25**). Due to differences in sexual risk behaviour observed between HC groups (**Table 5.23**), we also adjusted for condom use since last visit in the linear regression model. Alpha diversity remained significantly different between the hormonal contraceptive methods, with the alpha diversity of the vaginal microbiota of participants on COCPs being lower than that of participants on either Net-En (adj. p=0.031) or NuvaRing (adj. p=0.011). Using a linear mixed-effects (lme) model including visit and HC use as fixed effects and participant ID as random effect, the alpha diversity of

participants on COCP was lower than in participants on Net-En (p=0.003) and NuvaRing (p=0.00789). The vaginal pH was also lower in participants on COCP at time of sampling compared to participants on Net-En or NuvaRing (mean pH: 4.54 vs. 4.77 and 4.87) and this difference was significant between COCP and NuvaRing (p=0.005) (**Table 5.23**). Using lme modelling, this was also significant between COCP and Net-En (p=0.028). There were no significant changes in the alpha diversity of the vaginal microbiota of participants within each HC group before and after HC use although a slight decrease in diversity in the COC (median SI (IQR): 0.81 (0.39-1.76) versus 0.75 (0.30-1.60)) and Net-En (median SI (IQR): 1.70 (0.77-2.13) versus 1.57 (0.78-2.07)) users and a slight increase in NuvaRing users (median SI (IQR): 1.33 (0.50-2.10) versus 1.59 (0.62-2.06)) were observed (p>0.05, Wilcoxon Signed Rank test).

Table 6.24. Alpha diversity according to hormonal contraceptive method at crossover and exit visits.

	Shannon Index	
	P	P adj.
All hormonal contraceptive methods	**0.004**	**0.004**
COCP vs. Net-En	**0.002**	**0.002**
COCP vs. NuvaRing	**0.001**	**0.004**
Net-En vs. NuvaRing	0.498	0.498

P values generated using Kruskal-Wallis test with Dunn's post hoc test using the Benjamini-Hochberg (BH) method for multiple comparisons testing (MTC).

Table 6.25. Linear regression analyses of hormonal contraceptive method at crossover and exit visits adjusting for baseline alpha diversity.

	Shannon Index	
	P	P adj.
All hormonal contraceptive methods	**0.003**	**0.008**
COCP vs. Net-En	**0.008**	**0.032**
COCP vs. NuvaRing	**0.006**	**0.008**
Net-En vs. NuvaRing	0.998	0.887

P values generated using analysis of covariance (ANCOVA) with Tukey's post hoc test.

No differences in the prevalence of bacterial STIs, BV or HSV-2 serology were observed between the groups. Of note, there was a significantly higher prevalence of yeast in participants on NuvaRing (26.0%) compared to participants on both Net-En (17.4%) and COCP (8.51%) at the time of sampling

(p=0.050). Throughout the study fifteen partipants reported symptoms of vaginal infections including vaginal discharge or ichting. Yet, none of these symptoms were reported at the time of mucosal sampling.

No overall differences in microbiota composition (beta diversity) were found between the participants on the three different contraceptive methods (p=0.111, R2=0.018) or in the dispersion of the bacterial community within groups (p=0.662, F=0.413) (**Figure 5.17**). Since menstruation can have an effect on the vaginal microbiota (Eschenbach et al., 2000b; Gajer et al., 2012; Hickey et al., 2013; Srinivasan et al., 2010; Santiago et al., 2012), we looked at the dates of the latest menstrual period as reported by the participants. The time past since last menstrual period was very similar for participants on COCPs (median 20 days, IQR: 10-68 days) and NuvaRing (median 21 days, IQR: 9-70 days, p=0.947) while slightly longer for Net-En (median 37 days. IQR 11-154 days), albeit not statistically significant (COCP vs. Net-En, p=0.947; NuvaRing vs. Net-En, p=0.244).

5.3.6 Differentially abundant taxa between contraceptive methods

As in the intention-to-treat and according to protocol analyses at crossover, we looked at the mean relative abundance of the thirty most prevalent taxa according to hormonal contraceptive method at crossover and exit. Once again, the same species previously associated with the vaginal milieu were dominating the three groups (e.g. *Lactobacillus* spp., *Gardnerella vaginalis, Megasphaera*, BVAB1, *Prevotella* spp., *Atopobium vaginae* and *Sneathia*) (**Table 5.26**). Combined *L. iners* and *L. crispatus* represented a mean relative abundance of 76.6% in participants using COCP with *L. iners* being the most common (mRA 47.1%). In participants on Net-En, *L. crispatus* and *L. iners* combined had a mean relative abundance of 61.3% with *L. crispatus* and *L. iners* being present at a similar level (mRA 27.7% versus 33.6%). In participants on NuvaRing with *L. crispatus* and *L. iners* together accounted for 62.2% overall with *L. iners* having a mean relative abundance almost twice as high as *L. crispatus* (40.9% versus 21.3%).

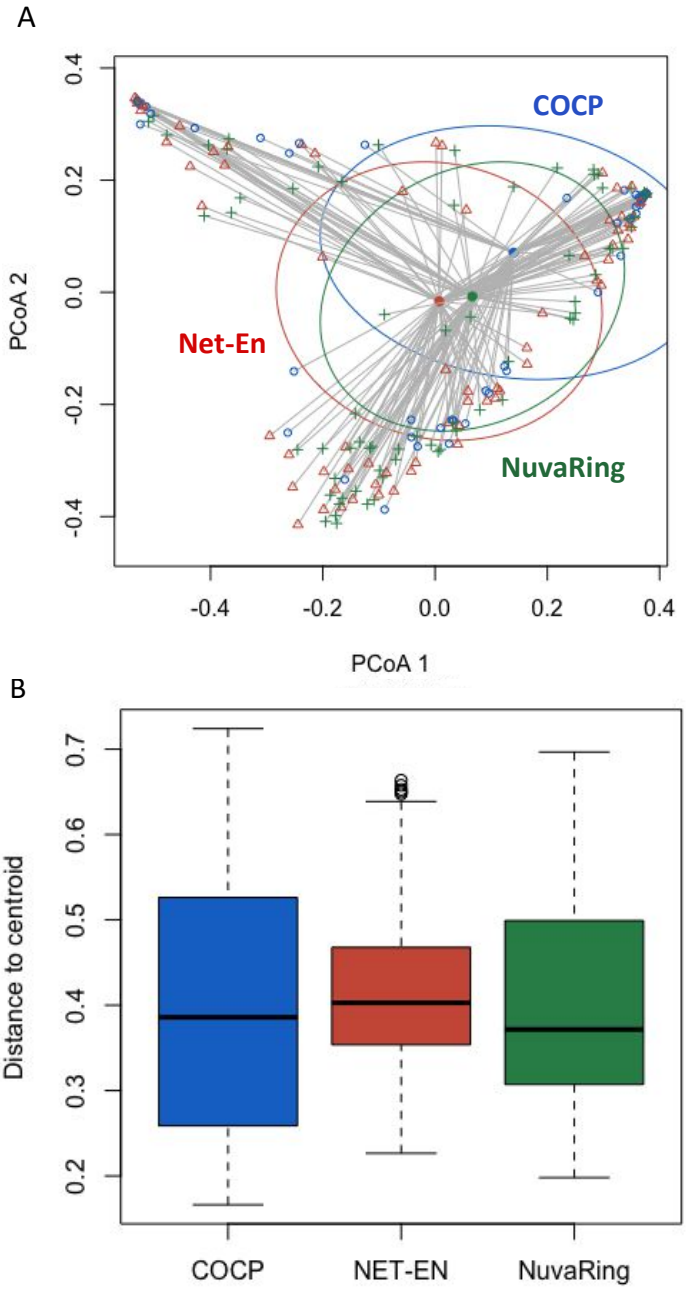


Figure 6.17. Beta diversity within hormonal contraceptive group at crossover and exit. A) Principal Coordinates Analysis (PCoA) of beta diversity calculated using weighted UniFrac according to hormonal contraceptive group with standard deviations ellipses around each group centroid. B) Barplot depicting the distance to the centroid for each sample in each hormonal contraceptive group.

Table 6.26. Top 30 most abundant taxa according to hormonal contraceptive method at crossover and exit.

COCP			Net-En			NuvaRing		
Genus	**Species**	**mRA**	**Genus**	**Species**	**mRA**	**Genus**	**Species**	**mRA**
Lactobacillus	*iners*	47.1%	*Lactobacillus*	*iners*	33.6%	*Lactobacillus*	*iners*	40.9%
Lactobacillus	*crispatus_acidophilus*	29.5%	*Lactobacillus*	*crispatus_acidophilus*	27.7%	*Lactobacillus*	*crispatus_acidophilus*	21.3%
Gardnerella	*vaginalis*	11.1%	*Gardnerella*	*vaginalis*	11.6%	*Gardnerella*	*vaginalis*	12.8%
Shuttleworthia	BVAB1	11.6%	*Shuttleworthia*	BVAB1	7.6%	*Leptotrichiaceae**		7.5%
Megasphaera		6.6%	*Prevotella*	*amnii*	6.1%	*Megasphaera*		6.1%
Prevotella	*amnii*	2.3%	*Megasphaera*		5.6%	*Shuttleworthia*	BVAB1	4.9%
Atopobium	*vaginae*	2.1%	*Leptotrichiaceae**		5.9%	*Prevotella*	*amnii*	4.5%
*Leptotrichiaceae**		1.9%	*Prevotella*	*timonensis*	2.8%	*Prevotella*	*timonensis*	2.8%
Prevotella	*timonensis*	1.6%	*Prevotella*		2.2%	*Sneathia*		2.9%
Prevotella		1.3%	*Sneathia*		2.2%	*Atopobium*	*vaginae*	2.0%
Sneathia		1.0%	*Atopobium*	*vaginae*	2.2%	*Prevotella*		1.5%
Lactobacillus	*jensenii*	1.0%	*Prevotella*	*bivia*	1.8%	*Lactobacillus*	*jensenii*	1.3%
Aerococcus	*christensenii*	0.8%	*Lactobacillus*	*jensenii*	2.0%	*Prevotella*	*bivia*	1.0%
Prevotella	*melaninogenica*	0.8%	*Aerococcus*	*christensenii*	1.4%	*Staphylococcus*		0.9%
Streptococcus	*infantis_mitis_oralis*	0.7%	*Leptotrichia*		1.6%	*Streptococcus*	*infantis_mitis_oralis*	0.9%
Veillonella	*montpellieriensis*	0.8%	*Clostridium*	BVAB2	0.7%	*Anaerococcus*	*prevotii_tetradius*	0.8%
Lactobacillus	*johnsonii_gasseri_taiwanensis*	0.6%	*Anaerococcus*	*prevotii_tetradius*	0.7%	*Clostridium*	BVAB2	0.7%
Prevotella	*bivia*	0.4%	*Streptococcus*	*infantis_mitis_oralis*	0.8%	*Aerococcus*	*christensenii*	0.5%
Dialister	*succinatiphilus_propionicifaciens*	0.3%	*Prevotella*	*melaninogenica*	0.7%	*Prevotella*	*disiens*	0.5%
Streptococcus	*agalactiae*	0.4%	*Mycoplasma*	*hominis*	0.6%	*Veillonella*	*montpellieriensis*	0.5%
Clostridium	BVAB2	0.3%	*Dialister*	*succinatiphilus_propionicifaciens*	0.4%	*Finegoldia*	*magna*	0.4%
Gardnerella		0.3%	*WAL_1855D*		0.5%	*Gemella*	*asaccharolytica*	0.5%
Parvimonas	*micra*	0.2%	*Fusobacterium*	*nucleatum*	0.4%	*Parvimonas*	*micra*	0.4%
Anaerococcus	*prevotii_tetradius*	0.2%	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*	0.4%	*Gemella*	*morbillorum_cluster*	0.6%
Dialister	*micraerophilus*	0.2%	*Parvimonas*	*micra*	0.3%	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*	0.4%
Fusobacterium	*nucleatum*	0.2%	*Veillonella*	*montpellieriensis*	0.4%	*Mycoplasma*	*hominis*	0.4%
Prevotella	*corporis*	0.2%	*Finegoldia*	*magna*	0.3%	*Fusobacterium*	*nucleatum*	0.4%
Prevotella	*disiens*	0.2%	*Peptoniphilus*	*harei_asaccharolyticus_grossensis*	0.3%	*Gardnerella*		0.4%
WAL_1855D		0.1%	*Prevotella*	*corporis*	0.3%	*Dialister*	*succinatiphilus_propionicifaciens*	0.3%
Finegoldia	*magna*	0.1%	*Peptostreptococcus*	*anaerobius*	0.3%	*Peptoniphilus*	*harei_asaccharolyticus_grossensis*	0.3%

OTUs merged at lowest taxonomic level. mRA: mean relative abundance.
*Note: this taxa only annotated down to family level

To identify differentially abundant taxa by HC method at crossover and exit, once again metagenomeSeq analysis was applied. The relative abundances of six taxa were significantly different between samples taken from participants on COCP or Net-En at the time of sampling (n=116) (**Figure 5.18A** and **Table 5.27**). These included *Jeotgalicoccus,* which was more abundant in participants on COCP and *Dialister micraerophilus*, *Fusobacterium equinum*, *Peptostreptococcus*, *Peptostreptococcus anaerobius*, *Campylobacter* and *BVAB3_M_indolicus* which were more abundant in participants on Net-En. Comparison of the microbial composition of the vaginas of participants on COCP to that of participants on the NuvaRing revealed six taxa differentially abundant between the groups at crossover and exit (n=124) (**Figure 5.18B** and **Table 5.27**). All six taxa, *Arcanobacterium*, *Peptostreptococcus anaerobius*, *Prevotella bivia*, *BVAB3_M_indolicus*, *Prevotella disiens* and *Fusobacterium equinum* had a higher relative abundance in the vaginal microbiota of participants on the NuvaRing. Only one taxa, *Actinomyces hongkongensis* (adj. p=3.87e-09, coeff -1.755), was differentially abundant between participants on the Net-En and NuvaRing methods (n=146) when combining crossover and exit visits with relative abundance being higher in participants on Net-En.

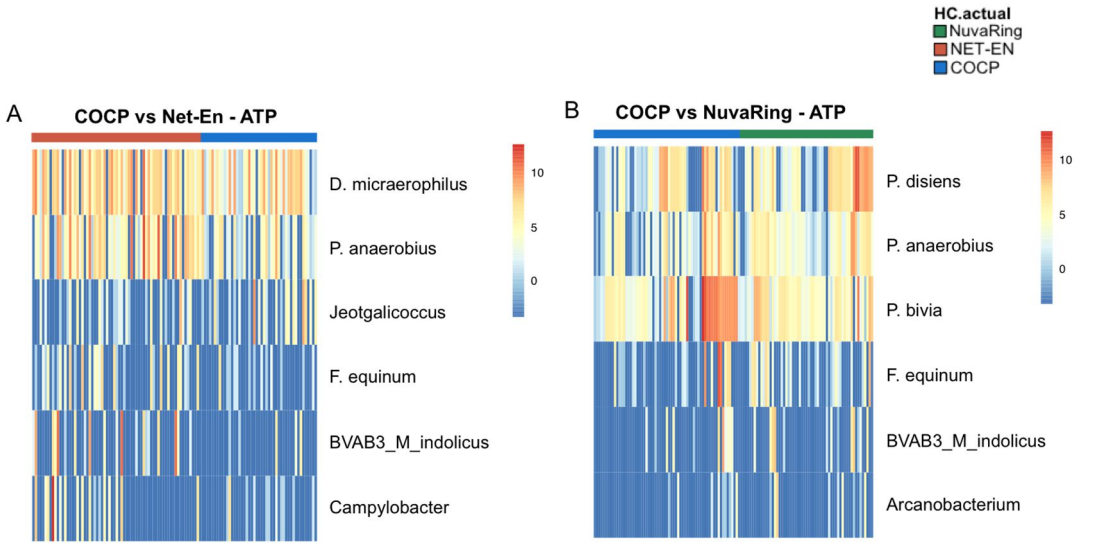


Figure 6.18. Differentially abundance taxa by hormonal contraceptive group at crossover and exit using metagenomeSeq. Bacterial taxa significantly differentially abundant and/or frequent by hormonal contraception category (A: COCP versus Net-En; B: COCP versus NuvaRing; C: Net-En versus NuvaRing) in vaginal samples from crossover and exit visits analysed using metagenomeSeq (FDR ≤ 0.05, fold-change ≥ 1.25, taxa present in ≥ 20% of samples in at least one of the two groups being compared). The heatmap shows unsupervised clustering of samples (columns) by Bray-Curtis distances split by contraceptive method. Log2-transformed standardized read counts are illustrated by the colour key.

Table 6.27. Differentially abundant taxa between participants different contraceptives at crossover and exit using metagenomeSeq.

	Coeff	P adj.	Family	Genus	Species
COCP vs Net-En	-1.413	0.0022	Staphylococcaceae	*Jeotgalicoccus*	NA
	1.321	0.0133	Veillonellaceae	*Dialister*	*micraerophilus*
	1.330	0.0087	Fusobacteriaceae	*Fusobacterium*	*equinum*
	1.454	0.0049	Peptostreptococcaceae	*Peptostreptococcus*	*anaerobius*
	1.521	0.0077	Campylobacteraceae	*Campylobacter*	NA
	2.029	0.0039	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
COCP vs NuvaRing	1.262	0.0076	Actinomycetaceae	*Arcanobacterium*	NA
	1.278	0.0136	Peptostreptococcaceae	*Peptostreptococcus*	*anaerobius*
	1.356	0.0426	Prevotellaceae	*Prevotella*	*bivia*
	1.366	0.0344	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
	1.577	0.0220	Prevotellaceae	*Prevotella*	*disiens*
	2.10	0.0014	Fusobacteriaceae	*Fusobacterium*	*equinum*

Random forest analysis was used to identify species predictive of hormonal contraceptive method. The ten most influential taxa in differentiating the vaginal microbiota of participants on COCP from Net-En at crossover and exit visits were *Peptostreptococcus anaerobius*, *Dialister succinatiphilus_propionicifaciens*, *Dialister micraerophilus*, *Prevotella* spp. (including *P. bivia*), *Peptoniphilus harei_asaccharolyticus_grossensis*, *L. iners*, *Finegoldia magna* and BVAB1 (AUC=0.67, sensitivity=0.56 and specificity=0.76 for the training set and a validation predicted error rate of 35.90% using the top five predictive taxa) (**Figure 5.19A** and **Table 5.28**). The top ten most predictive taxa for differentiating the vaginal microbiota of participants on COCP from NuvaRing at crossover and exit visits were *Anaerococcus prevotii_tetradius*, *Prevotella bivia*, *Peptostreptococcus anaerobius*, *Dialister micraerophilus*, *L. crispatus*, BVAB1, *Dialister succinatiphilus_propionicifaciens*, *Gardnerella vaginalis*, *Veillonella montpellieriensis* and *Aerococcus christensenii* (AUC=0.71, sensitivity=0.33, specificity=0.77 for the training set and a validation predicted error rate of 31.71% using the top five predictive taxa) (**Figure 5.19B** and **Table 5.28**). The error rate for the COCP and NuvaRing comparison, and thus the predictive model, improved when combining the samples from crossover and exit, suggesting that an increase in study participants could potentially strengthen this model. The ten most influential taxa in differentiating the vaginal microbiota

of participants on Net-En from NuvaRing crossover and exit visits were *Megasphaera*, *Staphylococcus*, *Prevotella* spp. (including *P. bivia*), *L. crispatus*, *Mycoplasma spermatophilum*, *Finegoldia magna*, *Gemella asaccharolytica*, *Gardnerella vaginalis*, and *U. parvum_urealyticum* (AUC=0.64, sensitivity=0.60 and specificity=0.57 for the training set and a validation predicted error rate of 53.06% using the top five predictive taxa) (**Figure 5.19C** and **Table 5.30**). As in the intention-to-treat and according to protocol analysis at crossover, the predictive model of Net-En versus NuvaRing remained weak with an error rate of 53.06%.

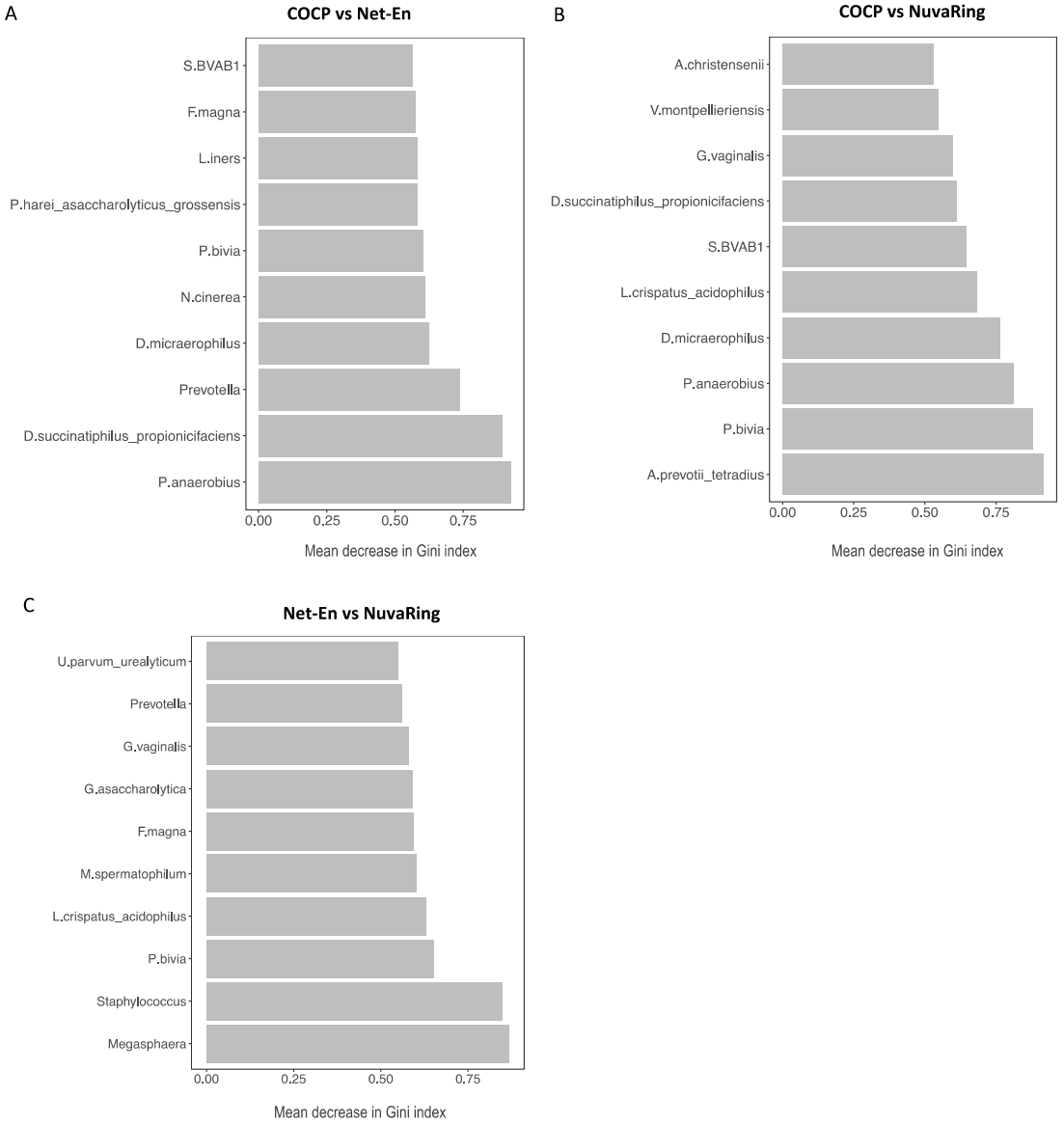


Figure 6.19. Random forest analysis according to protocol at crossover and exit. The top 10 most influential taxa by random forest analysis in predicting contraceptive method (A: COCP versus Net-En, B: COCP versus NuvaRing, C: Net-En versus NuvaRing) at crossover and exit. The x-axis indicates the mean decrease in Gini Index (length of bar represents predictive ability of each taxon).

Table 6.28. Taxa predictive of hormonal contraceptive method (COCP versus Net-En) at crossover and exit using random forest analysis.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_41	0.681	0.925	*Peptostreptococcus anaerobius*
OTU_14	2.281	0.894	*Dialister succinatiphilus_propionicifaciens*
OTU_9	1.607	0.739	*Prevotella*
OTU_39	-0.228	0.623	*Dialister micraerophilus*
OTU_13	4.387	0.609	*Neisseria cinerea*
OTU_11	1.779	0.604	*Prevotella bivia*
OTU_35	1.503	0.580	*Peptoniphilus harei_asaccharolyticus_grossensis*
OTU_1	-1.479	0.580	*Lactobacillus iners*
OTU_34	0.607	0.576	*Finegoldia magna*
OTU_3	0.984	0.564	S. BVAB-1

Training set size: 77 samples with 32 and 45 samples per class. Test set size: 39 samples with 15 and 24 samples per class. Validation predicted error: 35.9% (using top five features).

Table 6.29. Taxa predictive of hormonal contraceptive method (COCP versus NuvaRing) at crossover and exit using random forest analysis.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_28	3.689	0.917	*Anaerococcus prevotii_tetradius*
OTU_11	1.285	0.881	*Prevotella bivia*
OTU_41	2.302	0.813	*Peptostreptococcus anaerobius*
OTU_39	2.770	0.766	*Dialister micraerophilus*
OTU_2	2.635	0.682	*Lactobacillus crispatus_acidophilus*
OTU_3	1.448	0.645	S. BVAB-1
OTU_14	1.302	0.611	*Dialister succinatiphilus_propionicifaciens*
OTU_4	1.050	0.597	*Gardnerella vaginalis*
OTU_18	3.390	0.546	*Veillonella* montpellieriensis
OTU_16	-0.275	0.529	*Aerococcus christensenii*

Training set size: 83 samples with 30 and 53 samples per class. Test set size: 41 samples with 17 and 24 samples per class. Validation predicted error: 31.71% (using top five features).

Table 6.30. Taxa predictive of hormonal contraceptive method (Net-En versus NuvaRing) at crossover and exit using random forest analysis.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_5	0.660	0.868	*Megasphaera*
OTU_32	2.499	0.847	*Staphylococcus*
OTU_11	0.142	0.652	*Prevotella bivia*
OTU_2	0.641	0.629	*Lactobacillus crispatus_acidophilus*
OTU_95	3.578	0.602	*Mycoplasma spermatophilum*
OTU_34	1.088	0.594	*Finegoldia magna*
OTU_37	1.626	0.591	*Gemella asaccharolytica*
OTU_4	-0.030	0.578	*Gardnerella vaginalis*
OTU_9	-0.687	0.560	*Prevotella*
OTU_46	-1.042	0.551	*Ureaplasma parvum_urealyticum*

Training set size: 97 samples with 48 and 49 samples per class. Test set size: 49 samples with 21 and 28 samples per class. Validation predicted error: 53.06% (using top five features).

Using DESeq2 analysis for differential abundance analysis, twelve taxa were found to be differentially abundant in the vaginal microbiota of participants on COCP and Net-En at crossover and exit visits (**Figure 5.20A** and **Table 5.31**). These included *Prevotella bivia*, *Fusobacterium equinum*, *Sneathia*, *Peptostreptococcus anaerobius*, *Porphyromonadaceae*, *Sutterella*, *Campylobacter*, *BVAB3_M_indolicus*, *Anaerococcus prevotii_tetradius*, *Leptotrichiaceae*, *Anaerococcus lactolyticus* and finally *Streptococcus agalactiae*, which was the only taxa found to be more abundant in participants on COCP at time of sampling. Seventeen taxa were differentially expressed between participants on COCP and NuvaRing at crossover and exit (**Figure 5.20B** and **Table 5.31**) of which only *Prevotella melaninogenica* and *Campylobacter hominis* were more abundant in participants on COCP. The remaining fifteen taxa: *Prevotella bivia*, *Fusobacterium equinum*, *Enterobacteriaceae*, *Leptotrichiaceae*, *Porphyromonadaceae*, *Porphyromonas bennonis*, *Anaerococcus prevotii_tetradius*, *Mycoplasma hominis*, *Prevotella disiens*, *Sneathia*, *Campylobacter hominis*, *Peptostreptococcus anaerobius* and *Fusobacterium nucleatum* were more abundant in participants on NuvaRing. Twelve taxa were differentially abundant between the vaginal microbiota from participants on Net-En and NuvaRing at crossover and exit. Five of these *Bifidobacterium*, *Jonquetella anthropic, Prevotella melaninogenica*, *Mycoplasmataceae* and *Anaerococcus lactolyticus* had a higher relative abundance in participants on Net-En and the remaining seven

taxa *Staphylococcus*, *Enterobacteriaceae*, *Micrococcus*, *Gemella morbillorum_cluster*, *Corynebacterium glucuronolyticum*, *Streptococcus anginosus* and *Corynebacterium tuberculostearicum* were more abundant in participants using the NuvaRing (**Figure 5.20C** and **Table 5.31**).

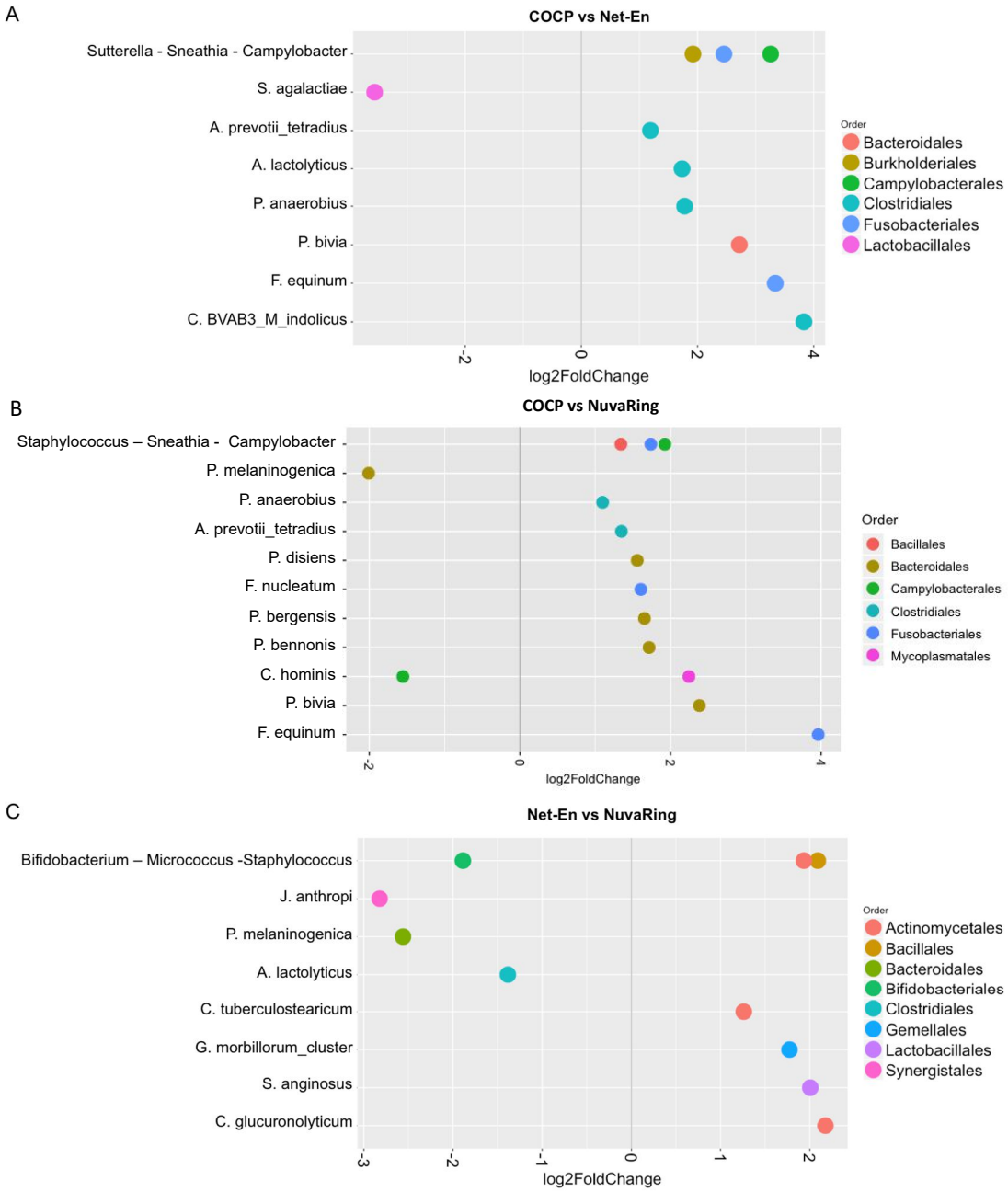


Figure 6.20. DESeq2 analysis of hormonal contraceptive methods at crossover and exit. Bacterial taxa significantly differentially abundant and/or frequent by hormonal contraceptive method at crossover and exit using DESeq2 (with an alpha of 0.05). A) Combined oral contraceptives (COCP) versus Net-En injection. B) COCP versus NuvaRing. C) Net-En injection versus NuvaRing. Taxa depicted at species level. Taxa without species level annotation (if >1) are grouped in the first line and order of genera corresponds to order of appearance of dot (from left to right). Figure only includes taxa with genus level annotation.

Table 6.31. Differentially abundant taxa between hormonal contraceptive methods at crossover and exit using DESeq2.

	Log2FC	P adj.	Family	Genus	Species
COCP vs Net-En	2.720	1.3e-05	Prevotellaceae	*Prevotella*	*bivia*
	-3.559	0.0004	Streptococcaceae	*Streptococcus*	*agalactiae*
	3.339	0.001	Fusobacteriaceae	*Fusobacterium*	*equinum*
	2.455	0.004	Leptotrichiaceae	*Sneathia*	NA
	1.777	0.005	Peptostreptococcaceae	*Peptostreptococcus*	*anaerobius*
	2.201	0.006	Porphyromonadaceae	NA	NA
	1.921	0.008	Alcaligenaceae	*Sutterella*	NA
	3.258	0.023	Campylobacteraceae	*Campylobacter*	NA
	3.830	0.032	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
	1.189	0.032	[Tissierellaceae]	*Anaerococcus*	*prevotii_tetradius*
	1.776	0.040	Leptotrichiaceae	NA	NA
	1.734	0.047	[Tissierellaceae]	*Anaerococcus*	*lactolyticus*
COCP vs NuvaRing	2.384	6.7e-06	Prevotellaceae	*Prevotella*	*bivia*
	3.962	4.1e-05	Fusobacteriaceae	*Fusobacterium*	*equinum*
	3.531	7.6e-05	Enterobacteriaceae	NA	NA
	2.8084	0.0002	Leptotrichiaceae	NA	NA
	2.412	0.0005	Porphyromonadaceae	NA	NA
	1.716	0.0050	Porphyromonadaceae	*Porphyromonas*	*bennonis*
	1.349	0.0066	[Tissierellaceae]	*Anaerococcus*	*prevotii_tetradius*
	2.245	0.0170	Mycoplasmataceae	*Mycoplasma*	*hominis*
	-2.009	0.0248	Prevotellaceae	*Prevotella*	*melaninogenica*
	1.558	0.0381	Prevotellaceae	*Prevotella*	*disiens*
	1.738	0.0460	Leptotrichiaceae	*Sneathia*	NA
	1.925	0.0460	Campylobacteraceae	*Campylobacter*	NA
	1.341	0.0460	Staphylococcaceae	*Staphylococcus*	NA
	1.654	0.0460	Prevotellaceae	*Prevotella*	*bergensis*
	-1.554	0.0460	Campylobacteraceae	*Campylobacter*	*hominis*
	1.098	0.0460	Peptostreptococcaceae	*Peptostreptococcus*	*anaerobius*
	1.606	0.0460	Fusobacteriaceae	*Fusobacterium*	*nucleatum*
Net-En vs NuvaRing	2.091	0.0003	Staphylococcaceae	*Staphylococcus*	NA
	-2.560	0.010	Prevotellaceae	*Prevotella*	*melaninogenica*
	2.181	0.017	Enterobacteriaceae	NA	NA
	1.934	0.045	Micrococcaceae	*Micrococcus*	NA
	-1.889	0.045	Bifidobacteriaceae	*Bifidobacterium*	NA
	1.774	0.045	Gemellaceae	*Gemella*	*morbillorum_cluster*
	2.177	0.045	Corynebacteriaceae	*Corynebacterium*	*glucuronolyticum*
	-3.586	0.045	Mycoplasmataceae	NA	NA
	2.006	0.045	Streptococcaceae	*Streptococcus*	*anginosus*
	-1.384	0.045	[Tissierellaceae]	*Anaerococcus*	*lactolyticus*
	1.261	0.046	Corynebacteriaceae	*Corynebacterium*	*tuberculostearicum*
	-2.823	0.046	Dethiosulfovibrionaceae	*Jonquetella*	*anthropi*

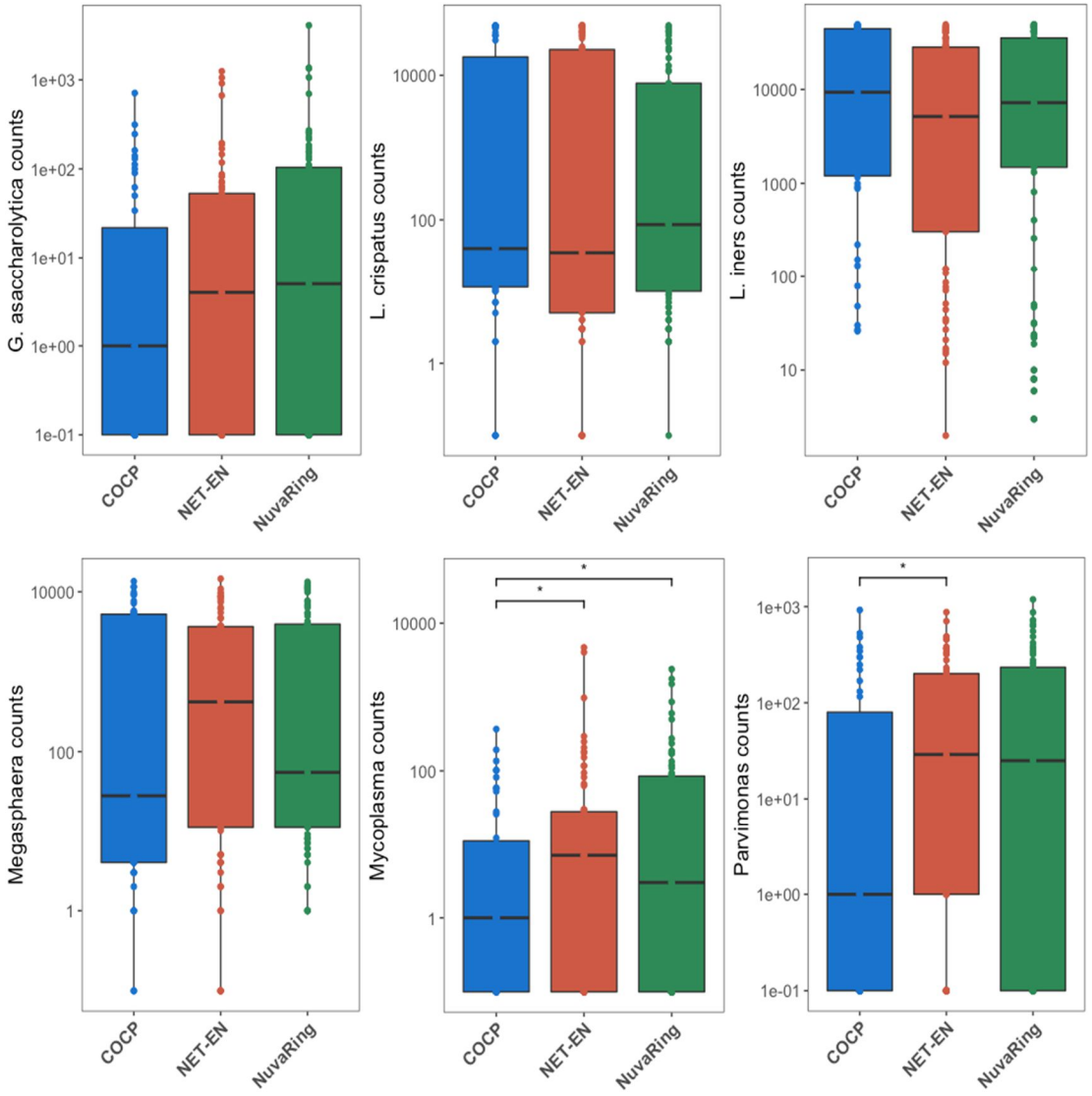
FC, fold change.

We also looked at the standardized read counts of specific bacteria of interest for HIV acquisition in each HC group in vaginal samples taken at both crossover and exit visits. Like the analysis of the crossover visit, no significant differences in the standardised read counts of *G. asaccharolytica*, *L. crispatus*, *Megasphaera*, *Sneathia* and *Veillonella montpellieriensis*, were observed between the participants on the different HC methods ($p<0.05$) (**Figure 5.21** and **Table 5.32**). In addition, the read counts of *L. iners* were no longer significantly different between the HC groups. The read counts of total lactobacilli were significantly higher in participants using COCP compared to participants on Net-En ($p=0.024$) or the NuvaRing ($p=0.041$) but not after adjusting for multiple comparisons. The read counts of *Parvimonas* remained significantly higher in participants using Net-En compared to participants on COCPs (adj. $p=0.041$) (**Figure 5.21** and **Table 5.32**). The read counts of *Mycoplasma* were significantly lower in participants on COCP compared to participants on either Net-En (adj. $p=0.043$) or NuvaRing (adj. $p=0.043$). The read counts for *P. bivia* were significantly higher in participants on NuvaRing compared to participants using COCPs (adj. $p=0.003$). This was also the case for participants on Net-En before adjustment for multiple comparisons ($p=0.043$). The read counts of *Prevotella* at the genus level were significantly higher in participants on Net-En (adj. $p=0.007$) and NuvaRing (adj. $p=0.012$) compared to participants using COCPs (**Figure 5.21** and **Table 5.32**).

Table 6.32. Standardized read counts of bacteria of interest according to hormonal contraceptive method at crossover and exit.

	COCP	Net-En	NuvaRing	P value
G. asaccharolytica	1 (0-24)	4 (0-53)	5 (0-104)	0.294
Lactobacillus	46,670 (5,420-48,843)	32,381 (1,460-46,909)	30,744 (3,093-47,770)	0.054
L. crispatus	40 (12-20,591)	35 (5-22,728)	86 (10-7,775)	0.783
L. iners	9,341 (1,203-44,372)	5,150 (305-28,243)	7,224 (1,483-35,346)	0.175
L. non-iners	0 (0-16)	0 (0-9)	0 (0-21)	0.107
Megasphaera	28 (4-5,237)	419 (11-3,668)	55 (11-3,941)	0.522
Mycoplasma	1 (0-11)	7 (0-28)	3 (0-85)	0.043
Parvimonas	1 (0-86)	29 (1-201)	25 (0-234)	0.060
Prevotella	223 (61-4,457)	2,059 (281-8,997)	1,874 (173-5,208)	**0.005**
P. amnii	6 (1-45)	8 (2-1,824)	8 (1-1,325)	0.372
P. bivia	10 (3-53)	38 (4-203)	46 (13-361)	**0.007**
P. melaninogenica	1 (0-10)	1 (0-3)	1 (0-4)	0.754
Sneathia	4 (1-133)	15 (1-371)	11 (1-483)	0.273
V. montpellieriensis	1 (0-4)	1 (0-13)	2 (0-28)	0.194

P values were generated using Kruskal-Wallis test adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method.



Continued on next page.

Continued from previous page.

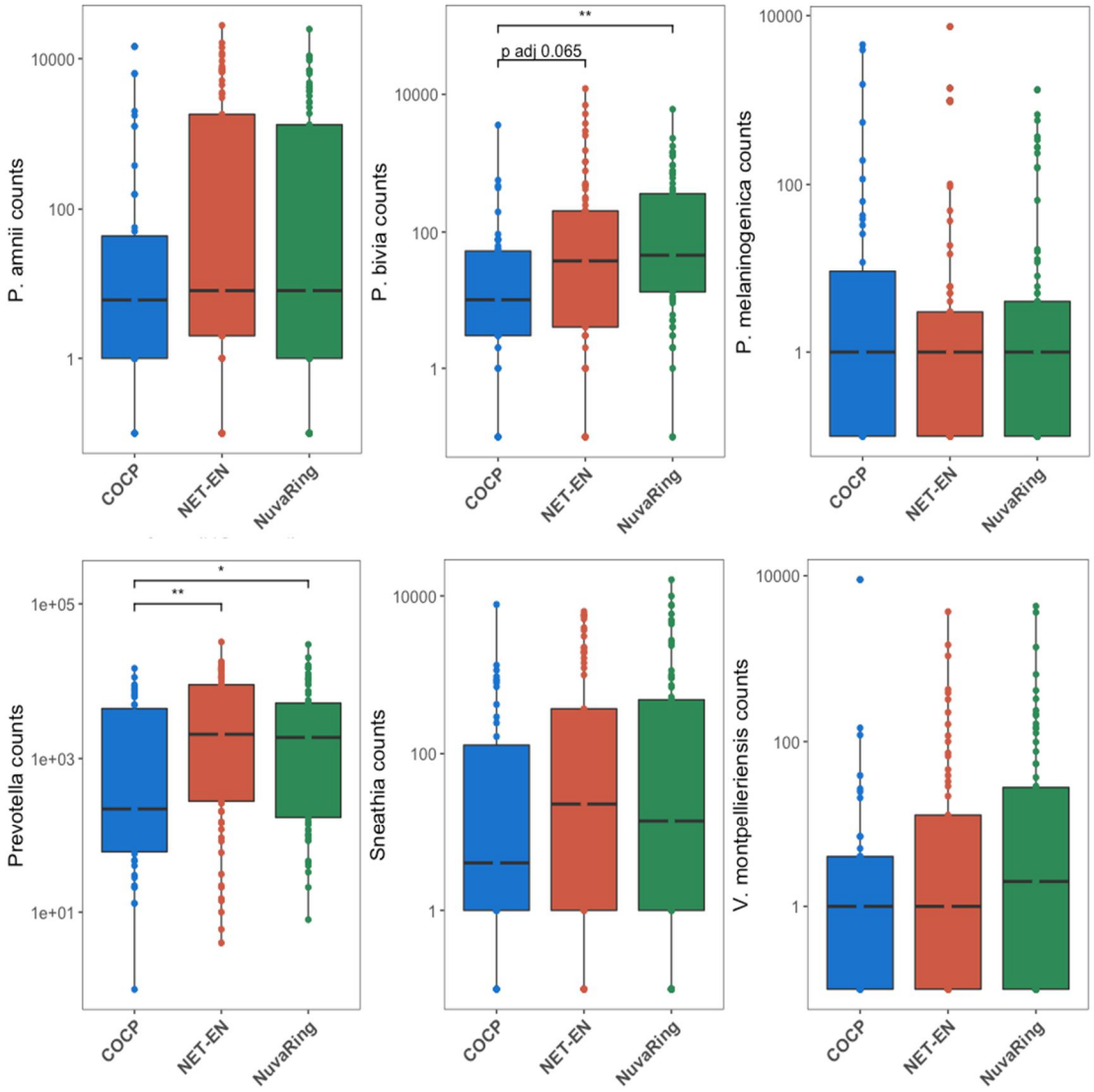


Figure 6.21. Differences in bacterial counts between hormonal contraceptive methods of bacteria of interest at crossover and exit. Boxplot showing the standardized read counts of bacteria of interest in 193 vaginal samples from 107 participants at either crossover (n=104) or exit (n=89) visit according to hormonal contraceptive method. P values were generated using unpaired Mann-Whitney-Wilcoxon tests adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. Significance codes: ns: adj. p>0.05, *: adj. p<0.05, **: adj. p<0.01, ***: adj. p<0.001.

5.3.7 Longitudinal analyses of vaginal microbiota according to contraceptive method

Across all three time points, 332 samples from 144 participants were available for analysis of the impact of hormonal contraceptives on the vaginal microbiota. In a paired analysis of matched samples from 26 participants who went either from COCP to Net-En or vice versa at any stage in the study, the alpha diversity of the vaginal microbiota of participants was significantly lower when on COCP

than the alpha diversity of their own vaginal microbiota at either the previous or following visit if they were on Net-En at that visit (median SI 0.86 vs. 1.55, p=0.017) (**Figure 5.22A** and **Table 5.33**). Furthermore, the alpha diversity of the vaginal microbiota of participants on COCP was also significantly lower than the alpha diversity of their own microbiota when on NuvaRing either before or after that visit (31 participants, median SI 0.65 vs. 1.24, p=0.033) (**Figure 5.22B** and **Table 5.33**). In contrast, there was no significant intra-participant change in alpha diversity of the vaginal microbiota when shifting between the Net-En and NuvaRing methods (57 participants, median SI 1.49 vs. 1.61, p=0.507) (**Figure 5.22C** and **Table 5.33**).

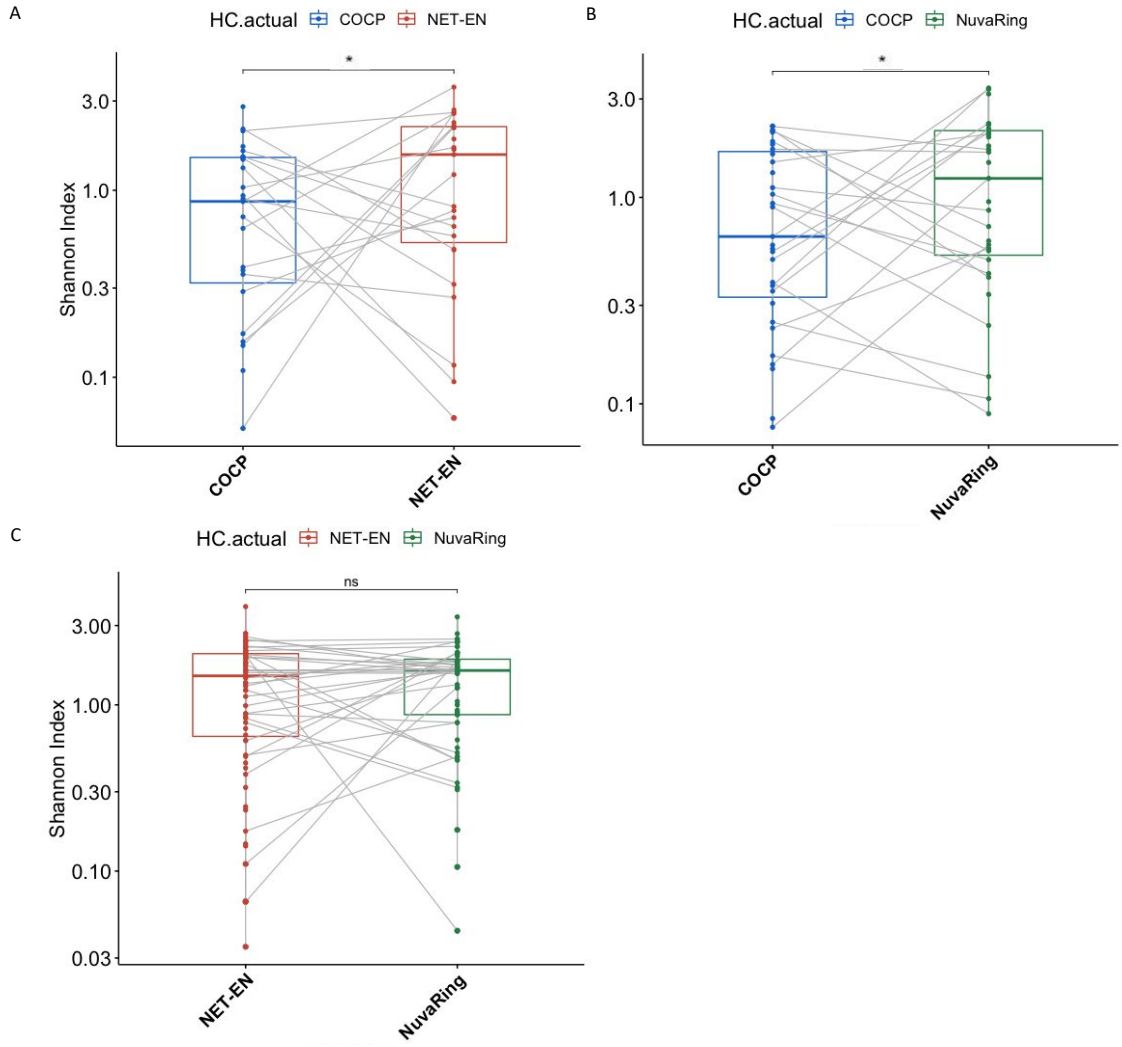


Figure 6.22. Alpha diversity within participants changing between specific hormonal contraceptive methods. Boxplots showing the alpha diversity measured by Shannon Index in A) 52 vaginal samples from 26 participants changing from COCP to Net-En or vice versa, B) 62 vaginal samples from 31 participants changing from COCP to NuvaRing or vice versa and C) 126 vaginal samples from 57 participants changing from Net-En to NuvaRing or vice versa. P values were generated using paired Wilcoxon Signed Rank tests. Y-axis is log10 transformed. ****=p<0.0001, ***=p<0.001, **=p<0.01, *=p<0.05, ns=p>0.05.

Table 6.33. Alpha diversity in participants changing between specific hormonal contraceptive methods.

	COCP (median (IQR))	Net-En (median (IQR))	NuvaRing (median (IQR))	P value
COCP vs. Net-En	0.87 (0.32-1.50)	1.55 (0.53-2.19)	-	**0.017**
COCP vs. NuvaRing	0.65 (0.33-1.67)	-	1.24 (0.53-2.11)	**0.033**
Net-En vs. NuvaRing	-	1.49 (0.65-2.03)	1.61 (0.87-1.88)	0.507

P values calculated using paired Wilcoxon Signed Rank tests.

The vaginal microbiota of participants using COCPs at any visit had a significantly lower alpha diversity at the time of sampling compared to the microbiota of participants who were on either Net-En or NuvaRing and of participants not using HC (adj. p=0.015) (**Figure 5.23**, **Table 5.34** and **Table 5.35**). Using lmd modelling, the alpha diversity of participants using COCP was significantly lower than in participants using Net-En or the NuvaRing (p=0.016 and p=0.015, respectively) and close to significant compared to participants not using HC (p=0.058). The microbiota of participants on the DMPA injectable also has a higher alpha diversity than COCP users, but not significantly so (adj. p=0.203, model: p=0.865). Information on hormonal contraceptive use was missing for six participants and participants on the contraceptive implant, Implanon, at screening were excluded from this analysis due to the low numbers (n=3). No overall differences in microbiota composition (beta diversity) were found between participants on the different contraceptive methods (including the DMPA injectable) or not using HC (adonis, p=0.245, R2=0.015) (**Figure 5.24**).

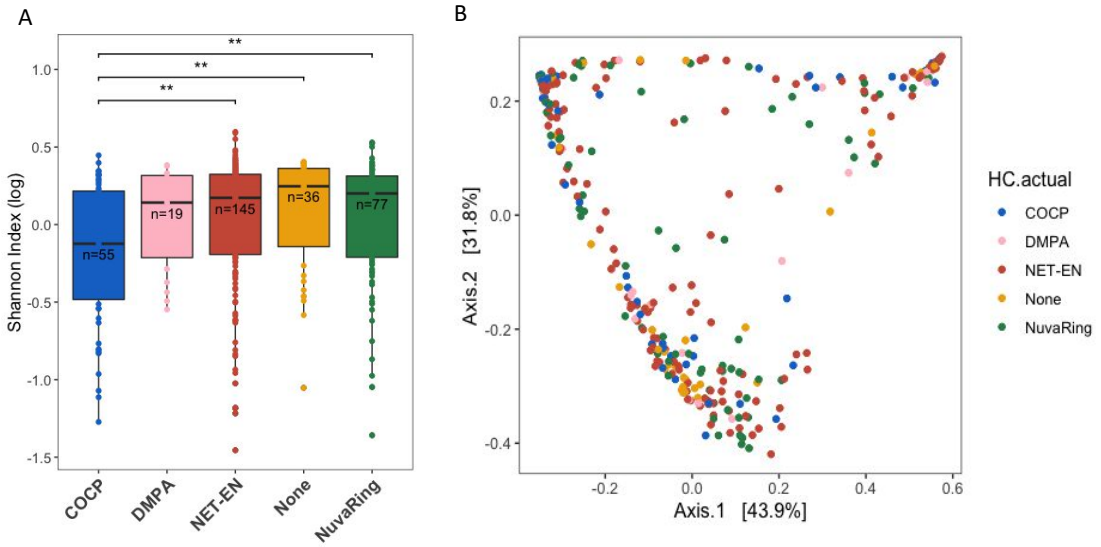


Figure 6.23. Alpha and beta diversity across all visits according to hormonal contraceptive method. **A**) Boxplot showing the alpha diversity measured using Shannon Index (log transformed values) of the microbiota from 334 vaginal samples from 144 participants from all three visits according to the hormonal contraceptive regime of the participant at the time of sampling (COCP: n=55; DMPA: n=19; Net-En: n=146; None: n=37; NuvaRing: n=77). P values were calculated using Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests and adjusted for multiple comparisons using Benjamini-Hochberg. Significance codes: ns: adj. p>0.05, *: adj. p<0.05, **: adj. p<0.01, ***: adj. p<0.001. B) Principal Coordinate Analysis (PCoA) of 332 vaginal samples from 144 participants from all three study visits using weighted UniFrac distances. Samples are coloured by the hormonal contraceptive regime of the participant at the time of sampling (six participants with missing values). Participants on the contraceptive implant, Implanon, were excluded due to low numbers (n=3).

Table 6.34. Alpha diversity according to hormonal contraceptive method at all visits - I.

	COCP (n=55)	**DMPA (n=19)**	**Net-En (n=146)**	**None (n=37)**	**NuvaRing (n=77)**	**P value**
Median SI (IQR)	0.75 (0.33-1.64)	1.39 (0.62-2.07)	1.49 (0.64-2.11)	1.77 (0.73-2.30)	1.59 (0.62-2.06)	**0.013**

P-value calculated using Kruskal-Wallis test adjusting for multiple comparisons using the Benjamini-Hochberg (BH) method. SI, Shannon Index.

Table 6.35. Alpha diversity according to hormonal contraceptive method at all visits - II.

	Shannon Index	
	P	P adj.
COCP vs. DMPA	0.081	0.203
COCP vs. Net-En	**0.003**	**0.015**
COCP vs. None	**0.003**	**0.015**
COCP vs. NuvaRing	**0.004**	**0.015**
DMPA vs. Net-En	0.797	0.886
DMPA vs. None	0.326	0.576
DMPA vs. NuvaRing	0.727	0.886
Net-En vs. None	0.345	0.576
Net-En vs. NuvaRing	0.909	0.909
None vs. NuvaRing	0.425	0.607

P values generated using Kruskal-Wallis test with Dunn's post hoc test using the Benjamini-Hochberg (BH) method for multiple comparisons testing (MTC). Six participants with missing values. Participants on the contraceptive implant, Implanon, were excluded due to low numbers (n=3). COCP: n=55; DMPA: n=19; Net-En: n=146; None: n=37; NuvaRing: n=77.

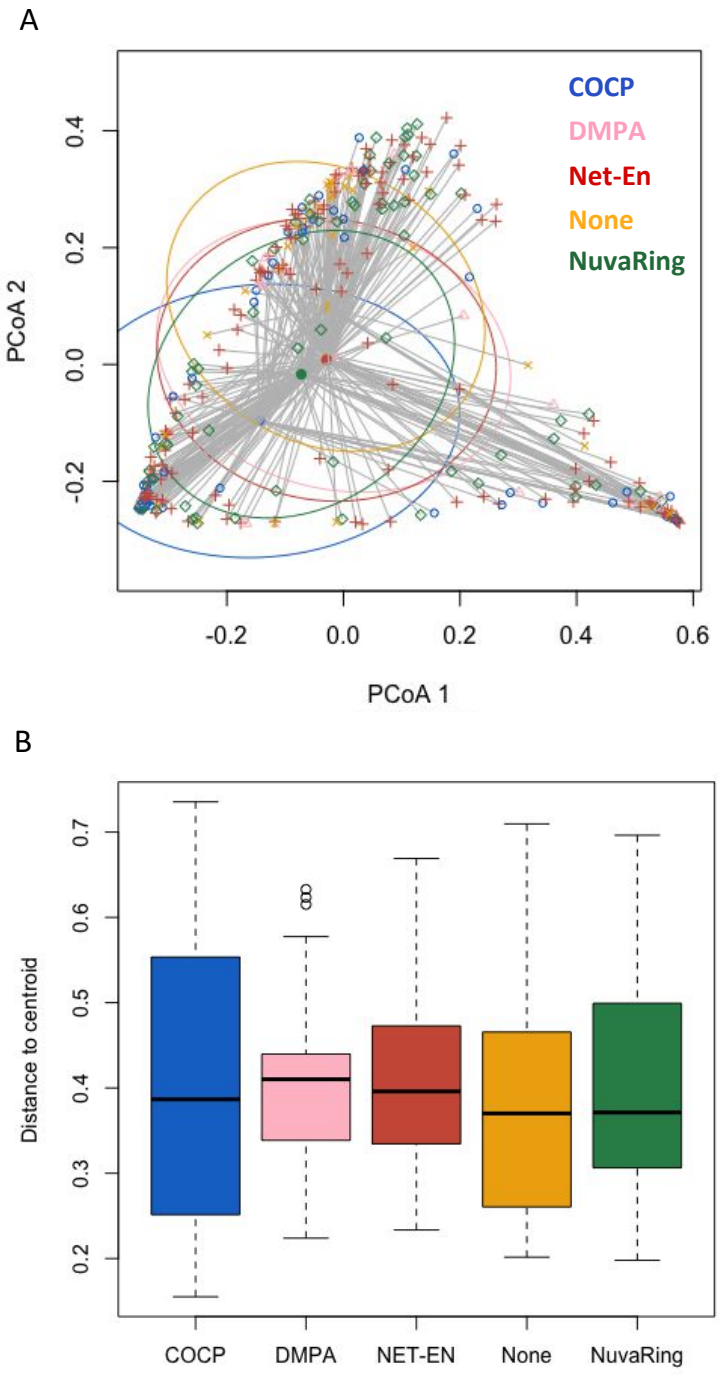
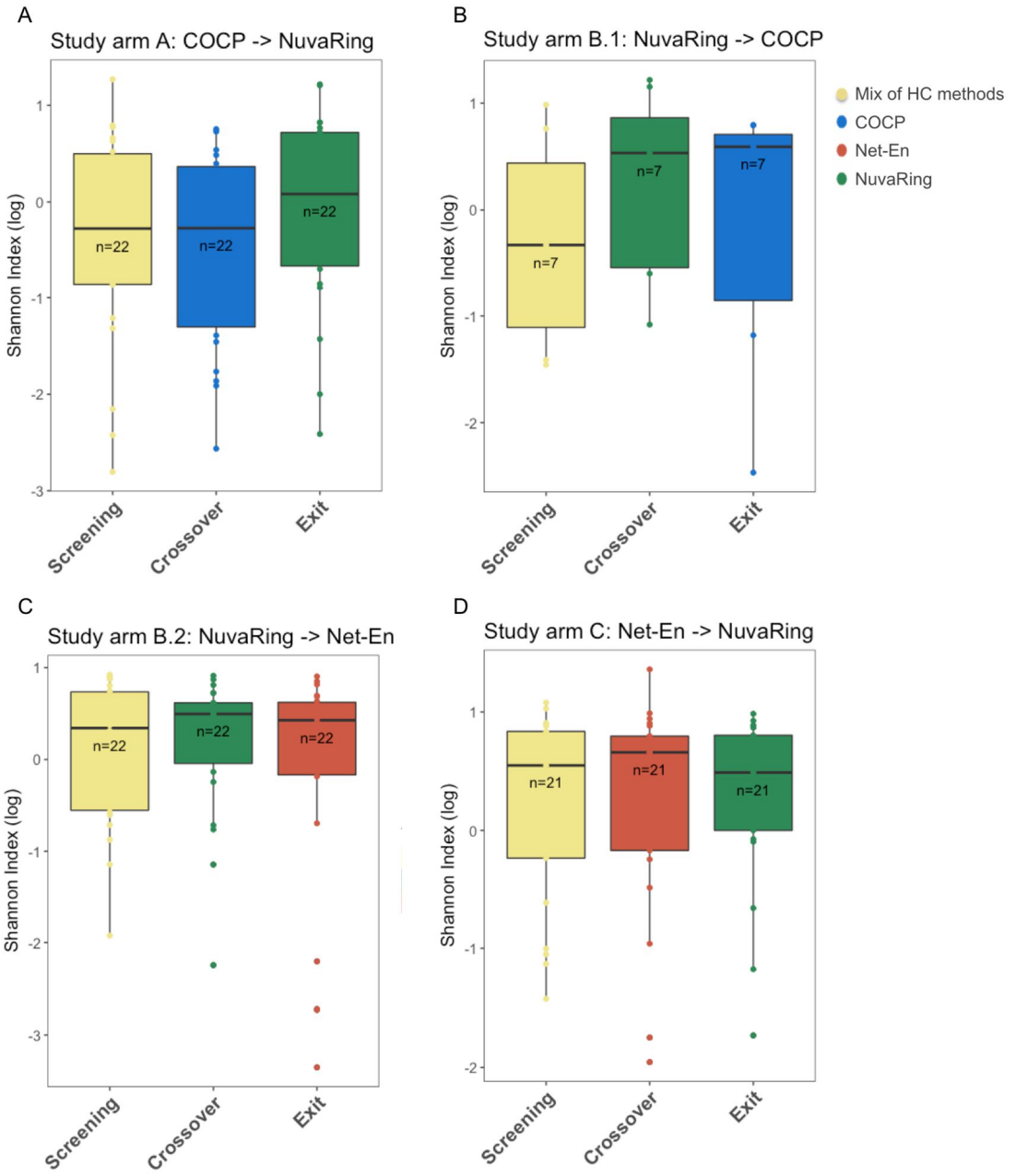


Figure 6.24. Beta diversity within hormonal contraceptive group across all visits. A) Principal Coordinates Analysis (PCoA) of beta diversity calculated using weighted UniFrac according to hormonal contraceptive group with standard deviations ellipses around each group centroid. B) Barplot depicting the distance to the centroid for each sample in each hormonal contraceptive group.

Due to the study design there are essentially four major groups in this cohort with relatively low numbers in each: 1. participants who were initially assigned to COCPs and who crossover to the NuvaRing as their second method (A, n=22), 2. participants first assigned to NuvaRing who chose COCP as their second contraceptive option (B.1, n=7), 3. participants first assigned to NuvaRing who chose Net-En as their second contraceptive option (B.2, n=22), and lastly 4. participants initially assigned to Net-En who switched to the NuvaRing as their second method (C, n=21) (**Figure 5.25**). The alpha diversity of the microbiota of the participants in each of these four groups did not differ over the course of the study period (A: p=0.244, B.1: p=0.847, B.2: p=0.062 and C: p=0.954) (**Figure 5.25**). These results could be due to low numbers as paired analyses showed significant differences between the alpha diversity of the vaginal microbiota of participants when on COCP compared to the NuvaRing or Net-En (section 5.3.3, **Figure 5.22**).

As described in Chapter 4, having a vaginal microbiota assigned to the diverse C1 community type was generally associated with a high level of stability and fewer shifts in community types compared to the two lactobacillus dominant community types while the *L. iners* dominant C3 community type was the least stable of the three community types. To address whether the use of different HC methods may have contributed to the observed shifts between community types in participants over time, we looked at the Fuzzy cluster distribution and BV prevalence within each HC group at the crossover and exit visit, respectively, compared to the distribution of community types and BV at the previous visit within the same participant (**Table 5.36**). A high degree of stability was observed within all three groups and no clear pattern in terms of transitions between community clusters was found. An increase in the *L. crispatus* dominant cluster in Net-En participants and an increase in the *L. iners* dominant community cluster in COCP participants were observed, but not statistically significant.



Group	Baseline	Crossover	Exit	P-value
A. COCP-NuvaRing	0.76 (0.43-1.65)	0.77 (0.27-1.44)	1.10 (0.52-2.05)	0.244
B1. NuvaRing-COCP	0.72 (0.35-1.63)	1.71 (0.58-2.47)	1.80 (0.45-2.47)	0.847
B2. NuvaRing-COCP	1.41 (0.58-2.09)	1.64 (0.97-1.85)	1.53 (0.85-1.86)	0.062
C. Net-En-NuvaRing	1.73 (0.79-2.31)	1.93 (0.84-2.22)	1.63 (1.00-2.24)	0.954

Figure 6.25. Diversity over time according to study arm. Boxplot showing the alpha diversity measured using Shannon Index (log transformed values) of the vaginal microbiota over time according to study arm and hormonal contraceptive use at the time of sampling: A) Study arm A: participants assigned to COCPs who crossed over to NuvaRing (n=22); B) Study arm B.1: participants assigned to NuvaRing who chose COCP as second option (n=6); C) Study arm B.2: participants assigned to NuvaRing who chose Net-En as second option (n=19) and D) Study arm C: participants assigned to Net-En who crossed over to NuvaRing (n=16). The alpha diversity values (measured using Shannon Index) for the baseline samples in each of the plots (A-D) have been combined irrespectively of hormonal contraceptive method used by the participants when screened. Below figure: Table of alpha diverisities measured with Shannon Index for each group (A, B1, B2 and C) at each visit. Values are depicted as median Shannon (interquartile range). P values were calculated using Friedman's paired test.

Table 6.36. Change in Fuzzy cluster and BV distribution over time according to hormonal contraceptive method.

	Screening	Crossover	P value	Crossover	Exit	P value
COCP						
Fuzzy cluster			0.429			0.873
C1	13	11		4	4	
C2	9	6		3	5	
C3	11	16		5	3	
BV prevalence			0.921			0.371
Positive	13	11		2	5	
Intermediate	2	2		0	0	
Negative	18	20		10	7	
Net-En						
Fuzzy cluster			0.331			0.776
C1	19	16		12	12	
C2	5	10		8	6	
C3	8	6		8	10	
BV prevalence			0.077			0.832
Positive	17	18		14	14	
Intermediate	5	0		2	1	
Negative	14	18		14	15	
NuvaRing						
Fuzzy cluster			0.811			0.705
C1	14	16		18	19	
C2	8	6		10	7	
C3	7	7		13	15	
BV prevalence			0.846			0.977
Positive	12	14		18	19	
Intermediate	2	1		1	1	
Negative	18	17		25	24	

P values generated using Chi-squared test (Fisher's exact test when expected values < 5).

To further address the impact of HC on the bacterial community, we applied a multi-state modelling approach. A multi-state model (MSM) describes how an individual moves between a series of states in continuous time (Jackson, 2011). For this study, we fitted a MSM model in which community type as determined by Fuzzy clustering and weighted UniFrac distances represented the state of each individual at each of the three time points (0, 16 and 32 weeks). As we sought to detect the impact of HC on the risk of moving within these states, we included HC method as the time-dependent explanatory variable. The number of participants with no transitions due to only one time point available with adequate sequencing data was 49 and these were excluded from the model. In total, data from 179 paired, consecutive visits with vaginal cluster assignment for the microbiota at both time points from 104 participants was available for inclusion in the model. At first we applied the model using all of our six HC groups: COCP, DMPA injectable, the contraceptive implant Implanon, Net-En

injection, None (including naïve and no current HC use) and NuvaRing, however the model was not able to converge, most likely due to the high number of different HC categories. We therefore collapsed the injectables DMPA and Net-En and the implant (Implanon) (n=3) categories into one (Net-En_DMPA_Implanon). The model was still not able to converge so we further collapsed the COCP and None categories into one (COCP_None). This generated three overall categories: COCP_None, Net-En_DMPA_Implanon and NuvaRing, which we applied to the model. The distribution of Fuzzy clusters between the three categories did not significantly differ (p=0.332) (**Table 5.37**).

Table 6.37. Distribution of Fuzzy clusters in hormonal contraceptive groups.

	C1	C2	C3
COCP_None	28 (38.9%)	17 (23.6%)	27 (37.5%)
Net-En_DMPA_Implanon	62 (46.3%)	37 (27.6%)	35 (26.1%)
NuvaRing	35 (46.7%)	14 (18.7%)	26 (34.7%)

According to the final model, the probability of transitioning from C1 to C2 or C3 after about four months on COCP was 8.8% and 18.9%, respectively (**Table 5.38**). Switching from C2 to another community type when on COCP was much more likely, particularly switching to C3 with a probability of 33.1%. As expected C3 was the most unstable community and the probabilities of transitioning to C1 or C2 were 36.4% and 18.5%, respectively. The probability of transitioning from C1 to another community was similar in the Net-En group to the COCP group. The probability of transitioning from C2 or C3 to another community type was however a bit lower in this group (**Table 5.38**). Overall, the NuvaRing group showed the highest probabilities of transitioning from one community type to another with around a 40% probability of changing to C1 from either C2 and C3 and a 58% probability of staying in C1 compared to 72.2% and 70.6% in the COCP and Net-En group, respectively (**Table 5.38**). The confidence intervals for the hazard scores were however very wide (**Table 5.39**), and these results should therefore be interpreted with some cautions.

Table 6.38. Estimated transition probabilities *P(t)* after 16 weeks according on hormonal contraceptive category.

To / From	**C1** (CI)	**C2** (CI)	**C3** (CI)
COCP_None			
C1	0.722 (2.7e-01,1.77)	0.088 (1.4e-59,0.47)	0.189 (8.6e-02,1.04)
C2	0.131 (4.8e-02,1.77)	0.538 (1.4e-59,0.77)	0.331 (9.0e-02,1.04)
C3	0.364 (1.7e-01,2.19)	0.185 (3.2e-59,0.39)	0.451 (2.6e-01,1.77)
Net-En_DMPA_Implanon			
C1	0.706 (8.6e-05,0.79)	0.045 (2.4e-02,0.853)	0.249 (1.3e-01,0.39)
C2	0.148 (8.6e-05,0.36)	0.638 (4.0e-01,0.856)	0.214 (1.0e-01,0.41)
C3	0.176 (9.6e-05,0.38)	0.195 (1.0e-01,0.435)	0.629 (3.9e-01,0.79)
NuvaRing			
C1	0.584 (2.8e-01,3.397)	0.153 (8.4e-35,0.506)	0.263 (5.5e-02,4.323)
C2	0.405 (1.2e-01,3.237)	0.487 (7.2e-35,0.799)	0.109 (2.1e-02,3.397)
C3	0.403 (1.3e-01,3.237)	0.135 7.2e-35,0.562)	0.463 (5.1e-02,3.397)

Rows correspond to "from-state" and columns to "to-state". CI, confidence interval.

Table 6.39. Hazard ratios of transitioning from one community cluster to another according to hormonal contraceptive category.

	HR	**Lower CI**	**Upper CI**
COCP_None			
C1 to C2	0.486	7.3e-03	0.325
C1 to C3	1.355	0.290	6.311
C2 to C1	5306	4.8e-63	5.8e+69
C2 to C3	0.301	0.059	1.533
C3 to C1	0.511	0.152	1.721
C3 to C2	0.793	0.145	4.343
Net-En_DMPA_Implanon			
C1 to C2	0.099	2.6e-07	37762
C1 to C3	1.173	0.247	5.581
C2 to C1	2.1e+05	0.000	Inf
C2 to C3	0.418	0.099	1.770
C3 to C1	0.352	0.086	1.441
C3 to C2	0.830	0.148	4.669
NuvaRing			
C1 to C2	2.853	0.065	125.8
C1 to C3	1.820	0.255	13.00
C2 to C1	9.4e+05	0.000	Inf
C2 to C3	2.8e-07	0.000	Inf
C3 to C1	1.197	0.187	7.647
C3 to C2	0.509	0.009	30.34

HR, hazard ratio; CI, confidence interval.

5.4 Discussion

5.4.1 Hormonal contraception and the vaginal microbiota

Any influence of hormonal contraception, particularly progestin-only injectables, on HIV susceptibility is of large concern in a country such as South Africa where long-acting injectable contraceptive options are the method of choice and where HIV incidence rates amongst young women are very high. In this study the impact of three different HC options (Net-En, COCPs and NuvaRing) on the FGT

microbiota amongst South African adolescent females was analysed. To our knowledge this is the first study to analyse the effects of different HC methods on the vaginal bacterial community in a randomized trial using molecular methods. Furthermore, this study focuses on adolescent girls, a group at the highest risk of unintended pregnancies and of acquiring HIV. In this study, an intention-to-treat analysis at crossover showed that participants assigned to COCP had a significantly less diverse vaginal microbiota compared to participants assigned to either Net-En or NuvaRing. The same was observed in an according to protocol analysis at crossover. These differences were evident even when adjusting for reported condom use. In a longitudinal, paired analysis the alpha diversity of the vaginal microbiota was also significantly lower when a participant was on COCP compared to when the same participant was on NuvaRing or Net-En and there was a non-significant decrease in alpha diversity within COCP users from baseline to crossover. The beta diversity of the vaginal community also differed between the assigned study arms and between HC group at crossover. This was true also for the distribution of Fuzzy clusters with the *L. iners* dominant C3 community type being the most prevalent in the COCP group while the diverse C1 community type was the most common in both the Net-En and the NuvaRing group. Of interest, the alpha diversity of the vaginal microbiota of participants on either Net-En or the NuvaRing was comparable to the alpha diversity of participants not on HC while COCP was significantly lower. This could suggest that the difference between the three study arms analysed in this study stems more from a decrease in alpha diversity from being on COCP rather than an increase in alpha diversity from being on Net-En or NuvaRing although the alpha diversity increased within NuvaRing users. These results are in agreement with previous study showing that women using COCPs were more likely to be colonized by beneficial *Lactobacillus* species compared with women using condoms alone (aOR: 1.94; 95% CI: 1.25-3.02), while women using DMPA were not (aOR: 1.09; 95% CI: 0.63-1.86) (Brooks et al., 2017). Women using COCPs were also less likely to be colonized by BV-associated bacteria relative to women who used condoms (aOR 0.29, 95% CI: 0.13-0.64) (Brooks et al., 2017). Furthermore, in a study looking at factors associated with recurrence of BV after treatment in a cohort of reproductive age women in Australia, use of oestrogen-

containing contraceptives was found to be protective with the risk of BV recurrence being halved (aHR = 0.51; 95% CI: 0.33–0.78) (Bradshaw et al., 2013a). Taken together, these data suggest that use of COCP may have a protective effect on the vaginal microbiota. Using Kruskal-Wallis test for this analysis is somewhat flawed due to violation of the assumptions of the test as it includes multiple samples from the same adolescents. A linear mixed-effects model was thus generated with visit and participant ID included in the model. The results were in line with the Kruskal-Wallis data.

To date, there is limited evidence suggesting combined contraceptive vaginal rings (CCVRs) have significant impact on the vaginal microbiota. However, most of the studies conducted on the impact of contraceptive vaginal rings on the FGT microbial environment have been based on Nugent scoring and bacterial culturing (Huang et al., 2015b; Veres et al., 2004; Creinin et al., 2008). In a newly published open-label NuvaRing study by Crucitti et al. (2018), the impact of NuvaRing use on the vaginal microbiota over the course of 12 weeks was evaluated by qPCR of key vaginal bacteria (i.e. *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae*) in 120 Rwandan women (Crucitti et al., 2018). The presence and mean $\log_{10}$ concentrations of *Lactobacillus* species in vaginal secretions increased significantly whereas the concentration of *G*. *vaginalis* and presence of *A*. *vaginae* decreased significantly (Crucitti et al., 2018). In this study, we did not observe the same pattern with the 16S sequencing data, however we measured the relative abundance of bacteria rather than their absolute quantities. It would be interesting to perform qPCR analysis our samples to get a more quantitative measurement of the bacteria in question.

Most studies to date investigating the association of injectable hormonal contraception, HIV susceptibility and vaginal microbiota have focused on DMPA and to a lesser degree on Net-En injectables (Polis et al., 2016; Morrison et al., 2015). These studies have hinted at slight differences between the two injectables in their impact on HIV risk with Net-En potentially having a lower HIV risk than DMPA injectables. Injectables containing different progestins could have different effects on the immune system and the FGT ecosystem for instance due to variations in dose (150 mg for DMPA versus 200 mg for Net-En), peak

serum progestin concentrations, methods of absorption, metabolism, and level of oestrogen suppression (Kuhl, 2005; Stanczyk et al., 2013; Africander et al., 2011; Heffron et al., 2018). Thus, they may also have different effects on the FGT microbiota, immunology and HIV susceptibility. Studying the impact of the Net-En injectable and comparing it to the DMPA injectable is therefore of great importance, as one type of progestin-only injection could prove safer to use for women at risk of HIV than another while providing the ease of not having to take a pill every day or to insert a monthly ring. In the limited analyses possible in this study, DMPA and Net-En injectables did not significantly differ from each other with regard to the alpha diversity of the vaginal microbiota. There may also be differences between specific oestrogens used in HC worth exploring. The two COCPs (Nordette and Triphasil) used in this study both contain ethinyl oestradiol and levonorgestrel, hindering any such analysis in this thesis.

5.4.2 Importance of specific species

Species with the capacity to make biofilm, such as *G. vaginalis*, have been suggested to play a central role in BV leading to high levels of persistence and recurrence (Swidsinski et al., 2014). Promotion of biofilm growth seems more effective in presence of *Prevotella bivia*, showing a higher ability to enhance *G. vaginalis* growth than other BV-associated anaerobes (Machado et al., 2013). BVAB1 has also been linked to persistent BV in adolescents from the same setting as this current study (WISH study, Chapter 3) (Lennard et al, 2018). These data underline the importance of studying the symbiotic relationships between the vaginal bacteria to improve our understanding of BV pathology and susceptibility to STIs.

While certain bacterial species have been associated with an optimal vaginal environment and protection against pathogenic bacteria and viruses, others have been associated with increased risk of STIs including HIV (Gosmann et al., 2017; McClelland et al., 2018). We therefore sought to determine whether hormonal contraception was associated with any differences in the relative abundance and read counts of specific bacteria implicated in risk of STIs. Using differential abundance testing and random forest analyses, we found that species associated with BV and risk of HIV was significantly more abundant in, and

predictive of, participants on Net-En (e.g. *Prevotella*, *Sneathia*, *Dialister* and *Parvimonas*) or NuvaRing (e.g. *Prevotella, Mycoplasma* and *Parvimonas*) compared to COCP while *L. iners* was more common in the COCP group. In agreement with our data, a study in Rwandan female sex workers of which the vaginal microbiota of 174 women was characterized by phylogenetic microarray, COCP users had lower semi quantitative vaginal abundance of *Prevotella*, *Sneathia/Leptotrichia amnionii*, and *Mycoplasma* species (Borgdorff et al., 2015). These results are concerning for adolescents in sub-Saharan Africa as COCPs are rarely used and long-acting reversible contraceptives are preferred (Ross & Agwanda, 2012; Sibeko et al., 2011).

The genus *Arcanobacterium* was more abundant in the vaginal microbiota of both participants on Net-En and NuvaRing compared to COCP. This particular genus has not been described in relation to the FGT microbiota before but is a constituent of the normal skin and throat microbiota. Species of this genus have been associated with human infections, including pharyngitis and cutaneous infections, which are caused by *Arcanobacterium haemolyticum* (Mackenzie et al., 1995; MACLEAN et al., 1946). Another species from this genus, *Arcanobacterium hippocoleae*, has previously been isolated from vaginal discharge of mares and related to vaginitis and placentitis (Hoyles et al., 2002; Bemis et al., 2008). These data suggest that species of the *Arcanobacterium* genera could be opportunistic pathogens with potential pro-inflammatory capabilities. The bacteria *Actinomyces hongkongensis* was found to be more abundant in participants on Net-En compared to both COCP and NuvaRing users in this study. Of interest, *Actinomyces* species have been suggested to play a role in intrauterine contraceptive device (IUCD)-associated pelvic actinomycosis (Woo et al., 2003).
The relative abundance of *N. gonorrhoea* was significantly higher in the vaginal microbiota of participants on NuvaRing compared to participants on COCP and Net-En. This was in concordance with the prevalence of *N. gonorrhoea* being significantly higher in the NuvaRing participants compared to COCP and Net-En participants at crossover. This data suggest that use of NuvaRing can increase risk of STIs, *N. gonorrhoea* in particular. The prevalence of *C. trachomatis* was similar in the Net-En and NuvaRing group and lower in the COCP group, albeit not significantly. The NuvaRing group also had a high level of *Candida* as

detected by microscopy compared to the other two groups. Although most clinical studies to date have not demonstrated a increased incidence of yeast infections with vaginal ring use but rather implicated COCP with an increased risk of vaginal candidiasis (van de Wijgert et al., 2013), our results are line with newly published data from a randomised trial on the NuvaRing in Rwandan women (Kestelyn et al., 2018). In their study the baseline prevalence of vaginal yeasts was 5% while the percentage of women with incident vaginal yeasts at one or more follow up visits came to 22% (Kestelyn et al., 2018). In vitro data have shown that yeast cells are capable of adhering to the surface of the NuvaRing potentially facilitating the development and recurrence of *Candida* infections (Camacho et al., 2007). Thus, awareness should be placed on the potential increased risk of candidiasis with use of CCVRs.

5.4.3 Sexual risk behaviour

Potential modulators of the vaginal microbiota, such as sexual risk behaviour and intravaginal practices, are expected to vary with socioeconomic status and demographic and makes the analyses of the effects of individual factors, such as hormonal contraception, challenging. Women who rely on certain hormonal contraceptive methods potentially behave differently from women using other types of hormonal contraception. In regards to HIV risk, another concern is the potential of differential misreporting of sexual behaviour between HC users and non HC users which may generate artificially inflated risk estimates (Heffron et al., 2017; Smith et al., 2017; McCoy et al., 2014). The application of a randomized, crossover study design is therefore crucial in order to tease out the biological impact of various hormonal contraceptive methods on the FGT environment and HIV risk, which could otherwise be markedly influence by differences in behaviour and the reporting of set behaviour. The randomized design of this study therefore represents one of its major strengths. Yet, since this study is open-label and, in the case of daily administered COCPs, requires a high level of adherence from the individual participants, we had to take into account that a study participant could potentially alter her sexual behaviour between visits from the sexual behaviour recorded at baseline. At crossover, we did not observe any significant differences in reported sexual behaviour according to either

study arm or hormonal contraceptive method. Yet, when combining the data from crossover and exit we observed significant differences in reported sexual risk behaviour in regards to condom usage. At crossover, participants initially assigned to NuvaRing were given the choice of COCPs or Net-En as their second method. As such, the data from the final visit cannot be considered data from a randomized cohort. As expected based on the general preference among young women in South Africa (Sibeko et al., 2011; Ross & Agwanda, 2012), the majority of the participants chose Net-En over COCPs (33 versus 12). When we looked at the reported sexual behaviour exclusively from the exit visit, we observed a trend in bias in sexual behaviour with participants who chose COCP reporting a higher level of condom use since their last visit compared to the participants who chose Net-En as their second option. Amongst COCP participants, a lower number of sexual partners and a higher level of condom use at their most recent vaginal-penile intercourse (91.7% versus 67.7%) was also reported. Among the COCP participants, more than half (58.4%) reported condom use always or more than half the time. The same was reported only by a fourth (25.8%) of the participants who chose Net-En. These data could suggest that an impact of increased condom use was partly responsible for the lower alpha diversity and decreased levels of BV-associated bacteria observed in COCP users, yet the differences between contraceptive methods were still significant after adjusting for condom use.

5.4.4 Limitations

One limitation of this study is the relative small number of enrolled participants with only 131 participants randomized to our three study arms. Yet, the retention rate at the crossover visit was 82% (107 of 131) and 70% (92 of 131) at the exit visit, which is relatively high for a cohort of adolescents. Also, the longitudinal study design provided us with the opportunity to perform both within-subject assessment comparing the time periods before and after randomization to one of the three contraceptives in addition to the across study arms comparison of the difference between the study interventions. Thus, each participant can function as their own control and paired analyses are possible providing increased statistical power. Another limitation of this study is that the

screening visit in most cases did not represent a true, contraceptive naïve baseline control as most participants where on hormonal contraception before initiating the study. We therefore had to take the hormonal contraception used at screening into account. Furthermore, the study is only randomized at the first follow-up visit, and we cannot treat the exit visit as part of a randomized cohort. Due to the construction of the study design, the participants were only on two out of three hormonal contraceptive options, thus we are lacking Net-En to COCP and COCP to Net-En transition arms for comparison. Future randomized trials with a larger number of participants are needed to further tease out the biological impact of hormonal contraception on the FGT microbiota. Nonetheless, insights gained from studies like this can potentially contribute to the development of therapeutic strategies in modulating the microbiota composition for disease prevention.

5.4.5 Conclusions

In summary, our data suggest that COCPs use may exert a positive influence on genital health through an increase in lactobacilli and a decrease in BV-associated bacterial taxa with an accompanying decrease in overall bacterial diversity and vaginal pH. In contrast, the vaginal microbiota of participants on Net-En and NuvaRing had increased levels of bacteria associated with BV and HIV risk. The underlying biological mechanism of how hormonal contraception changes the vaginal microbiota and facilitates the acquisition of STIs is however still poorly understood.

Chapter 6: Hormonal-induced changes to the vaginal microbiota and its relationship to genital mucosal immunity in adolescents

6.1 Introduction

Use of hormonal contraception, particular progestin-only injectables, has been associated with increased HIV susceptibility in young women (Vodstrcil et al., 2013; van de Wijgert et al., 2013; Morrison et al., 2015). One mechanism by which hormonal contraceptives could impact HIV risk is by altering the bacterial community of the female genital tract (FGT). An optimal vaginal microbiota is generally dominated by *Lactobacillus* species which are believed to protect the FGT against pathogens through the production of bacteriocins, lactic acid, and hydrogen peroxide (Amabebe & Anumba, 2018). Bacterial vaginosis (BV) is a common vaginal condition associated with increased risk of sexually transmitted infections (STIs) and poor reproductive outcomes (Afolabi et al., 2016; Gupta et al., 2000; Myer et al., 2005a; Borgdorff et al., 2014). BV is characterized by the replacement of lactobacilli species by a diverse group of anaerobic bacteria including *Gardnerella vaginalis*, *Prevotella*, *Sneathia* and *Atopobium vaginae*. Hormonal contraceptive induced-dysbiosis could in return cause changes to the genital tract mucosa resulting in enhanced HIV susceptibility. Such potential alterations include increased genital tract inflammation (Chandra et al., 2013; Ghanem et al., 2005; Ildgruben et al., 2003), increased recruitment and activation of HIV target cells in the FGT (Byrne et al., 2015; Prakash et al., 2005, 2002; Zang et al., 2002) and changes to the vaginal epithelium (Miller et al., 2000a; Eschenbach et al., 2000a; Bahamondes et al., 2014; Mauck et al., 1999). Women with elevated genital tract inflammatory markers, including pro-inflammatory cytokines, are at increased risk of acquiring HIV (Masson et al., 2015a). An increasing number of studies have suggested that high microbial diversity and specific BV-associated bacteria induce an inflammatory response in the vaginal mucosa, potentially modifying susceptibility to STIs, while lactobacilli dominant communities have been associated with reduced inflammation (Lennard et al., 2017; Gautam et al., 2015; Anahtar et al., 2015; Gosmann et al., 2017; Rebbapragada et al., 2008). Women with BV have also been shown to harbour an increased level of mucosal HIV target cells expressing CCR5, a co-receptor required for HIV cell entry (Thurman et al., 2015). Furthermore, successful treatment of BV with oral metronidazole leads to a significant decrease in activated, cervical CD4+ T cells (Rebbapragada et al., 2008). The increased

inflammation and elevated levels of CCR5 expressing CD4+ T cells seen among women with BV could predispose women with vaginal dysbiosis to HIV acquisition (Alcaide et al., 2016). T helper type 17 (Th17) cells represent a major immune cell subset in the FGT and are highly permissive to HIV infection (Rodriguez-Garcia et al., 2014; McKinnon et al., 2011; Gosselin et al., 2011; Monteiro et al., 2011; Hed et al., 2010). Th17 cells are characterized by interleukin-17 (IL-17), IL-21 and IL-22 production and express surface markers such as CCR4, CCR6 and CD161 (Korn et al., 2009). The development of gut residing Th17 cells have been shown to be regulated by gut commensals (Sawa et al., 2011; Shaw et al., 2012; Zaph et al., 2008; Ivanov et al., 2008) suggesting an interplay of mucosal bacteria and this particular immune cell type. Much is yet to be learned about the interplay of microbial communities and mucosal immunity and how these interactions can affect health and disease. The aim of this study was to assess whether hormonal contraceptive-induced changes to the vaginal microbiota affect the numbers, activation status and co-receptor expression of genital immune cell populations, including HIV-permissive Th17 cells, and the inflammation status of the genital tract mucosa, to establish a biological basis of how hormonal contraception and the vaginal microbiota potentially alters HIV susceptibility.

6.2 Materials and methods

For this analysis, we included all 151 adolescent girls screened for the uCHOOSE-A-Star study (described in Chapter 2.1.1 (p. 46-48)). The study procedures were approved by the Human Research Ethics Committee of the University of Cape Town, and informed consent or assent (if <18 years) was obtained from all participants before initiation of the study. Informed consent was obtained from a parent or legal guardian of participants younger than 18 years old. Information on demographics, medical and reproductive history and sexual risk behaviours was collected. Cervical secretions were collected at each visit with a menstrual cup for cytokine analysis. The concentrations of interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, interferon (IFN)-γ, soluble CD40-ligand (sCD40L) and tumor necrosis factor (TNF)-α in Softcup® cervical secretions were measured by Luminex Milliplex assay using the Bio-Plex

Pro Human Th17 Cytokine Panel as described in Chapter 2.4.1 (p. 57). Vulvovaginal and lateral wall swabs were collected for STI, BV and candidiasis screening and vaginal pH measurement. An additional vaginal swab from the lateral wall was obtained for microbiota analysis. Genomic DNA was extracted from the swab and 16S rRNA amplicon sequencing libraries were generated as described in Chapter 2.2.1 and 2.2.3 (p. 51-55). Upstream bioinformatics pre-processing of sequencing reads was conducted as described in Chapter 2.2.4 (p. 55-56). A cervical cytobrush was collected for *ex vivo* flow cytometry analysis of cervical immune cells. Flow cytometry analysis was performed by Doctoral candidate Iyaloo Konstantinus, Division of Virology, University of Cape Town, on cytobrushes collected at all three visits as described in Chapter 2.4.2 (p. 58). All downstream statistical analyses were performed in RStudio as described in Chapter 2.3 and 2.4.3 (p. 55-56, 60).

6.3 Results

6.3.1 Inflammation and the vaginal microbiota

Cytokine measurements were conducted on all available cervical samples from all participants at all time points (two samples were mixed during processing and consequently discarded). Cytokine and microbiota data were available for 335 sample visits from 149 participants of which 324 sequencing samples were assigned to a community cluster using Fuzzy clustering as described in Chapter 4 (section 4.3.2) The average detection rate for all fifteen cytokines analysed was 77% (Appendix VIII). For the individual cytokines, a cut-off of 55% detectable samples was set. Two cytokines, IL-10 and IL-4, were excluded from downstream analysis due to low sensitivity (43% and 39% of samples detectable, respectively). The overall intra- and interplate quality control (QC) measures were found acceptable for the remaining cytokines (Appendix VIII). We performed unsupervised clustering of the concentrations of remaining thirteen cytokines (IL-1β, IL-6, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L and TNF-α) using partitioning around medoids (PAM) clustering to define the overall genital inflammation status of the participants in this cohort. Using PAM clustering with Euclidean distances, two distinct clusters (k=2) based on the maximum average silhouette width (0.336) appeared, with

56.4% (n=189) being classified as inflammation high ("High") and 43.6% (n=146) classified as inflammation low ("Low") (**Figure 6.1**). The alpha diversity was significantly higher in the high inflammation group compared to the low inflammation group (**Figure 6.1B**). A linear mixed-effects (lme) model, including visits and participant ID, confirmed this ($p<0.001$).

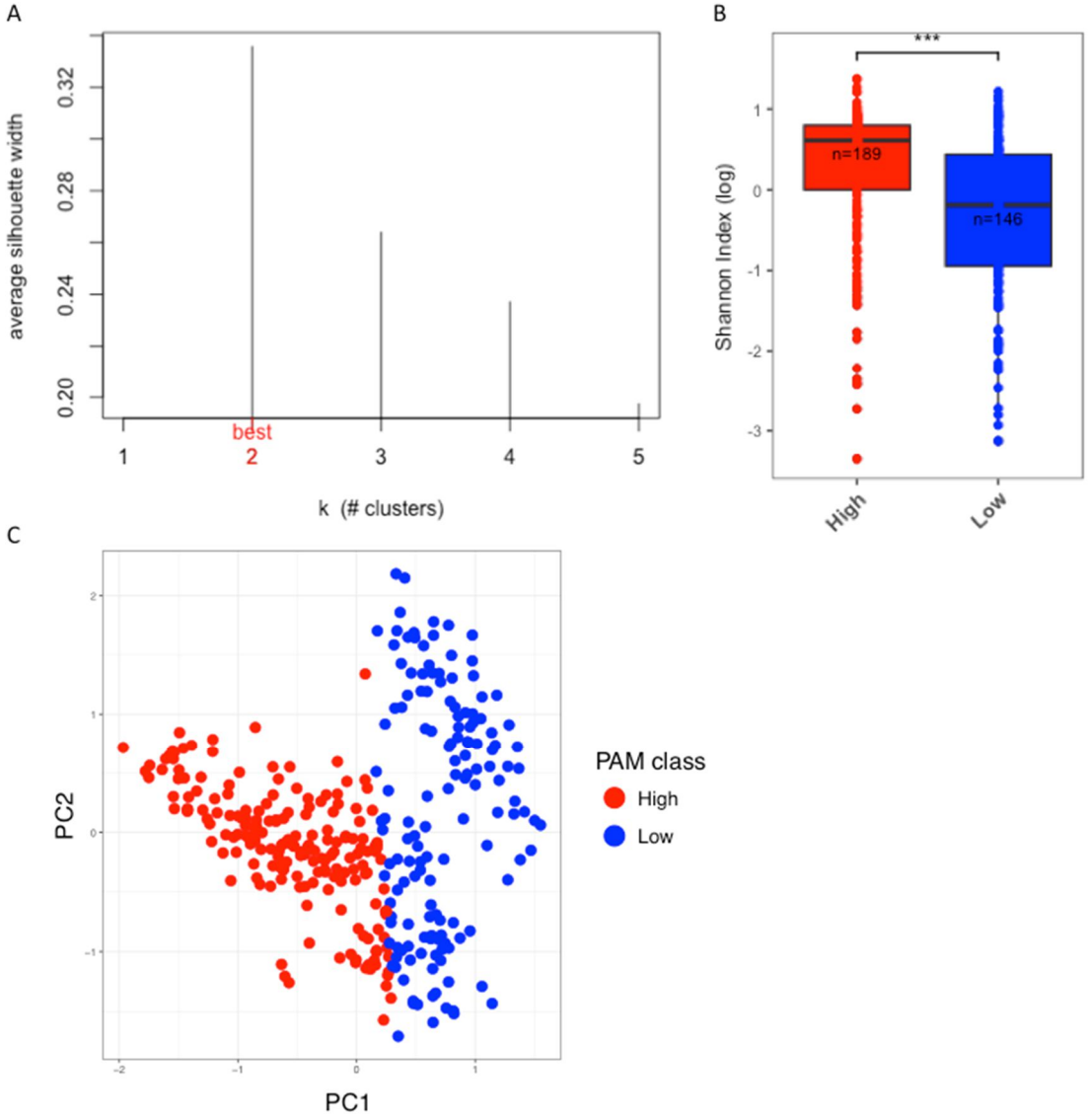


Figure 6.1. Cytokine clusters and alpha diversity. A). Barplot depicting the average silhouette width when applying different k's (1-5) for partitioning around medoids (PAM) clustering using Euclidean distances of the cytokine data. Optimal k was determined based on the highest average silhouette width. B) Boxplot showing the alpha diversity measured using Shannon Index (log transformed values) of the microbiota from 335 vaginal samples from 149 participants according to inflammation group (high versus low) identified by PAM clustering using Euclidean distances of the cytokine data. ***=p<0.001. C) PCoA of the cytokine data coloured by cytokine cluster (high, low) generated by PAM clustering with Euclidean distances.

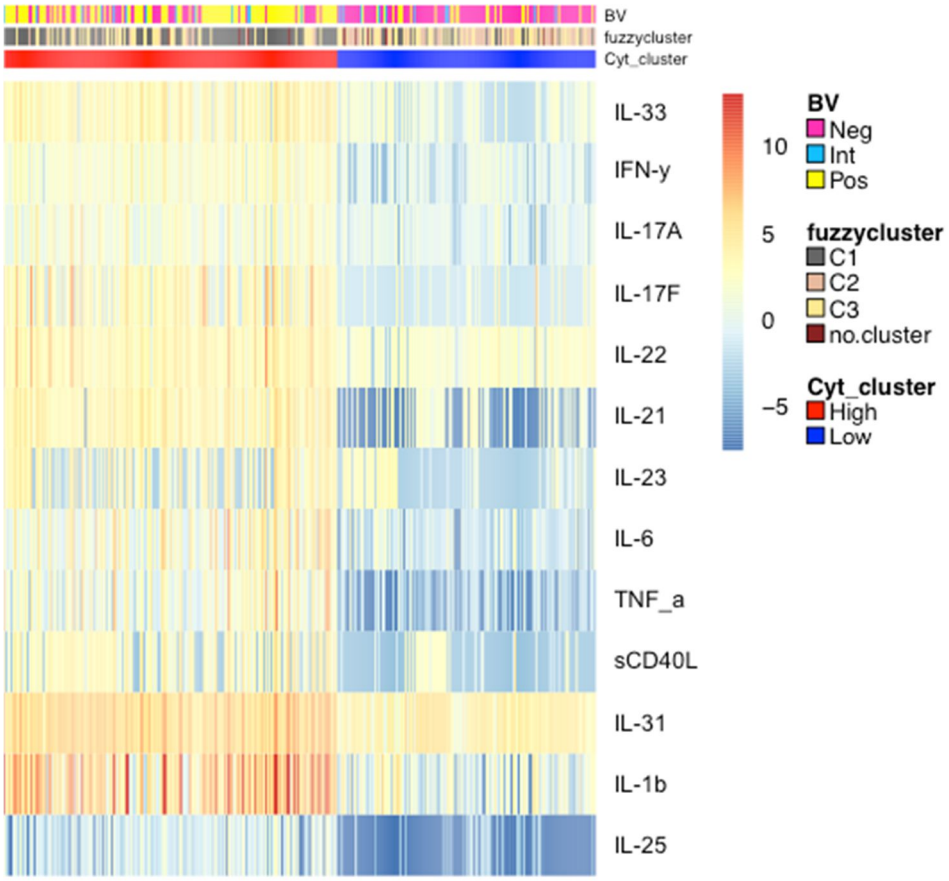


Figure 6.2. Cytokine clusters and the vaginal microbiota. Heatmap of cytokine concentrations of 13 cytokines from a Th17 bioplex panel from 335 samples from 149 participants using average linkage clustering with Euclidean distances. Annotation bars above the heatmap depict inflammation group generated by partitioning around medoids (PAM) clustering using Euclidean distances of the cytokine data (bottom bar), community cluster identified using Fuzzy clustering using weighted UniFrac distances (middle bar), BV status based on Nugent scoring (top bar). Log2-transformed cytokine concentrations are illustrated by the colour key. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from downstream analyses ("no.cluster" in figure, n=11).

We also found a significant difference in beta diversity between the high and low inflammation group (adonis, p=0.001, R=0.078) suggesting a significant difference in microbiota composition between these two groups (**Figure 6.3A**). Dispersion of the community structures of participants in the two groups did not significantly differ (betadisp, p=0.841, F=0.041) (**Figure 6.3B**) suggesting that there is no difference in the overall heterogeneity of species composition (dispersion) between the two groups. There were a significantly higher proportion of BV positive participants in the high inflammation group (p<2.2e-16) and high inflammation was associated with the diverse community type C1 as defined in Chapter 4 (**Figure 6.2** and **Table 6.1**). Using logistic regression, participants with the diverse C1 community was more likely to belong to the high inflammation group compared to both the *L. crispatus* dominant C2

community type (OR: 15.8; 95% range: 8.1-32.4, p=4.21e-15) and the *L. iners* dominant C3 community type (OR: 5.1; 95% range: 2.9-9.0, p=2.44e-08) after adjusting for STIs. Participants with a *L. crispatus* dominant type was less likely to be in the high inflammation group compared to participants in the *L. iners* dominant community type (OR: 0.32; 95% range: 0.16-0.62, p=0.001).

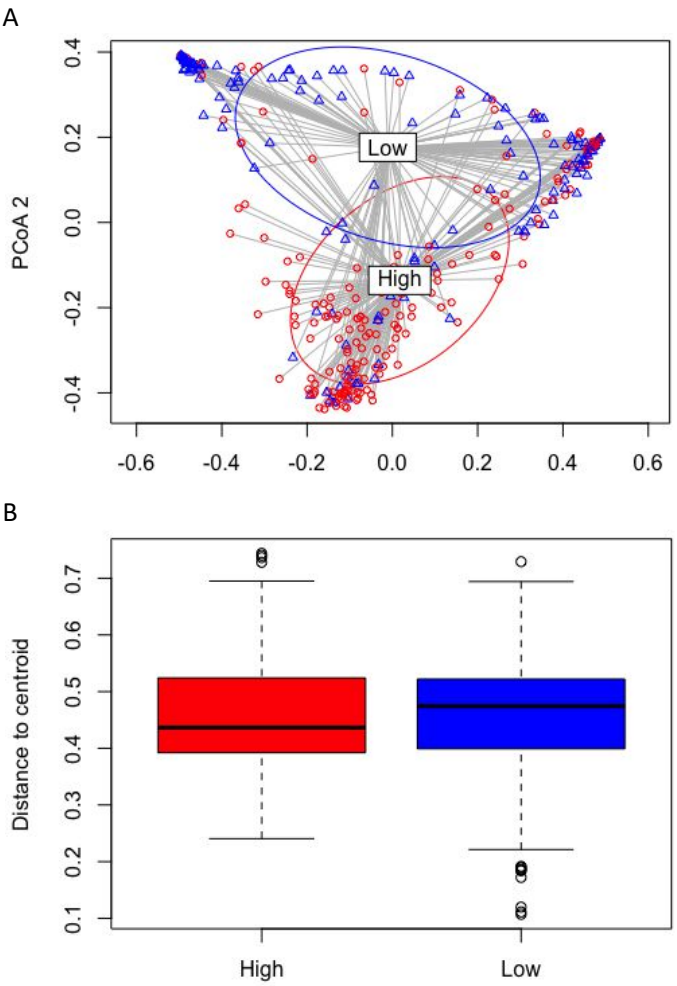


Figure 6.3. Cytokine clusters and beta diversity. A) Principal Coordinates Analysis (PcoA) of beta diversity calculated using weighted UniFrac according to cytokine cluster with standard deviations ellipses around each cytokine cluster centroid. B) Barplot depicting the distance to the centroid of the cytokine cluster for each sample in each cytokine cluster.

However, no differences in inflammation groups were observed in prevalence of positive HSV-2 serology or STIs and yeast infections (**Table 6.1**). There was also no significant difference in the distribution of hormonal contraceptive methods between the inflammation groups. Of interest however, when comparing samples taken only from participants while on of the three HC methods investigated in this cohort; COCP, Net-En and NuvaRing, the differences were

close to significant (p=0.056) and there was a significantly higher proportion of the inflammation high group in samples from participants on NuvaRing compared to participants on COCP (p=0.037) (**Table 6.2**). There was a trend towards a higher proportion of high inflammation in the samples from participants on NuvaRing versus participants using Net-En (p=0.076). There was a slightly higher level of intravaginal practices reported by participants in the high inflammation group, albeit not significantly so (**Table 6.1**). There were also no differences observed between the inflammation groups in regard to sexual risk behaviour (**Table 6.1**).

Table 6.1. Participant characteristics according to inflammation cluster.

	High (n=189)	**Low** (n=146)	**P value**
Fuzzycluster[1]			**<2.2e-16**
C1 (Diverse)	123 (66.5%)	28 (20.2%)	
C2 (*L. crispatus* dominated)	16 (8.6%)	58 (41.7%)	
C3 (*L. iners* dominated)	46 (24.9%)	53 (38.1%)	
BV prevalence			**<2.2e-16**
BV positive	118 (62.4%)	24 (16.4%)	
BV intermediate	13 (6.88%)	10 (6.85%)	
BV negative	58 (30.7%)	112 (76.7%)	
Vaginal pH, mean (sd)	5.02 (4.51-5.52)	4.58 (3.98-5.17)	**1.5e-12**
Shannon Index, median (IQR)	1.84 (1.00-2.22)	0.83 (0.39-1.55)	**3.7e-11**
Age at screening, median years (IQR)	17 (16-18)	17 (16-18)	0.747
STI prevalence			
Any STI(s)	72 (38.1%)	48 (32.9%)	0.383
Ct	50 (26.5%)	32 (21.9%)	0.407
Ng	20 (10.6%)	14 (9.59%)	0.908
Tv	14 (7.41%)	5 (3.42%)	0.185
Mg	6 (3.17%)	4 (2.74%)	1.000
HSV-2 serology[2]	56 (29.6%%)	52 (35.6%%)	0.286
Yeast cells present	32 (16.9%)	26 (17.8%)	0.948
Use of hormonal contraception[3]			0.288
None	20 (10.6%)	16 (11.3%)	
Net-En	78 (41.5%)	65 (45.8%)	
COCP	26 (13.8%)	28 (19.7%)	
DMPA	11 (5.85%)	8 (5.63%)	
NuvaRing	51 (27.1%)	24 (16.9%)	
Implanon	2 (1.06%)	1 (0.70%)	
Intra-vaginal practices[4]			
Douching	1 (1.27%)	0 (0.00%)	1.000
Washing with water	14 (17.7%)	5 (4.69%)	0.137
Washing with soap	10 (12.7%)	3 (7.81%)	0.144
Cloth	4 (5.06%)	0 (0.00%)	0.128
Drying	7 (8.86%)	1 (1.56%)	0.075
Medication	4 (5.06%)	3 (7.81%)	1.000
Tampon use	1 (1.27%)	0 (0.00%)	1.000
Herbs	1 (1.27%)	0 (0.00%)	1.000
Sexual risk behaviour[5]			
Age of sexual debut, median (IQR)	15 (14-16)	15 (14-16)	0.975
Sexual partners, median (IQR)	1 (1-1)	1 (1-1)	0.078
Multiple sexual partners, n (%)	3 (17.5%)	2 (15.4%)	0.896
New partner, n (%)	25 (14.6%)	16 (12.3%)	0.698

General condom use			0.811
Never	18 (10.5%)	11 (8.46%)	
Almost never	15 (8.77%)	10 (7.69%)	
Not sure	21 (12.3%)	22 (16.9%)	
Almost always	67 (39.2%)	49 (37.7%)	
Always	50 (29.2%)	38 (29.2%)	
Condom use during last PV intercourse			
Yes	100 (58.5%)	84 (64.6%)	0.294
PV sex acts per week, median (IQR)	1 (1-2)	1 (1-2)	0.899
Intergenerational sex with older partner (≥5 years)			0.886
No	126 (73.3%)	94 (72.3%)	
I don't think so	4 (2.3%)	3 (2.3%)	
Not sure	23 (13.4%)	16 (12.3%)	
I think so	3 (1.7%)	1(0.8%)	
Yes	16 (9.3%)	16 (12.3%)	
Transactional sex	1 (0.6%)	0 (0.0%)	1.000
Penile-anal intercourse	3 (1.7%)	1(0.8%)	0.637

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups, unpaired Mann-Whitney-Wilcoxon test for comparison of medians and unpaired Student's t test for comparison of means. BV, bacterial vaginosis; Ct, Chlamydia trachomatis*; HSV-2, herpes simplex virus type 2; IQR, interquartile range; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. No Fuzzy cluster assignment for eleven samples (four in "High" and seven in "Low").
2. One equivocal result.
3. Missing data from five participants (1 from "High" and 4 from "Low").
4. Information only collected at screening. High: n=79, Low: n=64.
5. Missing data from 34 participant visits (18 from "High" and 16 from "Low").

Table 6.2. Distribution of high and low inflammation groups according to hormonal contraception.

	COCP (n=54)	Net-En (n=143)	NuvaRing (n=75)	P value
Inflammation group				0.056
High	26 (48.1%)	78 (54.5%)	51 (68.0%)	
Low	28 (51.9%)	65 (45.5%)	24 (32.0%)	

When looking at the differences of the individual cytokines between the three community types, four cytokines, IL-21, IL-23, IL-31 and IL-33 did not significantly differ between community types C2 (*L. crispatus*-dominated) and C3 (*L. iners*-dominated) and IL-17A did not differ between C1 and C3 (**Figure 6.4**, **Figure 6.5** and **Table 6.1**). All other cytokines measured differed significantly between the three community types. For all cytokines, except IL-17A, the levels were significantly higher in C1 (diverse cluster) compared to both C2 and C3 and the cytokine levels were higher in C3 versus C2 in all cases but the four mentioned above (**Figure 6.4**, **Figure 6.5** and **Table 6.3**). The same outcome was found using lme model analysis ($p<0.05$). The cytokine concentrations for all thirteen cytokines were significantly positively correlated with alpha diversity ($p<0.001$) (**Figure 6.6** and **Table 6.4**). The strongest correlations were observed for IL-1β ($r=0.58$) and TNF-α ($r=0.52$) for which the strength of the correlation

was moderate while the strength of the correlations were weak for the remaining eleven cytokine (rho values ranging from 0.23-0.38).

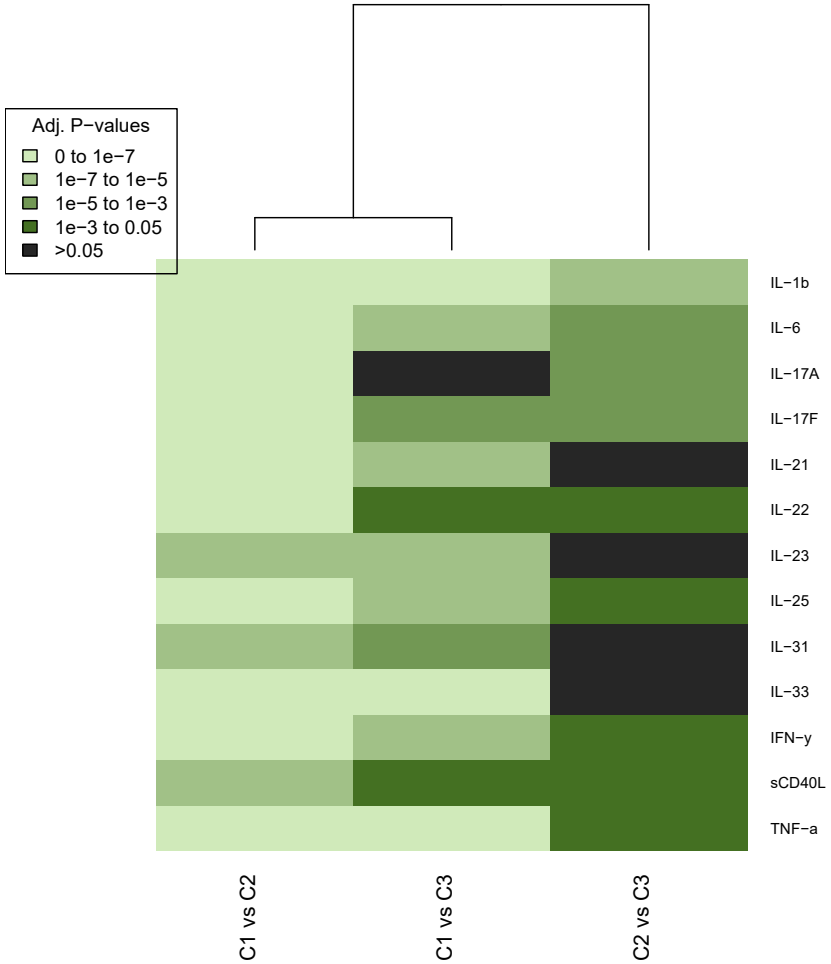


Figure 6.4. Community types and cytokine levels. Heatmap summary of cytokines levels in 324 samples by vaginal microbiota community type (C1, C2, C3) highlighting cytokines differing significantly between clusters. Differences in the individual cytokines between community clusters were assessed using unpaired Mann-Whitney-Wilcoxon test adjusted for multiple comparisons using the Benjamini-Hochberg method. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from the analyses (n=11).

Table 6.3. Differences in cytokine levels according to Fuzzy cluster.

	C1	C2	C3	P value	Adj. P	C1 vs. C2		C1 vs. C3		C2 vs. C3	
	(median (IQR))	(median (IQR))	(median (IQR))			P	Adj. P	P	Adj. P	P	Adj. P
IL-1β	60.4 (14.4-221.8)	0.51 (0.14- 2.50)	2.87 (0.77-24.1)	**1.3e-29**	**4.9e-28**	**6.0e-25**	**2.3e-23**	**1.2e-14**	**1.2e-13**	**3.4e-06**	**6.4e-06**
IL-6	2.84 (1.00-14.3)	0.34 (0.17-0.59)	0.61 (0.37-5.30)	**8.5e-15**	**9.0e-14**	**3.7e-14**	**2.9e-13**	**4.4e-06**	**7.8e-06**	**0.0003**	**0.0005**
IL-17A	2.04 (1.07-5.55)	0.94 (0.63-1.29)	1.56 (0.92-3.90)	**4.2e-09**	**1.2e-08**	**1.7e-09**	**5.5e-09**	0.092	0.099	**2.3e-05**	**4.0e-05**
IL-17F	7.37 (0.83-16.8)	0.34 (0.34-1.54)	2.02 (0.34-9.48)	**1.7e-12**	**8.4e-12**	**1.1e-12**	**6.2e-12**	**0.0002**	**0.0004**	**0.0005**	**0.0007**
IL-21	10.7 (4.43-25.4)	2.87 (0.02-6.47)	4.35 (0.01-10.7)	**2.1e-11**	**4.5e-11**	**1.5e-10**	**6.0e-10**	**6.7e-07**	**1.6e-06**	0.183	0.188
IL-22	9.10 (6.05-16.6)	4.65 (3.19-7.02)	6.12 (3.91-11.3)	**2.9e-09**	**9.2e-09**	**8.9e-10**	**3.2e-09**	**0.0008**	**0.001**	**0.018**	**0.021**
IL-23	7.00 (0.12-16.9)	0.12 (0.12-3.59)	0.12 (0.12-7.22)	**1.3e-08**	**1.7e-08**	**2.6e-07**	**7.1e-07**	**3.1e-06**	**6.0e-06**	0.703	0.703
IL-25	0.37 (0.08-1.28)	0.01 (0.01-0.13)	0.06 (0.01-0.37)	**1.5e-13**	**3.8e-13**	**1.3e-12**	**6.1e-12**	**4.7e-07**	**1.2e-06**	**0.007**	**0.009**
IL-31	47.6 (26.1-99.9)	26.1 (16.2-41.9)	27.4 (17.2-53.8)	**4.7e-07**	**4.7e-07**	**8.3e-07**	**1.8e-06**	**0.0001**	**0.0002**	0.131	0.138
IL-33	9.51 (4.07-21.2)	2.92 (0.75-3.65)	3.65 (0.54-6.85)	**4.9e-14**	**1.9e-13**	**2.7e-12**	**1.2e-11**	**1.2e-08**	**3.7e-08**	0.072	0.080
IFN-γ	3.46 (1.82-8.15)	1.19 (0.47-2.11)	1.71 (0.60-3.65)	**1.7e-13**	**4.8e-13**	**4.8e-13**	**3.1e-12**	**1.3e-06**	**2.6e-06**	**0.008**	**0.010**
sCD40L	4.91 (0.07-15.6)	0.07 (0.07-2.35)	0.90 (0.07-7.35)	**9.7e-07**	**1.1e-06**	**7.1e-07**	**1.63e- 6**	**0.003**	**0.003**	**0.011**	**0.014**
TNF-α	2.69 (0.90-9.43)	0.16 (0.01-0.35)	0.17 (0.05-0.90)	**1.2e-25**	**1.8e-24**	**1.3e-20**	**2.52e-19**	**6.3e-16**	**8.15e-15**	**0.041**	**0.047**

Cytokine concentrations in pg/ml. P values calculated using the Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. The data is from 324 samples from 149 participants. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from the analyses (n=11).

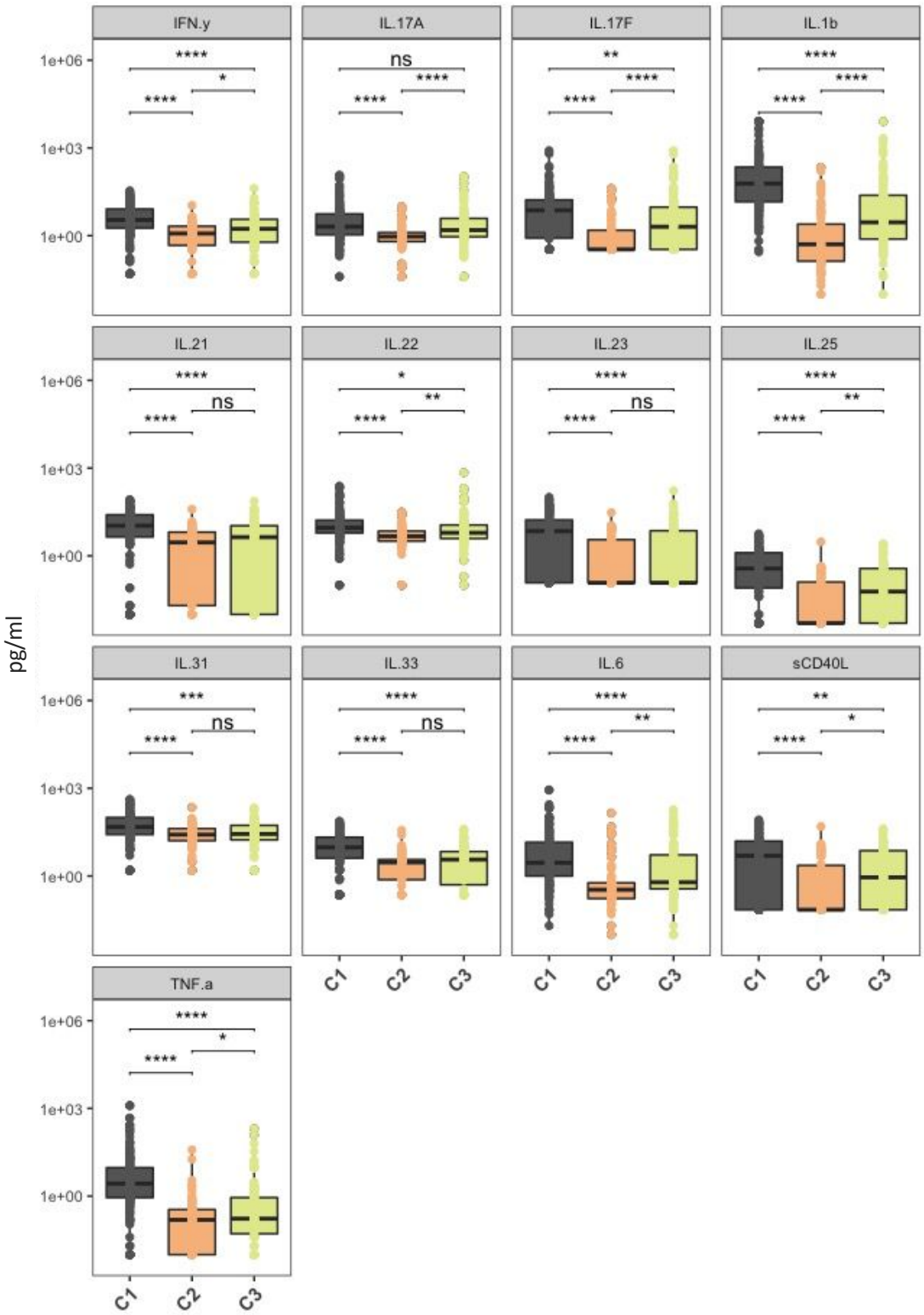


Figure 6.5. Cytokine levels according to Fuzzy cluster. Boxplots showing the concentration of 13 cytokines in pg/ml in 324 samples from 149 participants stratified by community type determined by Fuzzy clustering using weighted UniFrac. P values were generated using the unpaired Mann-Whitney-Wilcoxon test adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. Y-axis is log10 transformed. ****=adj. p<0.0001, ***= adj. p<0.001, **=adj. p<0.01, *=adj. p<0.05, ns=adj. p>0.05. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from the analyses (n=11).

pg/ml

Figure 6.6. Correlation plots of cytokines concentrations and alpha diversity. Correlation plots showing the concentration of 13 cytokines in pg/ml according to alpha diversity measured with Shannon Index in 335 vaginal samples from 149 participants. The Spearman correlation coefficient (rho, r) and the p values for each individual cytokine depicted on the figure. Y axis log10 transformed.

Table 6.4. Correlation coefficients for correlation of cytokine concentrations and alpha diversity.

	rho	P value
IL-1β	0.58	<2.2e-16
IL-6	0.38	5.5e-13
IL-17A	0.23	1.9e-05
IL-17F	0.32	1.7e-09
IL-21	0.27	3.9e-07
IL-22	0.27	7.6e-07
IL-23	0.29	4.4e-08
IL-25	0.36	1.0e-11
IL-31	0.23	2.3e-05
IL-33	0.35	3.6e-11
IFN-γ	0.32	1.2e-09
sCD40L	0.23	2.4e-05
TNF-α	0.52	<2.2e-16

The Spearman correlation coefficient (rho, r) and the p values for each individual cytokines.

We analysed the differences in the individual cytokine levels between the three hormonal contraceptives investigated in this study. In an according to protocol analysis at crossover and exit, nine cytokines (i.e. IL-1β, Il-6, IL-17A, IL-21, IL-22, IL-25, IL-33, IFN-γ and TNF-α) were significantly elevated in NuvaRing participants versus participants using COC of which two, IL-1β and Il-6, remained significant after adjusting for multiple comparisons (**Figure 6.7** and **Table 6.5**). Three cytokines (i.e. IL-1β, Il-6 and IL-17A) were significantly elevated in participants on NuvaRing compared to Net-En but not after adjusting for multiple comparisons (**Figure 6.7** and **Table 6.5**). A similar pattern was observed in an intention-to-treat and according to protocol analysis at crossover (Appendix VIII). In a paired analysis of matched samples from 26 participants who went either from COCP to Net-En or vice versa, no significant intra-individual differences in cytokine levels were observed (**Figure 6.8A** and **Table 6.6**). In contrast, the concentration of all thirteen analysed cytokines were significantly elevated within participants when using the NuvaRing compared to when using COCP (31 participants, **Figure 6.8B** and **Table 6.7**) or Net-En (57 participants, **Figure 6.8C** and **Table 6.8**).

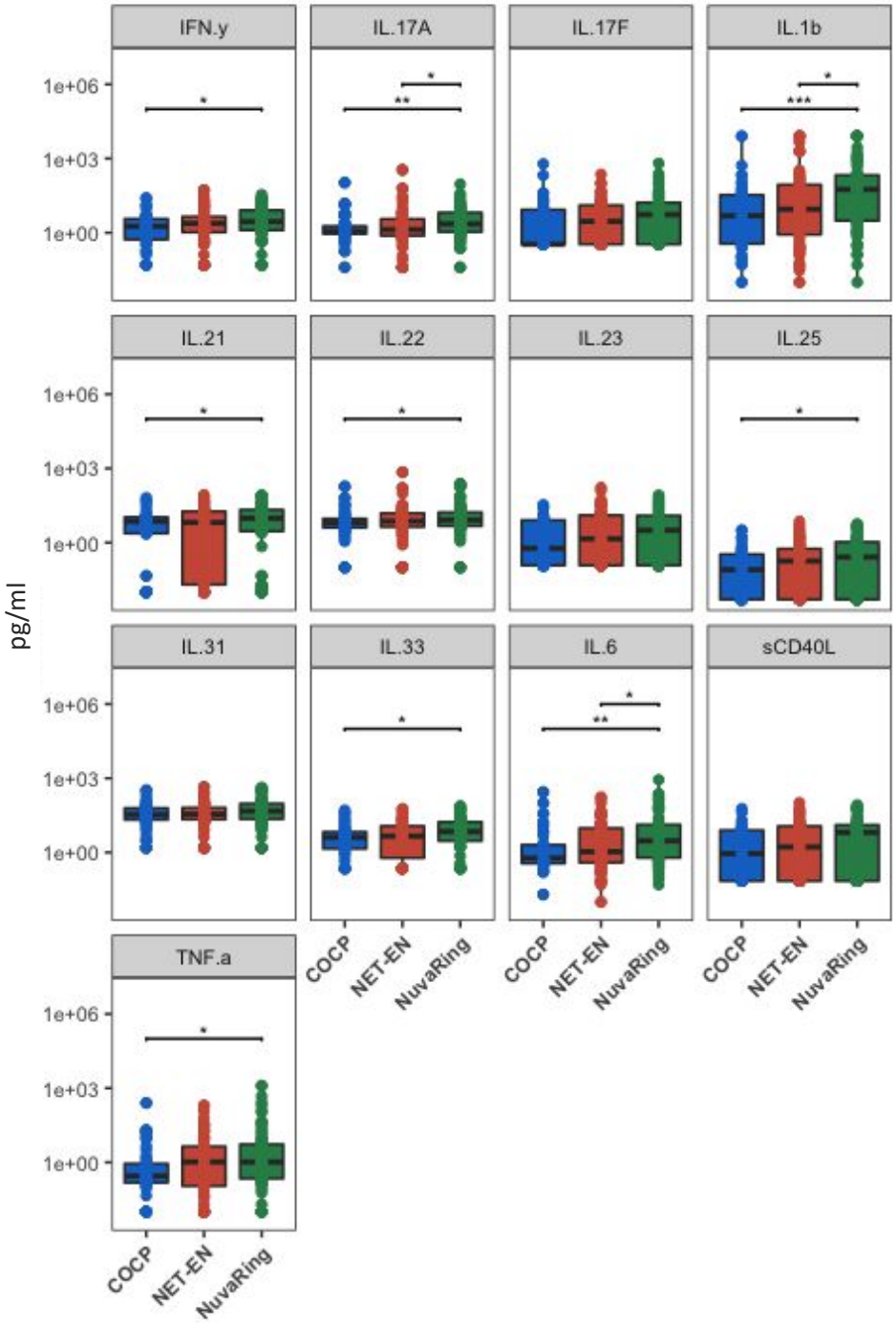


Figure 6.7. Cytokine levels according to hormonal contraceptive method. Boxplots showing the levels of 13 cytokines in pg/ml in 195 samples from 107 participants stratified by hormonal contraceptive method. P values were generated using the unpaired Mann-Whitney-Wilcoxon test. Y-axis is log10 transformed. ****=p<0.0001, ***=p<0.001, **=p<0.01, *=p<0.05, ns=p>0.05.

Table 6.5. Differences in cytokine concentrations according to hormonal contraception across all visits.

	COCP	Net-En	NuvaRing	P value	Adj. P value	COCP vs. Net-En		COCP vs. NuvaRing		Net-En vs. NuvaRing	
	(median (IQR))	(median (IQR))	(median (IQR))			P	Adj. P	P	Adj. P	P	Adj. P
IL-1β	4.87 (0.36-34.2)	8.91 (0.84-88.4)	56.2 (3.04-213)	**0.002**	**0.025**	0.206	0.335	**0.001**	**0.020**	**0.027**	0.129
IL-6	0.59 (0.36-2.04)	1.07 (0.39-9.66)	2.96 (0.61-13.4)	**0.004**	**0.029**	0.275	0.377	**0.001**	**0.026**	**0.031**	0.129
IL-17A	1.14 (0.90-1.86)	1.33 (0.76-3.60)	2.27 (1.08-6.55)	**0.009**	**0.040**	0.764	0.785	**0.005**	0.068	**0.017**	0.121
IL-17F	0.34 (0.34-8.58)	2.88 (0.34-12.9)	5.42 (0.34-16.6)	0.208	0.215	0.518	0.612	0.100	0.245	0.223	0.344
IL-21	7.22 (2.35-10.7)	6.50 (0.02-18.3)	9.66 (2.87-21.4)	0.110	0.172	0.527	0.612	**0.037**	0.129	0.175	0.308
IL-22	6.43 (4.01-9.38)	7.30 (4.29-15.5)	8.19 (4.65-16.8)	0.119	0.172	0.562	0.612	**0.040**	0.129	0.182	0.308
IL-23	0.59 (0.12-8.15)	1.42 (0.12-12.9)	3.15 (0.12-12.4)	0.215	0.215	0.268	0.377	0.074	0.203	0.546	0.612
IL-25	0.08 (0.01-0.34)	0.18 (0.01-0.57)	0.26 (0.01-1.06)	0.067	0.146	0.154	0.299	**0.023**	0.129	0.282	0.377
IL-31	33.9 (21.0-62.3)	35.6 (21.7-65.0)	46.8 (22.4-94.9)	0.212	0.215	0.998	0.998	0.153	0.299	0.125	0.286
IL-33	4.08 (1.43-6.85)	4.58 (0.90-11.8)	7.00 (2.92-17.2)	**0.047**	0.123	0.565	0.612	**0.019**	0.121	0.078	0.203
IFN-γ	1.81 (0.54-3.65)	2.38 (1.04-4.48)	2.81 (1.27-8.15)	0.100	0.172	0.290	0.377	**0.036**	0.129	0.229	0.344
sCD40L	0.90 (0.07-7.95)	1.67 (0.07-7.95)	6.44 (0.07-13.0)	0.148	0.193	0.641	0.676	0.063	0.190	0.166	0.308
TNF-α	0.28 (0.15-0.89)	1.04 (0.11-4.43)	1.03 (0.23-5.38)	**0.041**	0.123	0.152	0.299	**0.007**	0.070	0.385	0.484

Cytokine concentrations in pg/ml. P values calculated using the Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. The data is from 195 samples taken at crossover and exit from 107 participants. COCP: n=47; Net-En: n=71; NuvaRing: n=77.

Figure 6.8. Cytokine levels within participants changing between specific hormonal contraceptive methods. Boxplots showing the concentration of 13 cytokines in pg/ml in A) 52 cervical samples from 26 participants changing from COCP to Net-En or vice versa, B) 62 cervical samples from 31 participants changing from COCP to NuvaRing or vice versa and C) 126 cervical samples from 57 participants changing from Net-En to NuvaRing or vice versa. P values were generated using paired Wilcoxon Signed Rank test. Y-axis is log10 transformed. ****=p≤0.0001, ***=p≤0.001, **=p≤0.01, *=p≤0.05, ns=p>0.05

Table 6.6. Cytokine levels in participants changing from COCP to Net-En or vice visa.

	COCP (median (IQR))	Net-En (median (IQR))	P value	Adj. P
IL-1β	5.71 (0.35-49.7)	5.92 (0.56-53.4)	0.820	0.888
IL-6	0.49 (0.36-2.03)	0.65 (0.44-2.84)	0.697	0.888
IL-17A	1.14 (0.85-2.14)	1.17 (0.80-1.99)	0.904	0.904
IL-17F	0.34 (0.34-10.1)	0.34 (0.34-6.83)	0.780	0.888
IL-21	7.60 (2.53-10.7)	7.45 (2.51-16.1)	0.649	0.888
IL-22	6.73 (3.87-11.7)	6.80 (4.34-9.00)	0.338	0.888
IL-23	1.06 (0.12-9.07)	0.77 (0.12-12.9)	0.732	0.888
IL-25	0.08 (0.01-0.49)	0.13 (0.01-0.37)	0.514	0.888
IL-31	32.0 (21.0-58.9)	36.9 (24.3-55.5)	0.767	0.888
IL-33	3.65 (0.73-5.85)	3.86 (2.50-10.0)	0.346	0.888
IFN-γ	1.81 (1.06-4.46)	1.71 (1.04-3.82)	0.420 0.532	0.888
sCD40L	1.29 (0.07-7.95)	1.71 (0.07-7.43)	0.399	0.888
TNF-α	0.23 (0.15-0.89)	0.40 (0.14-2.31)		0.888

Cytokine concentrations in pg/ml. P values calculated using paired Wilcoxon Signed Rank test adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. The data is from 52 samples taken from 26 participants.

Table 6.7. Cytokine levels in participants changing from COCP to NuvaRing or vice visa.

	COCP (median (IQR))	NuvaRing (median (IQR))	P value	Adj. P
IL-1β	2.4 (0.41-29.4)	51.3 (2.82-395)	**0.0002**	**0.001**
IL-6	0.51 (0.36-1.39)	1.96 (0.47-8.72)	**0.001**	**0.002**
IL-17A	1.29 (0.94-1.77)	1.97 (1.10-10.3)	**0.009**	**0.010**
IL-17F	0.34 (0.34-7.20)	7.11 (0.34-27.5)	**0.026**	**0.026**
IL-21	6.33 (0.59-10.5)	10.6 (4.21-26.4)	**0.001**	**0.002**
IL-22	6.58 (4.58-8.19)	10.8 (5.74-28.3)	**0.003**	**0.005**
IL-23	0.18 (0.12-6.83)	3.36 (0.71-12.7	**0.0002**	**0.001**
IL-25	0.05 (0.01-0.28)	0.33 (0.01-0.60)	**0.003**	**0.004**
IL-31	33.1 (18.7-65.8)	45.0 (26.1-92.8)	**0.010**	**0.011**
IL-33	3.65 (1.31-6.92)	8.04 (3.65-16.4)	**0.0003**	**0.001**
IFN-γ	1.94 (0.71-3.36)	3.14 (1.74-7.80)	**0.001**	**0.003**
sCD40L	0.86 (0.07-7.95)	8.34 (0.09-13.3)	**0.001**	**0.002**
TNF-α	0.30 (0.15-0.89)	0.97 (0.24-3.49)	**0.004**	**0.005**

Cytokine concentrations in pg/ml. P values calculated using paired Wilcoxon Signed Rank test adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. The data is from 62 samples taken from 31 participants.

Table 6.8. Cytokine levels in participants changing from Net-En to NuvaRing or vice visa.

	Net-En (median (IQR))	NuvaRing (median (IQR))	P value	Adj. P value
IL-1β	4.81 (0.89-39.0)	59.9 (2.74 -25)	**2.8e-06**	**3.6e-05**
IL-6	0.59 (0.44-3.35)	4.24 (0.77-25.3)	**1.4e-05**	**9.3e-05**
IL-17A	1.31 (0.85-3.70)	2.70 (1.20-6.62)	**0.007**	**0.008**
IL-17F	0.86 (0.34-10.8)	6.38 (0.34-16.6)	**0.023**	**0.024**
IL-21	5.06 (2.49-11.1)	10.3 (4.16-21.4)	**0.0003**	**0.001**
IL-22	6.70 (4.59-13.4)	8.65 (5.10-17.5)	**0.004**	**0.005**
IL-23	0.77 (0.12-8.51)	3.56 (0.12-14.5)	**0.021**	**0.022**
IL-25	0.08 (0.01-0.37)	0.37 (0.01-1.18)	**0.002**	**0.004**
IL-31	35.6 (26.1-54.4)	52.1 (22.4-106)	**0.002**	**0.003**
IL-33	3.50 (0.73-9.29)	7.58 (3.65-21.1)	**0.0002**	**0.001**
IFN-γ	2.11 (1.02-3.46)	3.26 (1.27-8.15)	**0.002**	**0.003**
sCD40L	1.65 (0.07-6.96)	5.99 (0.07-11.4)	**0.004**	**0.006**
TNF-α	0.47 (0.05-2.50)	1.14 (0.32-5.47)	**0.002**	**0.003**

Cytokine concentrations in pg/ml. P values calculated using paired Wilcoxon Signed Rank test adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. The data is from 126 samples from 57 participants (65 combinations).

6.3.2 Differentially abundant taxa between high and low inflammation groups

In a metagenomeSeq analysis, the relative abundances of 29 taxa (merged at lowest taxonomic level) were differentially abundant between the high and low inflammation group. Bacteria such as *Gardnerella vaginalis*, *Sneathia*, BVAB 1-3, *Megasphaera*, *Dialister* spp., *Atopobium vaginae, Aerococcus christensenii*, *Mobiluncus mulieri*s, *Prevotella* spp., *Gemella asaccharolytica, Mycoplasma hominis*, *Arcanobacterium* and *Parvimonas micra* were shown to have a significantly higher relative abundance in the high inflammation group compared to the low inflammation group (**Figure 6.9** and **Table 6.9**). In contrast, *Clostridium perfringens* and several *Lactobacillus* species including both *L. iners* and *L. crispatus*, had a higher relative abundance in the low inflammation group compared to the high inflammation group.

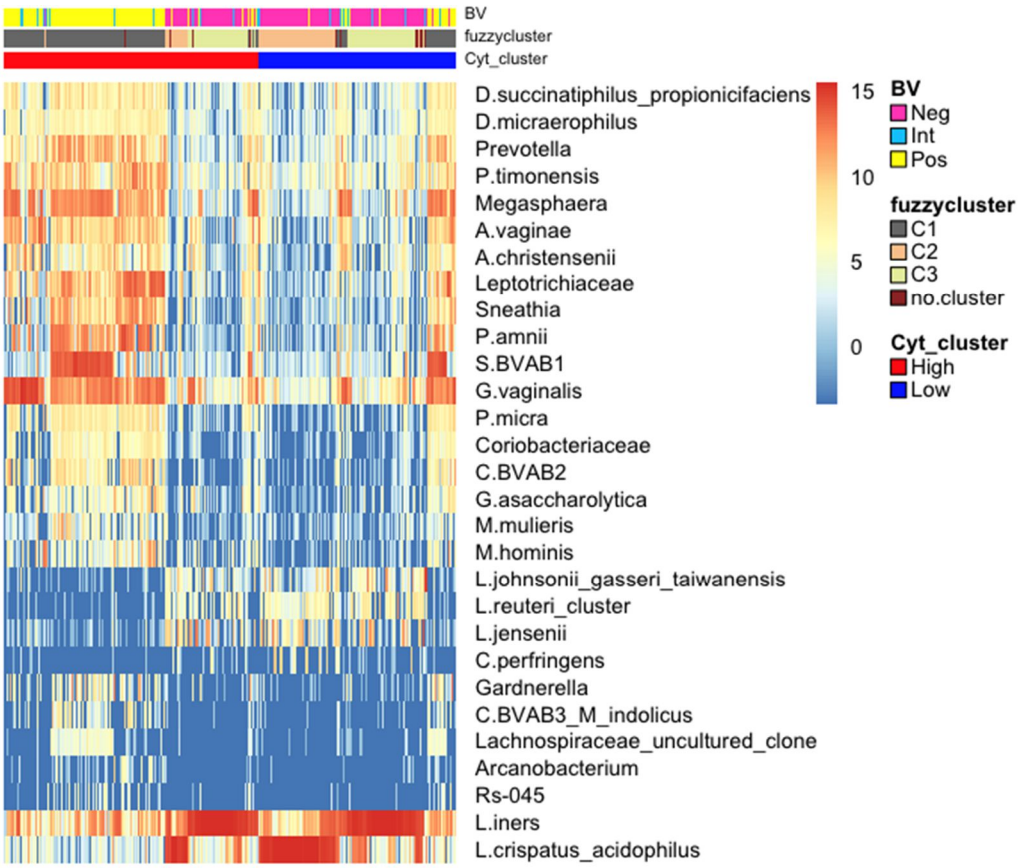


Figure 6.9. Differentially abundant taxa between low and high inflammation groups. Heatmap of bacterial taxa significantly differentially abundant and/or frequent by inflammation category in 335 vaginal samples from 149 participants analysed using metagenomeSeq (FDR ≤ 0.05, coefficient ≥ 1.25, taxa present in ≥ 20% of samples in at least one of the two groups being compared). The heatmap shows samples ordered by cytokine cluster followed by unsupervised clustering of samples (columns) by Bray-Curtis distances. Log2-transformed standardized read counts are illustrated by the colour key. Annotation bars above the heatmap depict cytokine derived inflammation group generated by partitioning around medoids (PAM) clustering using Euclidean distances of the cytokine data (Cyt_cluster, bottom bar), community cluster identified using Fuzzy clustering using weighted UniFrac distances (fuzzycluster, middle bar), BV status based on Nugent scoring (BV, top bar). Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from downstream analyses ("no.cluster" in figure, n=11).

Table 6.9. Differentially abundant taxa between low and high inflammation groups.

Coeff	P adj.	Family	Genus	Species
-3.635	1.8e-16	Leptotrichiaceae	*Sneathia*	NA
-3.601	3.6e-14	Leptotrichiaceae	NA	NA
-3.083	6.2 e-12	Bifidobacteriaceae	*Gardnerella*	*vaginalis*
-3.079	3.3e-14	Clostridiaceae	*Clostridium*	*BVAB2*
-2.905	2.1e-08	Veillonellaceae	*Megasphaera*	NA
-2.838	6.2e-12	Coriobacteriaceae	*Atopobium*	*vaginae*
-2.829	1.3e-07	Prevotellaceae	*Prevotella*	*amnii*
-2.624	8.4e-14	[Tissierellaceae]	*Parvimonas*	*micra*
-2.493	1.4e-12	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
-2.468	2.8e-09	Bifidobacteriaceae	*Gardnerella*	NA
-2.269	8.5e-10	Mycoplasmataceae	*Mycoplasma*	*hominis*
-2.260	2.7e-09	Prevotellaceae	*Prevotella*	*NA*

-2.176	8.0e-11	Gemellaceae	*Gemella*	*asaccharolytica*
-2.154	7.6e-12	Coriobacteriaceae	NA	NA
-1.985	0.0005	Lachnospiraceae	*Shuttleworthia*	BVAB1
-1.901	2.8e-08	Prevotellaceae	*Prevotella*	*timonensis*
-1.794	2.0e-06	Aerococcaceae	*Aerococcus*	*christensenii*
-1.608	2.4e-08	Veillonellaceae	*Dialister*	*succinatiphilus_propionicifaciens*
-1.552	7.0e-08	Lachnospiraceae	NA	NA
-1.538	5.7e-10	Veillonellaceae	*Dialister*	*micraerophilus*
-1.442	1.3e-07	Rs-045	NA	NA
-1.416	2.0e-06	Actinomycetaceae	*Mobiluncus*	*mulieris*
-1.325	4.4e-08	Actinomycetaceae	*Arcanobacterium*	NA
1.424	0.003	Lactobacillaceae	*Lactobacillus*	*iners*
1.911	1.4e-08	Clostridiaceae	*Clostridium*	*perfringens*
2.091	4.4e-06	Lactobacillaceae	*Lactobacillus*	*jensenii*
2.305	1.7e-09	Lactobacillaceae	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*
2.827	1.2e-16	Lactobacillaceae	*Lactobacillus*	*reuteri_cluster*
4.821	2.3e-17	Lactobacillaceae	*Lactobacillus*	*crispatus_acidophilus*

Similarly, the most predictive taxa of inflammation status determined using random forest analysis included several *Lactobacillus* species predictive of the low inflammation group with *L. crispatus* being the strongest predictor while taxa such as *Gardnerella vaginalis*, *Atopobium vaginae*, *Sneathia*, *Parvimonas micra* and *Dialister* spp. were found to be predictive of the high inflammation group (AUC=0.77, sensitivity=0.68, specificity=0.75 for the training set and a validated predicted error rate of 31.25% when using the top five features) (**Figure 6.10** and **Table 6.10**).

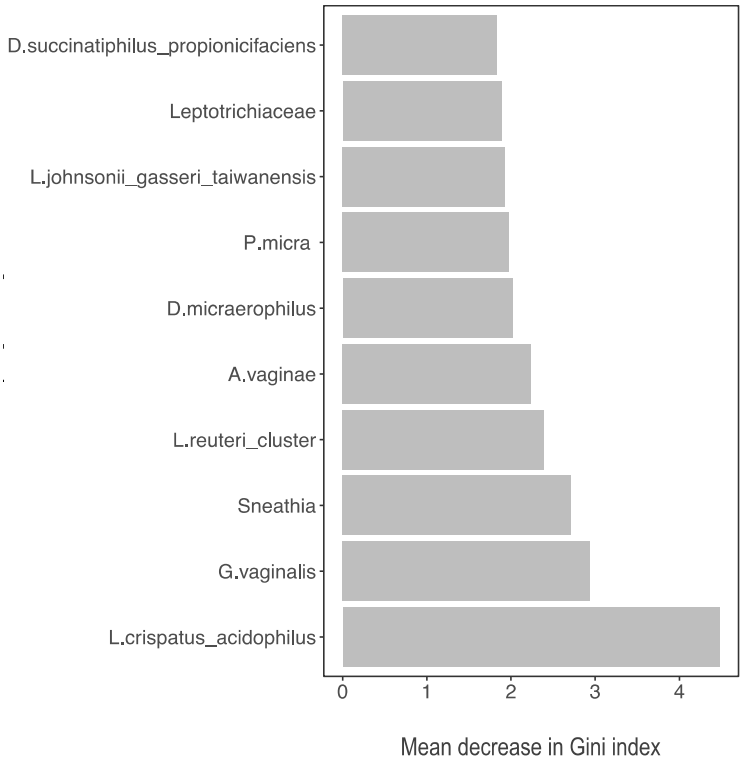


Figure 6.10. Taxa predictive of inflammation status. The top ten most influential taxa by random forest analysis in predicting inflammation group. The x-axis indicates the mean decrease in Gini index (length of bar represents predictive ability of each taxon).

Table 6.10. Top ten taxa predictive of inflammation group (high versus low) using random forest analysis.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_2	11.091	4.478	*Lactobacillus crispatus_acidophilus*
OTU_4	6.961	2.935	*Gardnerella vaginalis*
OTU_136	5.779	2.710	*Sneathia*
OTU_45	5.250	2.390	*Lactobacillus reuteri_cluster*
OTU_8	5.352	2.235	*Atopobium vaginae*
OTU_39	4.398	2.015	*Dialister micraerophilus*
OTU_24	5.284	1.976	*Parvimonas micra*
OTU_298	5.223	1.928	*Lactobacillus johnsonii_gasseri_taiwanensis*
OTU_6	3.930	1.886	*Leptotrichiaceae*
OTU_14	3.660	1.833	*Dialister succinatiphilus_propionicifaciens*

Training set size: 223 samples with 121 and 102 samples per class. Test set size: 112 samples with 68 and 44 samples per class. Validation predicted error: 31.25% (using top five features).

When applying DESeq2 for differential abundance testing, the standardized counts of the same taxa described in the metagenomeSeq analysis section were found to be differentially abundant between the high and low inflammation group. Additional taxa including *Peptostreptococcus anaerobius*, *Porphyromonas*

eunonis, *Fusobacterium nucleatum*, *Veillonella montpellieriensis*, *Moryella* and *Anaerococcus prevotii_tetradius* were found to be more abundant in the high inflammation group and *Acinetobacter baumanii*, *Corynebacterium* spp., and *Jeotgalicoccus* were found to be more abundant in the low inflammation group (alpha = 0.01) (**Figure 6.11** and **Table 6.11**).

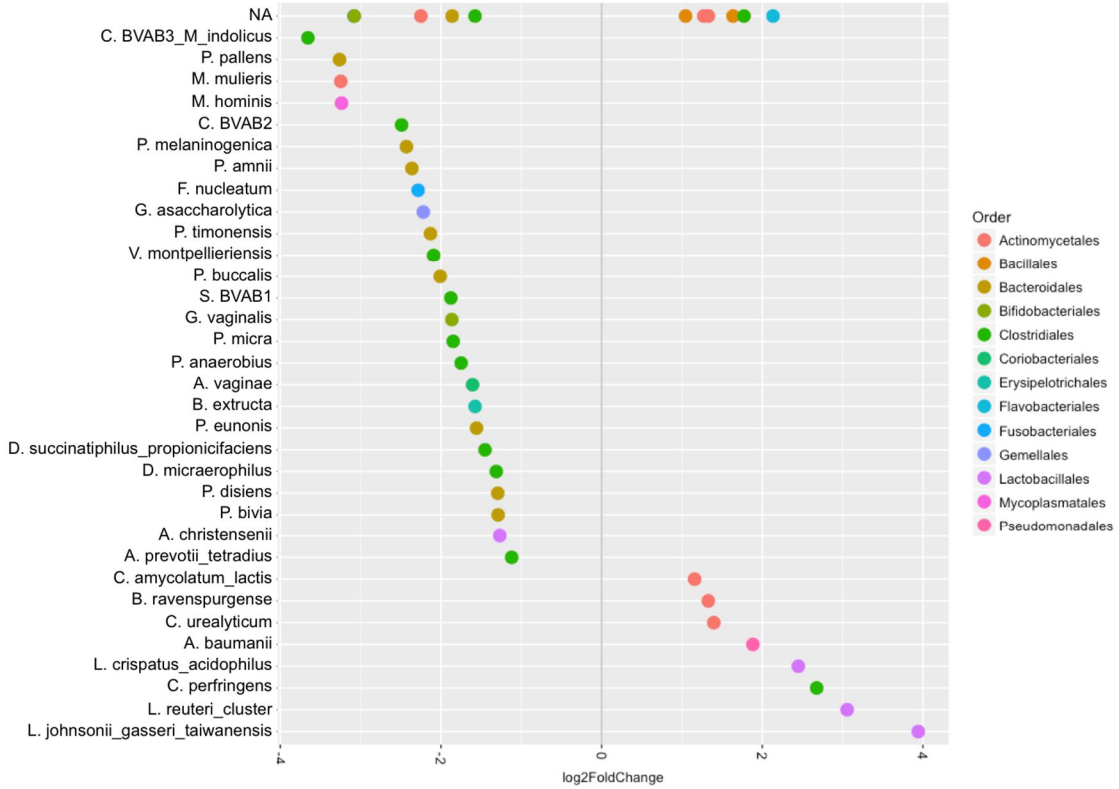


Figure 6.11. DESeq2 analysis of inflammation groups. Bacterial taxa significantly differentially abundant by inflammation category in 335 vaginal samples from 149 participants analysed using DESeq2 (alpha = 0.01). Taxa depicted at species level. Eleven taxa without species level annotation are grouped in the first line ("NA") and correspond to (in order of appearance of dot): Sneathia, Gardnerella, Arcanobacterium, Prevotella, Moryella, Staphylococcus, Micrococcus, Nesterenkonia, Jeotgalicoccus, Clostridium and Chryseobacterium. Figure only depicts taxa with genus level annotation.

Table 6.11. DESeq2 analysis of inflammation groups.

Log2FC	P adj.	Family	Genus	Species
-3.247	1.4e-19	Actinomycetaceae	*Mobiluncus*	*mulieris*
3.942	8.2e-18	Lactobacillaceae	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*
-3.033	8.3e-16	Leptotrichiaceae	NA	NA
-3.083	4.5e-15	Leptotrichiaceae	*Sneathia*	NA
-2.129	6.2e-15	Prevotellaceae	*Prevotella*	*timonensis*
-3.237	9.7e-15	Mycoplasmataceae	*Mycoplasma*	*hominis*
-2.009	2.0e-12	Prevotellaceae	*Prevotella*	*buccalis*
-1.749	5.0e-10	Peptostreptococcaceae	*Peptostreptococcus*	*anaerobius*

Continued on next page.

Continued from previous page.

Log2FC	P adj.	Family	Genus	Species
3.057	5.0e-10	Lactobacillaceae	*Lactobacillus*	*reuteri_cluster*
-1.864	5.6e-10	Bifidobacteriaceae	*Gardnerella*	*vaginalis*
-1.860	2.2e-09	Prevotellaceae	*Prevotella*	NA
-2.283	2.3e-09	Fusobacteriaceae	*Fusobacterium*	*nucleatum*
-1.312	7.8e-09	Veillonellaceae	*Dialister*	*micraerophilus*
2.450	1.1e-08	Lactobacillaceae	*Lactobacillus*	*crispatus_acidophilus*
-2.219	3.0e-08	Gemellaceae	*Gemella*	*asaccharolytica*
-2.36	4.6e-08	Prevotellaceae	*Prevotella*	*amnii*
-3.655	4.6e-08	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
-1.451	5.4e-08	Veillonellaceae	*Dialister*	*succinatiphilus_propionicifaciens*
-2.490	1.4e-07	Clostridiaceae	*Clostridium*	BVAB2
-1.557	2.0e-07	Porphyromonadaceae	*Porphyromonas*	*eunonis*
-3.078	2.9e-07	Bifidobacteriaceae	*Gardnerella*	NA
-2.429	8.2e-07	Prevotellaceae	*Prevotella*	*melaninogenica*
-1.847	8.3e-07	[Tissierellaceae]	*Parvimonas*	*micra*
1.884	1.3e-05	Moraxellaceae	*Acinetobacter*	*baumanii*
-1.607	1.3e-05	Coriobacteriaceae	*Atopobium*	*vaginae*
-1.119	1.4 e-05	[Tissierellaceae]	*Anaerococcus*	*prevotii_tetradius*
2.134	1.9e-05	[Weeksellaceae]	*Chryseobacterium*	NA
-1.877	2.7e-05	Lachnospiraceae	*Shuttleworthia*	BVAB1
-2.091	2.8e-05	Veillonellaceae	*Veillonella*	*montpellieriensis*
-1.580	4.0e-05	Porphyromonadaceae	NA	NA
-1.288	0.0001	Prevotellaceae	*Prevotella*	*bivia*
-1.293	0.0002	Prevotellaceae	*Prevotella*	*disiens*
-1.504	0.0002	Coriobacteriaceae	NA	NA
1.636	0.0003	Staphylococcaceae	*Jeotgalicoccus*	NA
1.158	0.0004	Corynebacteriaceae	*Corynebacterium*	*amycolatum_lactis*
1.773	0.001	Clostridiaceae	*Clostridium*	NA
-1.267	0.001	Aerococcaceae	*Aerococcus*	*christensenii*
-1.575	0.001	Lachnospiraceae	*Moryella*	NA
-3.262	0.001	Prevotellaceae	*Prevotella*	*pallens*
1.045	0.002	Staphylococcaceae	*Staphylococcus*	NA
-2.249	0.002	Actinomycetaceae	*Arcanobacterium*	NA
1.400	0.002	Corynebacteriaceae	*Corynebacterium*	*urealyticum*
-1.575	0.003	Erysipelotrichaceae	*Bulleidia*	*extructa*
1.328	0.003	Brevibacteriaceae	*Brevibacterium*	*ravenspurgense*
1.272	0.004	Micrococcaceae	*Micrococcus*	NA
-1.921	0.005	Lachnospiraceae	NA	NA
1.329	0.005	Micrococcaceae	*Nesterenkonia*	NA
2.677	0.006	Clostridiaceae	*Clostridium*	*perfringens*
-2.501	0.008	Rs-045	NA	NA

FC, fold change.

6.3.3 IL-17 and vaginal infections

Due to the relationship between HIV and Th17 cells, we conducted a similar analysis using the IL-17 cytokines IL-17A and IL-17F only. Using PAM clustering the data again grouped to two clusters (k=2) with a maximum silhouette width of 0.609 (**Figure 6.12A**). 176 samples were assigned to high IL-17 cluster and 159 to a low IL-17 cluster (**Figure 6.12C**).

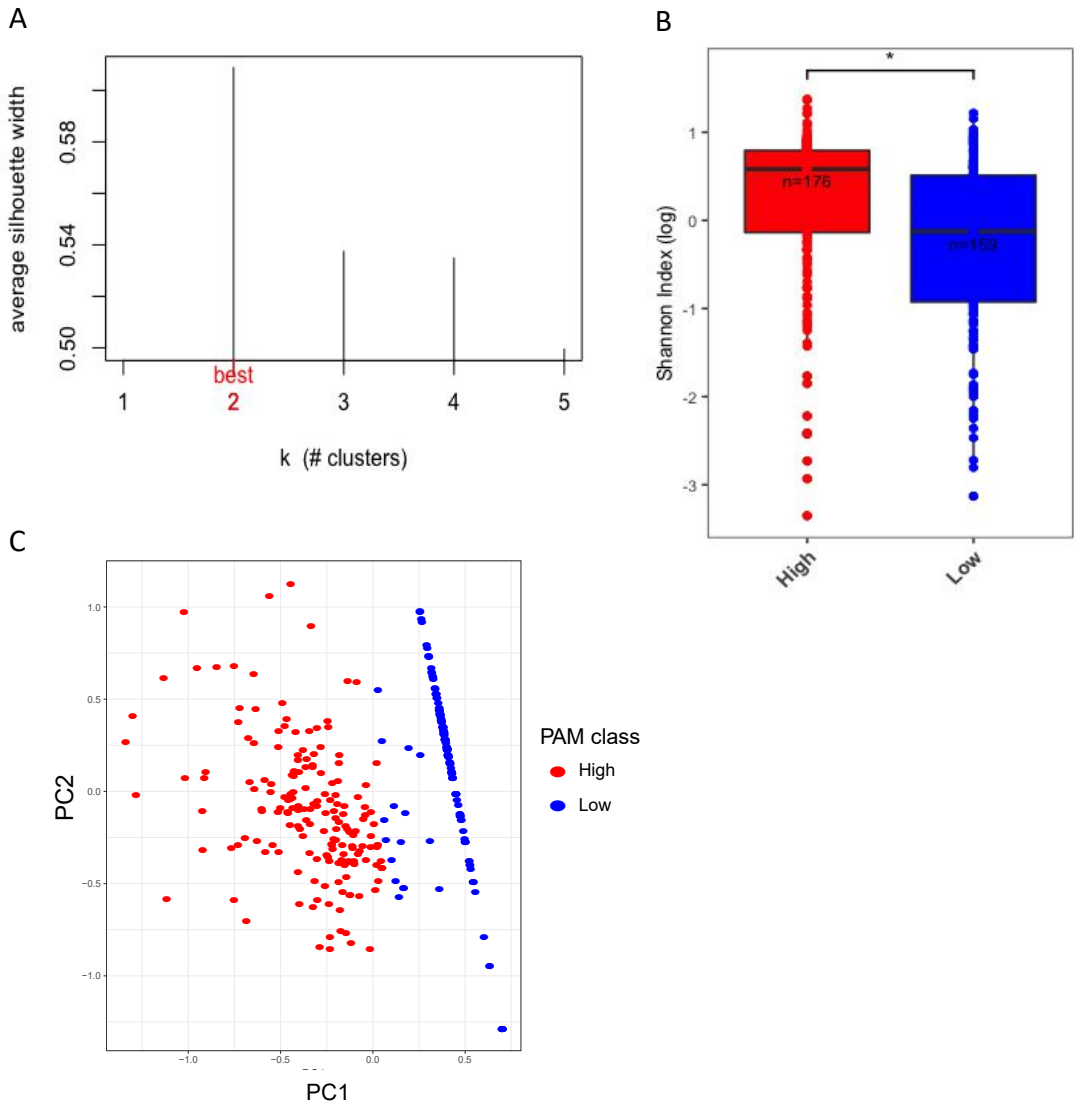


Figure 6.12. Defining IL-17 clusters. A) Barplot depicting the average silhouette width when applying different k's (1-5) for partitioning around medoids (PAM) clustering using Euclidean distances of the cytokine data. Optimal k was determined based on the highest average silhouette width. B) Boxplot showing the alpha diversity measured using Shannon Index (log transformed values) of the microbiota from 335 vaginal samples from 149 participants according to IL-17 group (high versus low) identified by PAM clustering using Euclidean distances of the cytokine data. *=p<0.05. C) PCoA of the cytokine data coloured by IL-17 cluster (high, low) generated by PAM clustering with Euclidean distances.

The high IL-17 group was significantly associated with community type C1 (62.6%) while the low IL-17 was most commonly associated with the *L. crispatus* dominated C2 community type (38.6%) (**Figure 6.14** and **Table 6.12**). The *L. iners* dominant C3 community type was similarly distributed between the two groups (**Table 6.12**). We found a significant difference in microbial community beta diversity between the high and low IL-17 clusters (adonis, p=0.001, R2=0.109) suggesting a significant difference in microbiota composition between these two groups (**Figure 6.13**). This remained significant after adjusting for presence of STIs (p=0.001, R2=0.109). Dispersion of the community structures of participants in the two groups did not significantly differ (betadisp, p=0.075, F=3.20) suggesting that there is no difference in the overall heterogeneity of species composition (dispersion) (**Figure 6.13**).

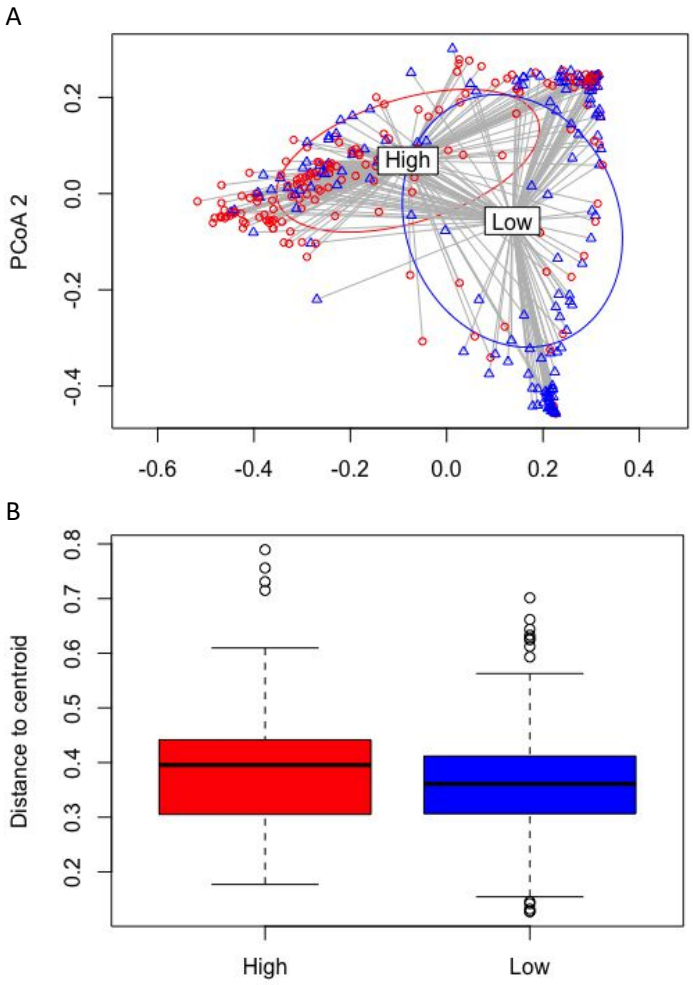


Figure 6.13. Beta diversity within IL-17 clusters. A) Principal Coordinates Analysis (PCoA) of beta diversity calculated using weighted UniFrac according to high and low IL-17 clusters with standard deviations ellipses around each study arm centroid. B) Barplot depicting the distance to the centroid for each sample in each IL-17 group.

Table 6.12. Participant characteristics according to IL-17 grouping.

	High IL-17 (n=176)	**Low IL-17** (n=159)	**P value**
Fuzzycluster[1]			**6.2e-12**
C1	107 (62.6%)	44 (28.8%)	
C2	15 (8.77%)	59 (38.6%)	
C3	49 (28.7%)	50 (32.7%)	
BV prevalence			**4.1e-09**
BV positive	100 (56.8%)	42 (26.4%)	
BV intermediate	15 (8.52%)	8 (5.03%)	
BV negative	61 (34.7%)	109 (68.6%)	
Vaginal pH, mean (sd)	5.02 (4.53-5.51)	4.61 (4.00-5.22)	**5.7e-11**
Shannon Index, median (IQR)	1.79 (0.87-2.21)	0.88 (0.40-1.67)	**1.3e-08**
STI prevalence			
Any STI(s)	74 (42.0%)	46 (28.9%)	**0.017**
Ct	51 (29.0%)	31 (19.5%)	0.059
Ng	19 (10.8%)	15 (9.43%)	0.817
Tv	13 (73.9%)	6 (3.78%)	0.234
Mg	6 (34.1%)	4 (2.52%)	0.753
HSV-2 serology[2]	53 (30.1%)	55 (34.6%)	0.489
Yeast cells present	41 (23.3%)	17 (10.7%)	**0.004**
Use of hormonal contraception[3]			0.694
None	22 (12.7%)	14 (8.92%)	
Net-En	71 (41.0%)	72 (45.9%)	
COCP	26 (15.0%)	28 (17.8%)	
DMPA	9 (5.20%)	10 (6.37%)	
NuvaRing	43 (24.9%)	32 (20.4%)	
Implanon	2 (1.16%)	1 (0.07%)	
Intra-vaginal practices[4]			
Douching	1 (1.33%)	0 (0.00%)	1.000
Washing with water	11 (14.7%)	8 (11.8%)	0.792
Washing with soap	8 (10.7%)	5 (7.35%)	0.691
Cloth	3 (4.00%)	1 (1.47%)	0.622
Drying	1 (1.33%)	0 (0.00%)	1.000
Medication	4 (5.33%)	3 (4.41%)	1.000
Tampon use	7 (9.33%)	1 (1.47%)	0.065
Herbs	1 (1.33%)	0 (0.00%)	1.000
Sexual risk behaviour[5]			
Sexual partners, median (IQR)	1 (1-1)	1 (1-1)	**0.048**
Multiple sexual partners, n (%)	4 (2.58%)	1 (0.69%)	0.372
New partner, n (%)	23 (15.9%)	18 (13.4%)	0.687
General condom use			0.822
Never	14 (9.03%)	15 (10.3%)	
Almost never	15 (9.68%)	10 (6.85%)	
Not sure	20 (12.9%)	23 (15.8%)	
Almost always	62 (40.0%)	54 (37.0%)	
Always	44 (28.4%)	44 (30.1%)	
Condom use during last PV intercourse			
Yes	86 (55.8%)	98 (67.1%)	0.059
PV sex acts per week, median (IQR)	1 (1-2)	1 (1-2)	0.701
Intergenerational sex with older partner (≥5 years)			0.492
No	113 (72.9%)	107 (73.3%)	
I don't think so	3 (1.94%)	4 (2.73%)	
Not sure	23 (14.8%)	16 (11.0%)	
I think so	3 (1.94%)	1 (0.68%)	
Yes	13 (8.39%)	19 (13.0%)	
Transactional sex	2 (1.29%)	0 (0.00%)	0.499
Penile-anal intercourse	2 (1.29%)	2 (1.37%)	1.000

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups, unpaired Mann-Whitney-Wilcoxon test for comparison of medians and unpaired Student's t test for comparison of means. BV, bacterial vaginosis; Ct, Chlamydia trachomatis*; HSV-2, herpes simplex virus type 2; IQR, interquartile range; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoeae*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. No Fuzzy cluster assignment for eleven samples (five in "High IL-17" and six in "Low IL-17").
2. One equivocal result.
3. Missing data from five participants (three from "High IL-17" and two from "Low IL-17").
4. Information only collected at screening. IL-17 high: n=75, IL-17 low: n=68.
5. Missing data from 34 participant visits (21 from "High IL-17" and 13 from "Low IL-17")

There were significant differences in alpha diversity (1.79 versus 0.88, p=1.3e-08) and vaginal pH (5.02 versus 4.61, p=5.7e-11) between the two clusters with the IL-17 high group being more diverse and having a higher vaginal pH (**Figure 6.12B** and **Table 6.12**, lme: p=0.036 and p<0.001, respectively). Alpha diversity and vaginal pH remained significantly associated with IL-17 group after adjusting for STIs and *Candida* using logistic regression (Shannon Index: OR 2.43; 95% confidence interval (CI): 1.81-3.30, p=6.5e-09; vaginal pH: OR 4.43, 95% CI: 2.80- 7.27, p=8.4e-10). There were no differences in the distribution of COCP, Net-En and NuvaRing users between the high and low IL-17 groups (p=0.482). In the IL-17 high group, 94.2% reported having had one or more sexual partners compared to 87.7% in the low IL-17 group (p=0.048) (**Table 6.12**). A close to but not significant difference in condom use at the last penile-vaginal intercourse between the two groups was observed with the low IL-17 group reporting higher level of condom use (61.7% versus 55.8%, p=0.059) (**Table 6.12**). No other differences between the IL-17 groups were observed regarding sexual risk behaviour. Also, no differences in intravaginal behaviour were observed despite a trend towards higher level of tampon use in the IL-17 high group (p=0.065) (**Table 6.12**).

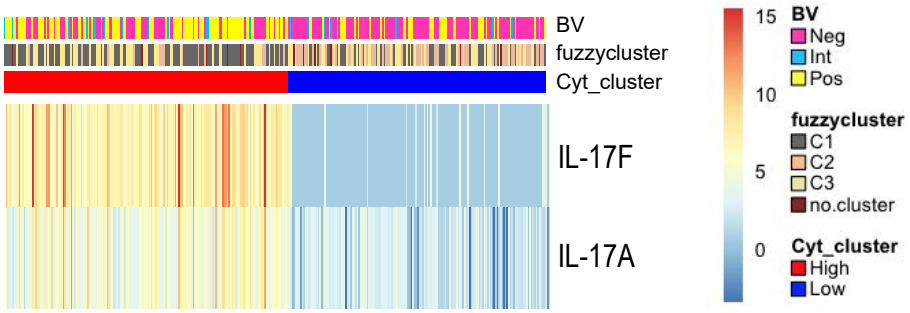


Figure 6.14. IL-17 levels and the vaginal microbiota. Heatmap of IL-17A and IL-17F cytokines from a Th17 bioplex panel using average linkage clustering with Euclidean distances. Annotation bars above the heatmap depict IL-17 group generated by partitioning around medoids (PAM) clustering using Euclidean distances of the cytokine data (Cyt_cluster, bottom bar), community cluster identified using Fuzzy clustering using weighted UniFrac distances (fuzzycluster, middle bar), BV status based on Nugent scoring (BV, top bar). Log2-transformed cytokine concentrations are illustrated by the colour key. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from downstream analyses ("no.cluster" in figure, n=11).

There were significant differences in BV and STI prevalence between the two groups (**Table 6.12**). Accordingly, there were higher levels of IL-17A and IL-17F in participants with one or more STI compared to STI negative participants (adj. p=0.0005 and adj. p=0.019, respectively) and in BV positive versus BV negative participants (adj. p=7.9e-05 and adj. p=6.4e-09, respectively) (**Table 6.13**). IL-1β (adj. p=0.019) and TNF-α (adj. p=0.022) were also significantly higher in participants with one or more STIs compared to STI negative participants (Appendix VIII). The IL-17A levels were significantly higher in *C. trachomatis* positive participants compared to *C. trachomatis* negative participants (adj. p=0.009). IL-17F levels were also significantly higher in the *C. trachomatis* positive participants before adjusting for multiple comparisons (p=0.025) (**Table 6.13**). IL-17A and IL-17F levels were higher in *N. gonorrhoea* positive compared to negative participants but not significantly so (**Table 6.13**). Yeast infected participants had significantly higher IL-17A and IL-17F levels compared to participants with no yeast cells detected by microscopy (adj. p=2.9e-04 and adj. p=6.5e-05, respectively) (**Table 6.13**). IL-22 was also significantly higher in participants with detectable fungal infections (adj. p=6.5e-05) while no difference in IL-21 was observed (Appendix VIII). We did not find any significant differences in IL-17A levels between *T. vaginalis* positive and negative participants, but significantly higher levels of IL-17F, IL-22, IL-25 and TNF-α in *T. vaginalis* positive compared to *T. vaginalis* negative participants (Appendix VIII). HSV-2 serology did not have any impact on cytokine concentrations (**Table 6.13**).

Table 6.13. IL-17A and IL-17F levels according to vaginal infections.

	IL-17A (median (IQR))	P	P adj.	IL-17F (median (IQR))	P	P adj.
BV		**7.9e-05**	**7.9e-05**		**3.9e-09**	**6.4e-09**
Positive	1.92 (1.07-5.35)			7.22 (0.82-15.8)		
Intermediate	2.12 (1.17-7.16)			6.52 (0.34-19.2)		
Negative	1.11 (0.78-2.32)			0.34 (0.34-5.59)		
STI		**3.9e-05**	**0.0005**		**0.0045**	**0.0193**
Yes	2.08 (1.08-5.63)			5.53 (0.34-15.8)		
No	1.23 (0.80-2.67)			1.38 (0.34-8.88)		
C. trachomatis		**0.0007**	**0.0091**		**0.0255**	0.163
Yes	2.22 (1.12-5.60)			5.29 (0.34-15.9)		
No	1.29 (0.84-3.19)			2.02 (0.34-10.1)		
N. gonorrhoeae		**0.015**	0.200		0.573	0.645
Yes	2.36 (1.10- 9.24)			4.04 (0.34-17.7)		
No	1.35 (0.84-3.75)			2.92 (0.34 -0.8)		
T. vaginalis		0.066	0.116		**0.023**	**0.099**
Yes	1.66 (1.36-11.3)			12.64 (0.34-66.8)		
No	1.35 (0.85-3.78)			2.92 (0.34-10.5)		
Yeast cells		**6.6e-05**	**2.9e-04**		**5.3e-06**	**6.5e-05**
Yes	3.56 (1.08-8.60)			14.2 (0.34-40.0)		
No	1.31 (0.84-3.19)			2.20 (0.34-8.88)		
HSV-2 serology		0.887	0.887		0.813	0.887
Yes	1.56 (0.83- 3.71)			2.22 (0.34-12.9)		
No	1.34 (0.87-3.89)			2.92 (0.34-11.3)		

Cytokine concentrations in pg/ml. P values calculated using Kruskal-Wallis test and unpaired Mann-Whitney-Wilcoxon test adjusted using Benjamini-Hochberg (BH) method. BV, bacterial vaginosis; HSV-2, herpes simplex virus type 2; STI, sexually transmitted infection.

6.3.4 Differentially abundant taxa between high and low IL-17 groups

Using metagenomeseq, 24 taxa were differentially relative abundant between the IL-17 low and IL-17 high group (**Figure 6.15** and **Table 6.14**). Except for *P. melaninogenica*, *M. muliereis*, *Dialister* and *L. iners*, the same taxa which were differentially abundant between high and low total cytokine groups in **Figure 6.9** were evident in the IL-17 analysis. *L. iners* was not found to be differentially abundant between the IL-17 groups corresponding to the distribution of the *L. iners* dominant community cluster (C3), which was found to be similar in both groups.

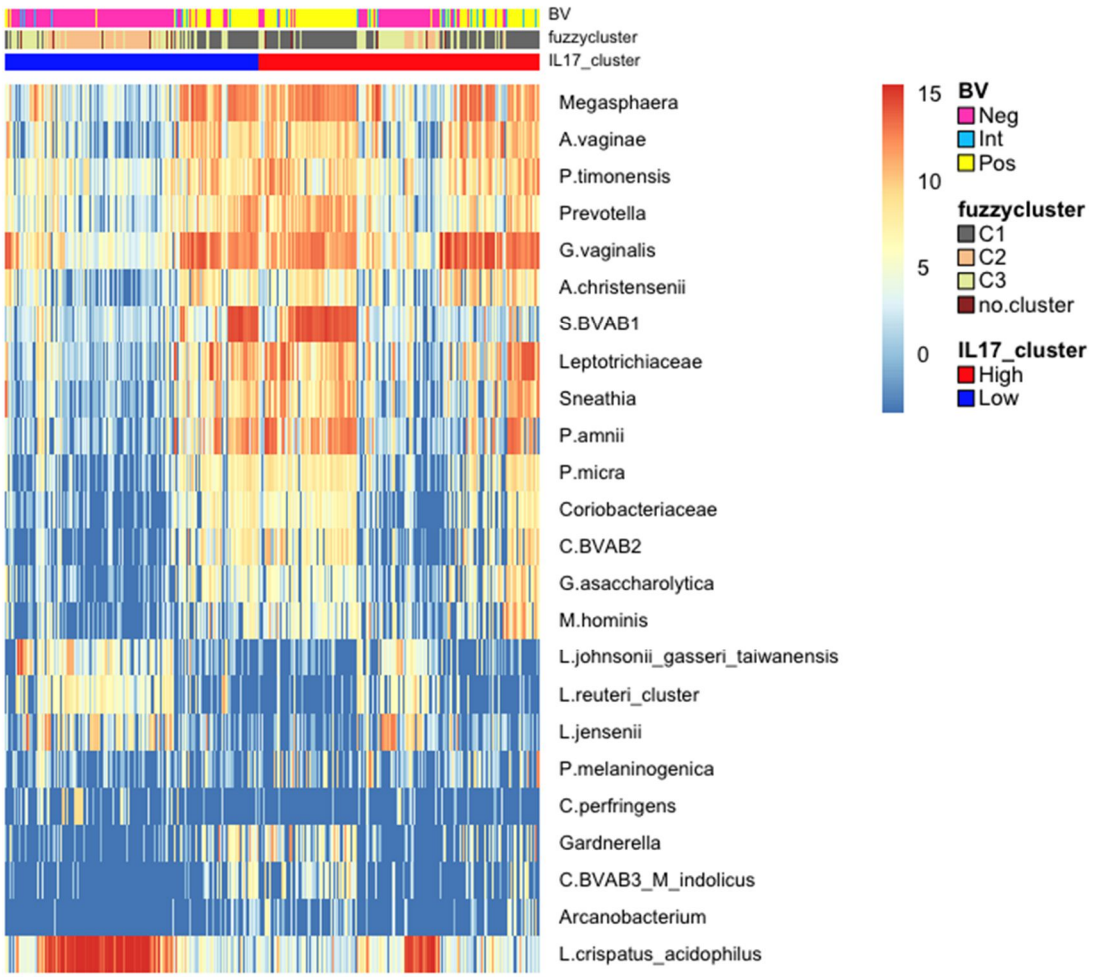


Figure 6.15. Differentially abundant taxa between low and high Il-17 groups. Bacterial taxa significantly differentially abundant and/or frequent by Il-17 category in 335 vaginal samples from 149 participants analysed using metagenomeSeq (FDR ≤ 0.05, coefficient ≥ 1.25, taxa present in ≥ 20% of samples in at least one of the two groups being compared). The heatmap shows samples ordered by cytokine cluster followed by unsupervised clustering of samples (columns) by Bray-Curtis distances. Log2-transformed standardized read counts are illustrated by the colour key. Annotation bars above the heatmap depict cytokine derived inflammation group generated by partitioning around medoids (PAM) clustering using Euclidean distances of the cytokine data (Cyt_cluster, bottom bar), community cluster identified using Fuzzy clustering using weighted UniFrac distances (fuzzycluster, middle bar), BV status based on Nugent scoring (BV, top bar). Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from downstream analyses ("no.cluster" in figure, n=11).

Table 6.14. Differentially abundant taxa between low and high Il-17 groups.

Coeff	P adj.	Family	Genus	Species
-2.429	1.3e-06	Leptotrichiaceae	NA	NA
-2.352	7.0e-07	Bifidobacteriaceae	*Gardnerella*	*vaginalis*
-2.346	4.3e-08	Coriobacteriaceae	*Atopobium*	*vaginae*
-2.284	1.0e-06	Leptotrichiaceae	*Sneathia*	NA
-2.189	4.3e-09	Mycoplasmataceae	*Mycoplasma*	*hominis*
-2.102	0.0001	Veillonellaceae	*Megasphaera*	NA
-2.069	9.2e-07	Clostridiaceae	*Clostridium*	BVAB2
-1.759	1.2e-06	[Tissierellaceae]	*Parvimonas*	*micra*
-1.671	1.1e-05	Aerococcaceae	*Aerococcus*	*christensenii*
-1.590	8.6e-07	Coriobacteriaceae	NA	NA
-1.549	0.0081	Prevotellaceae	*Prevotella*	*amnii*
-1.545	9.0e-06	Gemellaceae	*Gemella*	*asaccharolytica*
-1.478	0.0003	Prevotellaceae	*Prevotella*	NA
-1.438	4.3e-09	Actinomycetaceae	*Arcanobacterium*	NA
-1.431	8.0e-05	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
-1.414	0.0014	Bifidobacteriaceae	*Gardnerella*	NA
-1.386	0.0001	Prevotellaceae	*Prevotella*	*timonensis*
-1.350	0.0002	Prevotellaceae	*Prevotella*	*melaninogenica*
-1.348	0.0290	Lachnospiraceae	*Shuttleworthia*	BVAB1
1.280	0.0093	Lactobacillaceae	*Lactobacillus*	*jensenii*
1.790	7.7e-06	Lactobacillaceae	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*
2.098	4.7e-09	Lactobacillaceae	*Lactobacillus*	*reuteri_cluster*
2.197	1.4e-10	Clostridiaceae	*Clostridium*	*perfringens*
4.343	6.0e-14	Lactobacillaceae	*Lactobacillus*	*crispatus_acidophilus*

The top ten most predictive taxa in predicting IL-17 group according to random forest analysis were *L. crispatus*, *M. hominis*, *G. vaginalis*, *A. vaginae*, *A. christensenii*, *L. reuteri_cluster, D. micraerophilus*, *P. micra*, *Sneathia* and *L. iners* (Training AUC=0.69, sensitivity=0.54 and specificity=0.72 and a validation predicted error rate of 30.36% when using top five features) (**Figure 6.16** and **Table 6.15**). *L. crispatus* however, had by far the largest Mean Decrease Gini than the other taxa in the model.

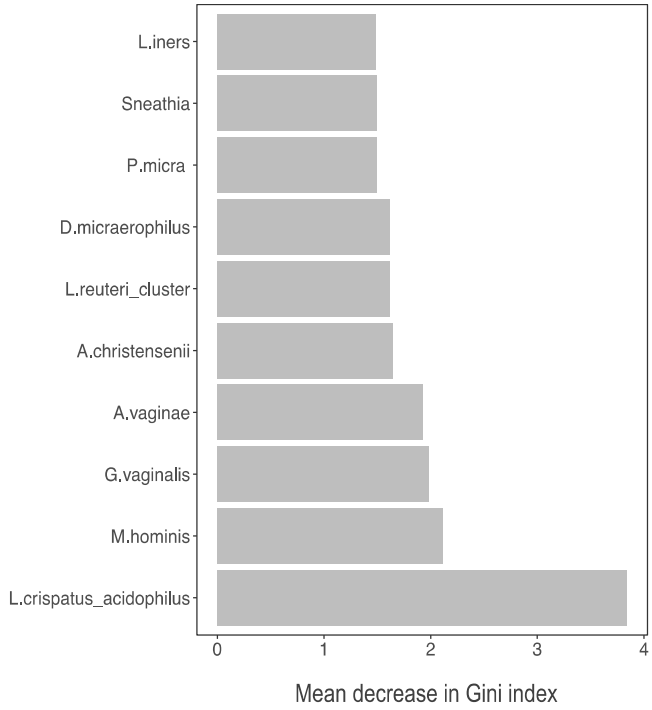


Figure 6.16. Random forest analysis of Il-17 groups. The top 10 most influential taxa by random forest analysis in predicting IL-17 group. The x-axis indicates the mean decrease in Gini Index (length of bar represents predictive ability of each taxon).

Table 6.15. Top ten taxa predictive of IL-17 group (high versus low) using random forest analysis.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_2	10.433	3.841	*Lactobacillus crispatus_acidophilus*
OTU_26	4.902	2.115	*Mycoplasma hominis*
OTU_4	3.444	1.979	*Gardnerella vaginalis*
OTU_8	4.437	1.927	*Atopobium vaginae*
OTU_16	3.615	1.649	*Aerococcus christensenii*
OTU_45	4.828	1.619	*Lactobacillus reuteri_cluster*
OTU_39	3.500	1.616	*Dialister micraerophilus*
OTU_24	5.599	1.499	*Parvimonas micra*
OTU_136	2.575	1.493	*Sneathia*
OTU_1	2.247	1.489	*Lactobacillus iners*

Training set size: 223 samples with 116 and 107 samples per class. Test set size: 112 samples with 60 and 52 samples per class. Validation predicted error: 30.36% (using top five features).

Using DeSeq2, the standardized read counts of thirty taxa (merged at lowest taxonomic level) were differentially abundant between the IL-17 groups (alpha 0.01) (**Figure 6.17** and **Table 6.16**). *M. hominis*, *Prevotella* spp. (including *P. melaninogenica*, *P. timonensis*, *P. buccalis* and *P. bivia*), *P. eunonis*, *G. asaccharolytica*, *Sneathia*, *F. nucleatum*, *D. micraerophilus*, *A. vaginae*, *P.*

anaerobius, *Arcanobacterium*, *A. christensenii*, *H. parainfluenzae*, *Moryella*, *G. vaginalis* and *P. micra* were more abundant in the high IL-17 group while non-*iners Lactobacillus* spp. (including *L. crispatus*, *L. johnsonii_gasseri_taiwanensis* and *L. reuteri_cluster*), *C. perfringens*, *Chryseobacterium*, *A. baumanii*, *Micrococcus*, *C. urealyticum*, *B. ravenspurgense* (also known as *B. massiliense*) and *Jeotgalicoccus* were more abundant in the low IL-17 group.

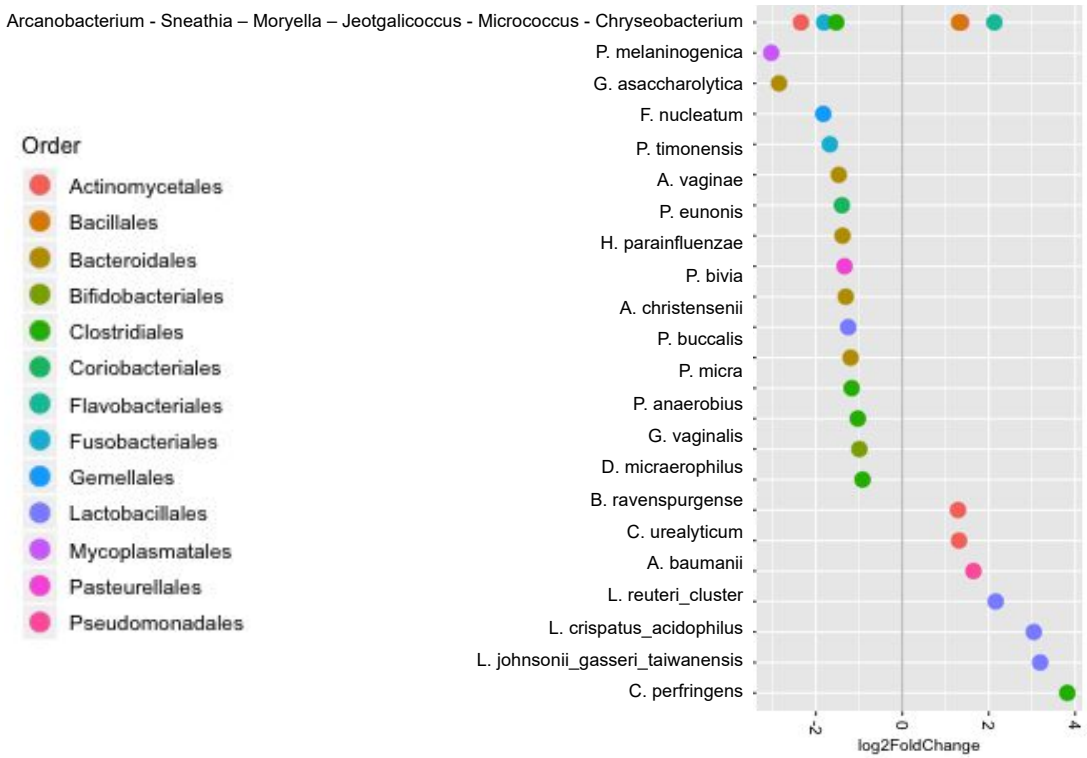


Figure 6.17. DESeq2 analysis of IL-17 groups. Bacterial taxa significantly differentially abundant by IL-17 group using DESeq2 (with an alpha of 0.01). Taxa depicted at species level. Taxa without species level annotation (if >1) are grouped in the first line and order of genera corresponds to order of appearance of dots (from left to right). Figure only includes taxa annotated at the genus level.

Table 6.16. DESeq2 analysis of IL-17 groups.

Log2FC	P adj.	Family	Genus	Species
3.044	7.8e-13	Lactobacillaceae	*Lactobacillus*	*crispatus_acidophilus*
-3.035	1.1e-12	Mycoplasmataceae	*Mycoplasma*	*hominis*
3.191	6.0e-12	Lactobacillaceae	*Lactobacillus*	*johnsonii_gasseri_taiwanensis*
-2.851	4.5e-09	Prevotellaceae	*Prevotella*	*melaninogenica*
-1.469	8.8e-07	Prevotellaceae	*Prevotella*	*timonensis*
-1.384	9.3e-06	Porphyromonadaceae	*Porphyromonas*	*eunonis*
-1.829	2.3e-05	Gemellaceae	*Gemella*	*asaccharolytica*
-1.808	2.4e-05	Leptotrichiaceae	*Sneathia*	NA
3.820	3.2e-05	Clostridiaceae	*Clostridium*	*perfringens*
-1.676	3.3e-05	Fusobacteriaceae	*Fusobacterium*	*nucleatum*
2.131	3.9e-05	[Weeksellaceae]	*Chryseobacterium*	NA
2.161	7.3e-05	Lactobacillaceae	*Lactobacillus*	*reuteri_cluster*
-1.196	0.0001	Prevotellaceae	*Prevotella*	*buccalis*
-1.308	0.0001	Prevotellaceae	*Prevotella*	*bivia*
-0.920	0.0002	Veillonellaceae	*Dialister*	*micraerophilus*
1.648	0.0002	Moraxellaceae	*Acinetobacter*	*baumanii*
-1.397	0.0003	Coriobacteriaceae	*Atopobium*	*vaginae*
-1.495	0.0004	Leptotrichiaceae	NA	NA
-1.029	0.0007	Peptostreptococcaceae	*Peptostreptococcus*	*anaerobius*
-2.345	0.0012	Actinomycetaceae	*Arcanobacterium*	NA
-1.250	0.0012	Aerococcaceae	*Aerococcus*	*christensenii*
-1.336	0.0018	Pasteurellaceae	*Haemophilus*	*parainfluenzae*
-1.529	0.0020	Lachnospiraceae	*Moryella*	NA
1.362	0.0024	Micrococcaceae	*Micrococcus*	NA
-0.994	0.0036	Bifidobacteriaceae	*Gardnerella*	*vaginalis*
-1.200	0.0045	Porphyromonadaceae	NA	NA
1.313	0.0054	Corynebacteriaceae	*Corynebacterium*	*urealyticum*
-1.171	0.0054	[Tissierellaceae]	*Parvimonas*	*micra*
1.291	0.0056	Brevibacteriaceae	*Brevibacterium*	*ravenspurgense*
1.313	0.0072	Staphylococcaceae	*Jeotgalicoccus*	NA

*NOTE: only annotated at order level

6.3.5 Specific taxa associated with individual cytokines

When looking at the correlation of the concentrations of individual cytokines and the standardized read counts of specific bacterial taxa, we found that bacteria associated with BV (e.g. BVAB2-3, *Dialister micraerophilus*, *Anaerococcus* spp., *Peptococcus*, *Sneathia*, *Atopobium vaginae* and *Gardnerella vaginalis*) and increased HIV susceptibility (e.g. *Parvimonas micra*, *Prevotella* spp., including *P. amnii* and *P. melaninogenica*, *Gemella asaccharolytica*, *Mycoplasma hominis*, *Sneathia*, *Veillonella* and *Megasphaera*) were correlated positively with the

concentration of several pro-inflammatory cytokines (including IFN-γ, IL-1 β, IL-6, IL-31 and IL-33) (adj. p<0.1, R>0.2) (**Figure 6.18**). TNF-α concentrations were positively correlated with *Anaerococcus* spp. (*A. obesiensis* and *A. prevotii_tetradius*), *Prevotella bergensis*, *Moryella*, *Streptococcus sanguinus_morbirenis* and *Peptococcus* (**Figure 6.18**). In contrast, the abundance of *L. crispatus* and *L. iners* was negatively associated with several of the analysed cytokines, including IL-1β, IL-21, IL-25, IL-31, IL-33 and sCD40L as well as IFN-γ and IL-23 in the case of *L. crispatus*. These results correspond well to the association observed between the three community clusters and the cytokine levels (**Figure 6.5**). The IL-17 cytokines showed slightly different associations and correlated positively with the abundance of taxa less commonly described in relation to the FGT. For IL-17A these included *Streptococcus anginosus*, *Atopobium minutum*, *Moryella*, *Peptococcus* and *Gemella asaccharolytica* and for IL-17F, these taxa included *Staphylococcus*, *Bacillus cereus*, *Corynebacterium striatum* and *Atopobium minutum* (**Figure 6.18**).

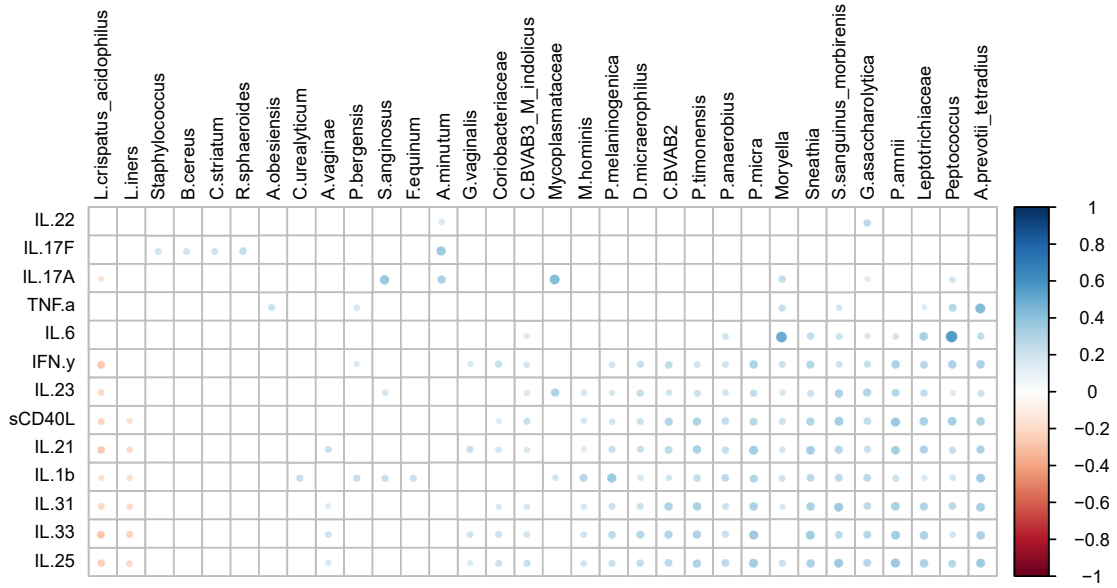


Figure 6.18. Correlation between cytokines and standardized read counts of individual bacteria. Significant positive (blue) or negative (red) correlations between standardized read counts of bacteria (merged at lowest taxonomic level) and vaginal concentrations of cytokines using Spearman's rank correlation. Adjusted p value of 0.1 set as cut-off. Only R values>0.2 displayed. Each OTU has ≥10 counts in ≥30% of samples.

6.3.6 CD4+CCR5+ T cells and the vaginal microbiota

We next analysed the association of the presence and activation status of HIV target cells in the cervix and the bacterial communities present in the FGT. The frequencies of CD4+ T cells expressing CCR5 in cervical cytobrushes were thus evaluated and related to the vaginal microbiota for 129 participants at the baseline visit. When comparing the frequencies of CCR5+CD4+ T cells in the three community clusters generated by Fuzzy clustering, no significant differences were found (**Figure 6.19** and **Table 6.17**). When looking at the frequency of activated (HLA-DR+CD38+) CD4+ T cells that were CCR5+, no significant differences were observed between the three community clusters either (**Figure 6.19** and **Table 6.17**). Accordingly, there where also no significant correlation between the CD4+CCR5+ (p=0.28) or CD4+CCR5+HLA-DR+CD38+ (p=0.52) T cell frequencies and alpha diversity (**Figure 6.20**). Additionally, we did not see any significant differences in the frequencies of CCR5+CD4+ T cells or activated CCR5+CD4+ T cells between BV positive, intermediate and negative participants at baseline (**Table 6.18**). Using linear regression, no association of BV status on CD4+CCR5+ frequency was found after adjusting for the presence of an STI. Having one or more STIs regardless of BV status was also not associated with CD4+CCR5+ frequency. However, being BV positive and positive for one or more STI was significantly associated with increased CD4+CCR5+ levels compared to being BV negative and STI negative (OR: 1.2e+11; 95% CI: 1.5e+03-9.3e+18, p=0.006). This association was specifically mediated by *C. trachomatis* infection (OR: 5.4e+16; 95% CI: 3.3e+08-8.7e+24, p=0.0001).

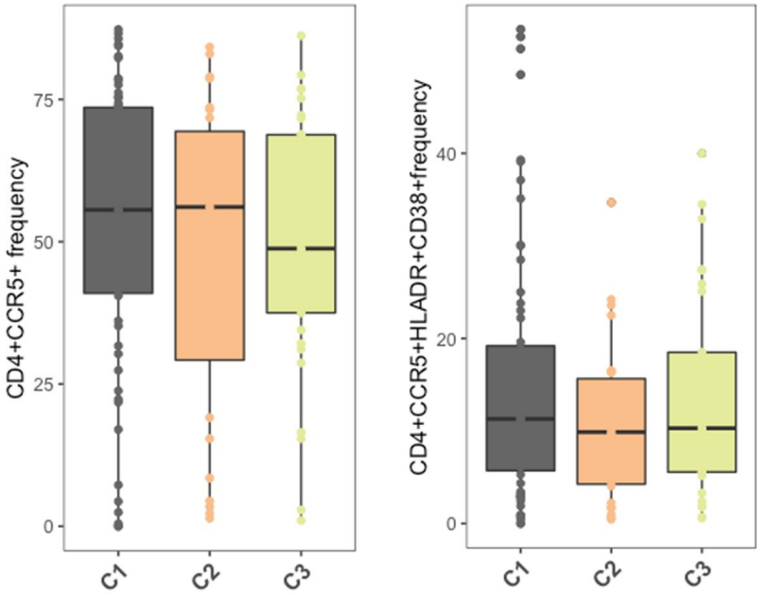


Figure 6.19. CD4+CCR5+ T cell frequency according to Fuzzy clusters. Boxplot depicting the CD4+CCR5+ and CD4+CCR5+HLA-DR+CD38+ cell frequencies (out of total CD4+ T cells) measured by flow cytometry in cervical cytobrushes from 123 participants according to community cluster (C1, n=63; C2, n=27; C3, n=33) defined using Fuzzy clustering with weighted UniFrac distances. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from the figure (n=6). P values were calculated using Kruskal Wallis test. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from the figure (n=6).

Table 6.17. CD4+CCR5+ T cell frequencies according to Fuzzy clusters at screening.

	C1 (n=63)	**C2** (n=27)	**C3** (n=33)	**P value**
CD4+CCR5+%, median (IQR)	55.6 (41.0-73.6)	56.1 (29.2-69.4)	48.8 (37.5-68.8)	0.622
CD4+CCR5+CD38+HLA-DR+%, median (IQR)	11.3 (5.71-19.2)	9.88 (4.26-15.7)	10.3 (5.56-18.5)	0.652

P values calculated using Kruskal Wallis test. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from the analyses (n=6).

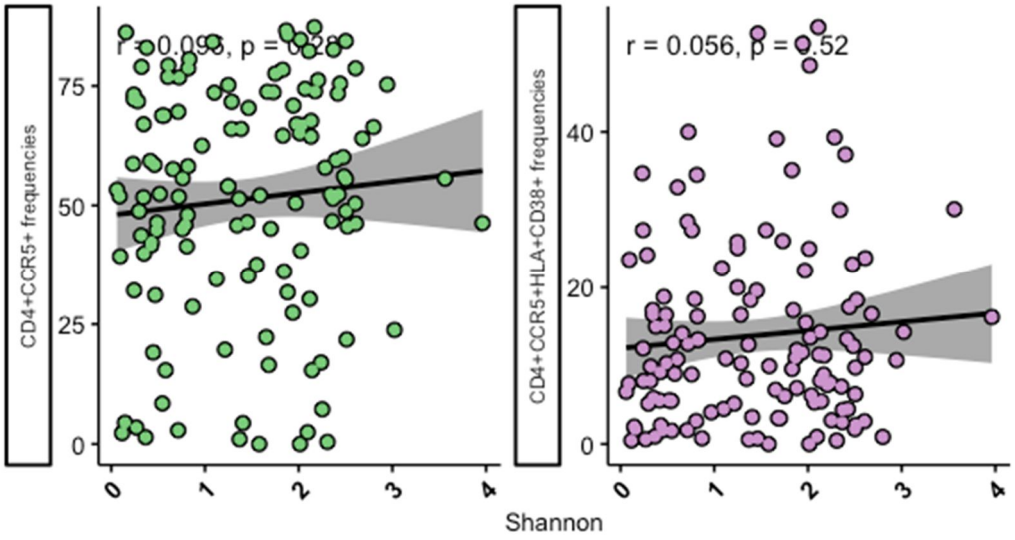


Figure 6.20. CD4+CCR5+ T cell frequencies and alpha diversity. Correlation plots of CD4+CCR5+ and CD4+CCR5+CD38+HLA-DR+ T cell frequencies (out of total CD4+ T cells) and alpha diversity measured by Shannon Index. In screening visits from 115 participants The Spearman correlation coefficient (rho, r) and the p values for the different study arms are depicted in the figure.

Table 6.18. CD4+CCR5+ T cell frequencies according to BV status at screening.

	BV positive (n=52)	**Intermediate** (n=18)	**BV negative** (n=59)	**P value**
CD4+CCR5+%, median (IQR)	58.7 (39.2-74.6)	46.5 (38.5-57.8)	53.3 (39.6-69.6)	0.274
CD4+CCR5+CD38+HLA-DR+%, median (IQR)	11.05 (5.1-17.7)	17.8 (6.6-23.5)	10.3 (5.3-16.5)	0.389

P values calculated using Kruskal Wallis test.

We then binarized the CD4+CCR5+ frequency into a high (n=64) and low (n=65) group (using the overall median of 53.3%) (**Table 6.19**). There were no significant differences between the two groups in regard to any of the measures analysed including community cluster distribution, vaginal pH, BV, STI and yeast prevalence, HC use, intravaginal practices or sexual risk behaviour (**Table 6.19**). Using logistic regression, no association of BV status on CD4+CCR5+ group was found after adjusting for the presence of an STI. However, being BV positive and positive for one or more STI was significantly associated with the high CD4CCR+ group (OR: 6.46, 95% CI: 1.33-33.5, p=0.023). Again, having BV and being positive for *C. trachomatis* meant being more likely to fall within the high CD4+CCR5+ group compared to being BV and *C. trachomatis* negative (p=0.002, OR=23.6,95% CI: 3.60-220.5). We found no significant differences in beta diversity between the high and low CD4+CCR5+ group (Adonis, p=0.775, R=0.004, betadisper, p=0.186, F=1.772) (**Figure 5.21**).

Table 6.19. Participant characteristics according to CD4+CCR5+ binarization.

	High CD4+CCR5+ (n=64)	Low CD4+CCR5+ (n=65)	P value
Fuzzycluster[1]			0.451
C1	33 (55.0%)	30 (47.6%)	
C2	14 (23.3%)	13 (20.6%)	
C3	13 (21.7%)	20 (31.7%)	
BV prevalence			0.547
BV positive	28 (43.8%)	24 (36.9%)	
BV intermediate	7 (10.9%)	11 (16.9%)	
BV negative	29 (45.3%)	30 (46.1%)	
Vaginal pH, mean (sd)	4.95 (4.37-5.52)	4.93 (4.30-5.57)	0.914
Shannon Index, median (IQR)	1.70 (0.72-2.17)	1.37 (0.48-2.10)	0.213
STI prevalence			
Any STI(s)	27 (42.2%)	27 (41.5%)	1.000
Ct	22 (34.4%)	19 (29.2%)	0.661
Ng	9 (14.1%)	7 (10.8%)	0.764
Tv	3 (4.7%)	9 (13.8%)	0.127
Mg	2 (3.1%)	1 (1.5%)	0.619
HSV-2 serology[2]	19 (29.7%)	14 (21.5%)	0.359
Yeast cells present	12 (18.8%)	7 (10.8%)	0.303
Use of hormonal contraception[3]			0.174
None	15 (23.4%)	15 (25.0%)	
Net-En	31 (48.4%)	37 (61.7%)	
COCP	6 (9.4%)	1 (1.7%)	
DMPA	10 (15.6%)	7 (11.7%)	
Implanon	2 (3.1%)	0 (0.0%)	
Intra-vaginal practices[4]			
Douching	1 (1.6%)	0 (0.0%)	1.000
Washing with water	7 (10.9%)	10 (16.1%)	0.554
Washing with soap	8 (12.5%)	5 (8.1%)	0.599
Cloth	1 (1.6%)	1 (1.6%)	1.000
Drying	1 (1.6%)	0 (0.0%)	1.000
Medication	3 (4.7%)	4 (6.6%)	0.715
Tampon use	4 (6.3%)	3 (4.8%)	1.000
Herbs	1 (1.6%)	0 (0.0%)	1.000
Sexual risk behaviour[5]			
Sexual partners, median (IQR)	1 (1-1)	1 (1-1)	0.434
Multiple sexual partners, n (%)	2 (3.8%)	2 (3.6%)	1.000
New partner(s), n (%)	18 (34.0%)	15 (27.3%)	0.585
General condom use			0.454
Never	7 (13.2%)	2 (3.6%)	
Almost never	5 (9.4%)	7 (12.7%)	
Not sure	6 (11.3%)	8 (14.5%)	
Almost always	18 (34.0%)	22 (40.0%)	
Always	17 (32.1%)	16 (29.1%)	
Condom use during last PV intercourse			
Yes	30 (57.7%)	35 (63.6%)	0.666
PV sex acts per week, median (IQR)	1 (1-2)	1 (1-2)	0.869
Intergenerational sex with older partner (≥5 years)			0.412
No	22 (41.5%)	17 (30.9%)	
I don't think so	1 (1.9%)	5 (9.1%)	
Not sure	18 (34.0%)	19 (34.5%)	
I think so	1 (1.9%)	3 (5.5%)	
Yes	11 (20.8%)	11 (20.0%)	
Transactional sex	0 (0.0%)	1 (1.8%)	1.000
Penile-anal intercourse	1 (1.9%)	2 (3.6%)	0.424

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups, unpaired Mann-Whitney-Wilcoxon test for comparison of medians and unpaired Student's t test for comparison of means. BV, bacterial vaginosis; Ct, Chlamydia trachomatis*; HSV-2, herpes simplex virus type 2; IQR, interquartile range; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. No Fuzzy cluster assignment for six samples (two in "High CD4+CCR5+" and four in "Low CD4+CCR5+").

2. One equivocal result.
3. Missing data from five samples, all from "Low CD4+CCR5+.
4. Missing data from three samples, all from "Low CD4+CCR5+.
5. Missing data from 21 samples (eleven from "High CD4+CCR5+" and ten from "Low CD4+CCR5+")

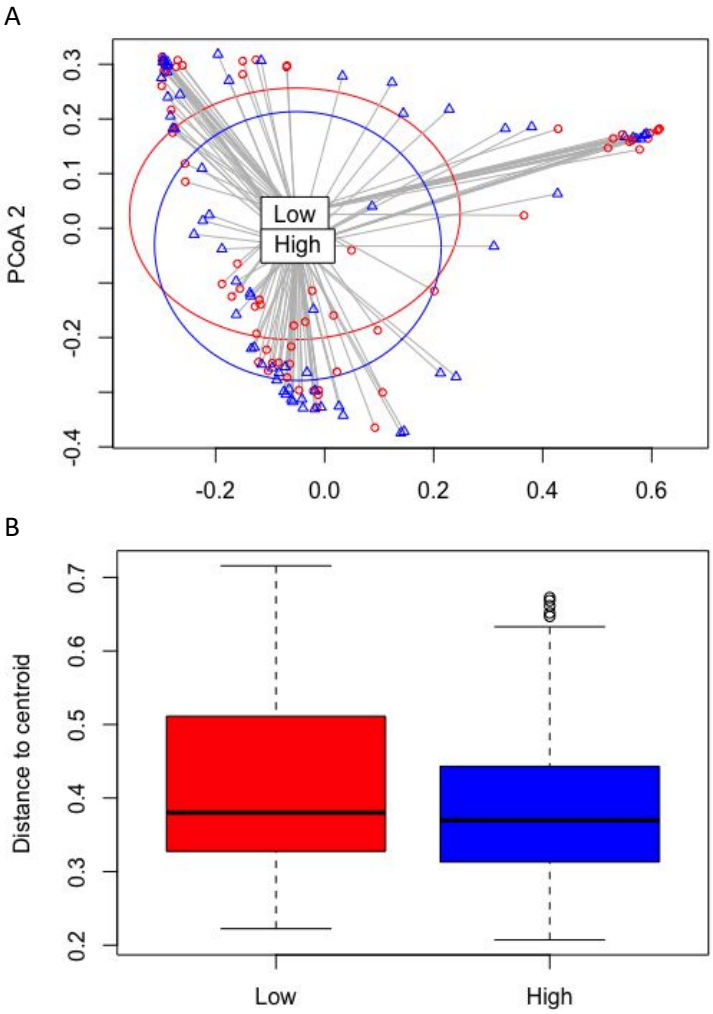


Figure 6.21. Beta diversity within CD4+CCR5+ clusters. A) Principal Coordinates Analysis (PcoA) of beta diversity calculated using weighted UniFrac according to high and low CD4+CCR5+ clusters with standard deviations ellipses around each study arm centroid. B) Barplot depicting the distance to the centroid for each sample in each CD4+CCR5+ group.

6.3.7 Differentially abundant taxa between high and low CD4+CCR5+ groups

No significant association between the frequency of CD4+CCR5+ T cells and any specific taxa were found (significance level: adj. p=0.1 and r=0.2). Using metagenomeSeq, four taxa (merged at lowest taxonomic level) were differentially relative abundant between the low and high CD4+CCR5+ groups (**Table 6.20**). *Mycoplasmataceae* had a higher relative abundance in the low CD4+CCR5+ group and *Methylobacterium radiotolerans*, BVAB3 and *Mobiluncus mulieris* had a higher relative abundance in the high CD4+CCR5+ group.

Table 6.20. Differentially abundant taxa between high and low CD4+CCR5+ group.

Coeff	P adj.	Family	Genus	Species
-2.221	0.0008	Mycoplasmataceae	NA	NA
1.253	3.8e-08	Methylobacteriaceae	*Methylobacterium*	*radiotolerans*
1.548	0.0190	Clostridiaceae	*Clostridium*	*BVAB3_M_indolicus*
1.703	0.0061	Actinomycetaceae	*Mobiluncus*	*mulieris*

The most predictive taxa in predicting CD4+CCR5+ group according to random forest analysis were *L. crispatus*, *P. timonensis*, *Megashaera*, *BVAB-1*, *Porphyromonas*, *P. eunonis*, *Bacteroidales*, *G. vaginalis*, *Arcanobacterium* and *L. iners* (Training AUC=0.36, sensitivity=0.57 and specificity=0.61 and a validation predicted error: 34.88% when using the top 5 features) (**Figure 6.22** and **Table 6.21**).

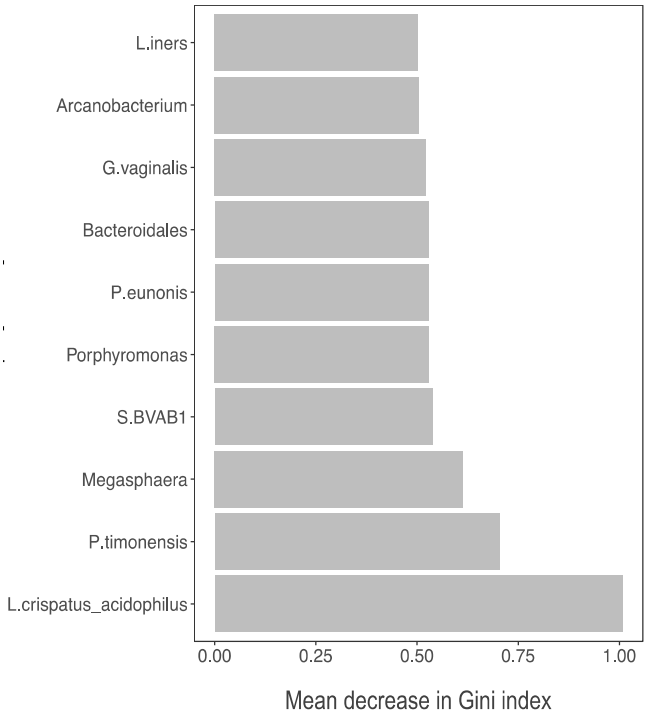


Figure 6.22. Random forest analysis of CD4+CCR5+ groups. The top 10 most influential taxa by random forest analysis in predicting CD4+CCR5+ group at baseline. The x-axis indicates the mean decrease in Gini Index (length of bar represents predictive ability of each taxon).

Table 6.21. Top ten taxa predictive of CD4+CCR5+ group (high versus low) using random forest analysis.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_2	2.738	1.008	*Lactobacillus crispatus_acidophilus*
OTU_10	-0.564	0.704	*Prevotella timonensis*
OTU_5	-2.011	0.614	*Megasphaera*
OTU_3	0.327	0.538	S. BVAB1
OTU_81	1.291	0.530	*Porphyromonas*
OTU_40	0.619	0.528	*Porphyromonas eunonis*
OTU_63	0.003	0.528	*Bacteroidales*
OTU_4	-0.938	0.522	*Gardnerella vaginalis*
OTU_125	2.962	0.504	*Arcanobacterium*
OTU_1	-2.848	0.503	*Lactobacillus iners*

Training set size: 86 samples with 42 and 44 samples per class. Test set size: 43 samples with 23 and 20 samples per class. Validation predicted error: 34.88% (using top five features).

Using DeSeq2, the standardized read counts eleven taxa (merged at lowest taxonomic level) were differentially abundant between the CD4+CCR5+ groups (alpha 0.01) (**Table 6.22**). *Mobiluncus mulieris*, *Bacteroidales*, *Prevotella bivia* and *Peptoniphilus lacrimalis* were more abundant in the high CD4+CCR5+ group while *Gemella morbillorum_cluster*, *Mycoplasmataceae*, *Chryseobacterium*, *Streptococcus* spp. and *Staphylococcus* were more abundant in the low CD4+CCR5 group.

Table 6.22. DESeq2 analysis of CD4+CCR5 groups.

Log2FC	P adj.	Family	Genus	Species
3.573	1.0e-07	Actinomycetaceae	*Mobiluncus*	*mulieris*
-3.534	1.9e-06	Gemellaceae	*Gemella*	*morbillorum_cluster*
-7.537	9.4e-06	Mycoplasmataceae	NA	NA
-3.087	0.0001	[Weeksellaceae]	*Chryseobacterium*	NA
-1.786	0.0090	Streptococcaceae	*Streptococcus*	*infantis_mitis_oralis*
2.248	0.0090	Bacteroidales*	NA	NA
-2.303	0.0155	Streptococcaceae	*Streptococcus*	*anginosus*
1.635	0.0155	Prevotellaceae	*Prevotella*	*bivia*
-1.351	0.0378	Staphylococcaceae	*Staphylococcus*	NA
-2.783	0.0378	Streptococcaceae	*Streptococcus*	*mutans*
1.309	0.0419	[Tissierellaceae]	*Peptoniphilus*	*lacrimalis*

*NOTE: only annotated at order level

6.3.8 Th17 cell frequency according to the vaginal microbiota

Th17 cells have been described as a major immune cell subset in the FGT (McKinnon et al., 2011; Rodriguez-Garcia et al., 2014) and an important SIV target cell in the genital mucosa of macaques (Stieh et al., 2016). Also Th17 cells are known to be important mediators of immunity to bacteria at mucosal surfaces (Khader et al., 2009). We therefore analysed the frequencies of Th17 cells defined based on chemokine receptiors (CD4+CCR6+CCR10-) in cervical cytobrushes and related these to the vaginal microbiota for 115 participants at screening. Since we only used chemokine receptors to define the populations, we will refer to these cells as Th17-like in the remainder of this dissertation. We did not observe any significant differences in Th17-like frequencies (out of the total CD4+ T cell population) (p=0.352) or in Th17-like counts (p=0.558) between the three vaginal community clusters (**Figure 6.23** and **Table 6.23**) or any significant correlations between the Th17-like frequencies (p=0.31) or Th17-like cell counts (p=0.40) and alpha diversity (**Figure 6.24**). We likewise did not find any differences in the frequencies of CCR5+ Th17-like cells (p=0.330) or activated (HLA-DR+CD38+) CCR5+ Th17-like cells (p=0.766) between the three clusters (**Figure 6.23** and **Table 6.23**). No significant association between the frequencies of Th17-like cells, Th17-like cell counts and any specific taxa were found (significance level: adj. p=0.1 and R=0.2).

Table 6.23. Th17-like frequencies according to Fuzzy clusters at screening.

	C1 (n=57)	**C2** (n=22)	**C3** (n=31)	**P value**
Th17-like%, median (IQR)	58.0 (44.1-66.5)	55.0 (43.8-64.03)	49.4 (43.2-61.2)	0.352
Th17-like count, median (IQR)	222 (90-553)	128 (60.0-420)	175 (78.0-441)	0.558
Th17-like CCR5+%, median (IQR)	70.5 (58.4-79.2)	63.5 (54.0-75.0)	64.5 (52.4-74.5)	0.330
Th17-like CCR5+HLA+CD38+%, median (IQR)	14.3 (6.7-28.8)	14.0 (6.8-20.9)	13.4 (6.2-23.5)	0.766

P values calculated using Kruskal Wallis test. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from the analyses (n=5).

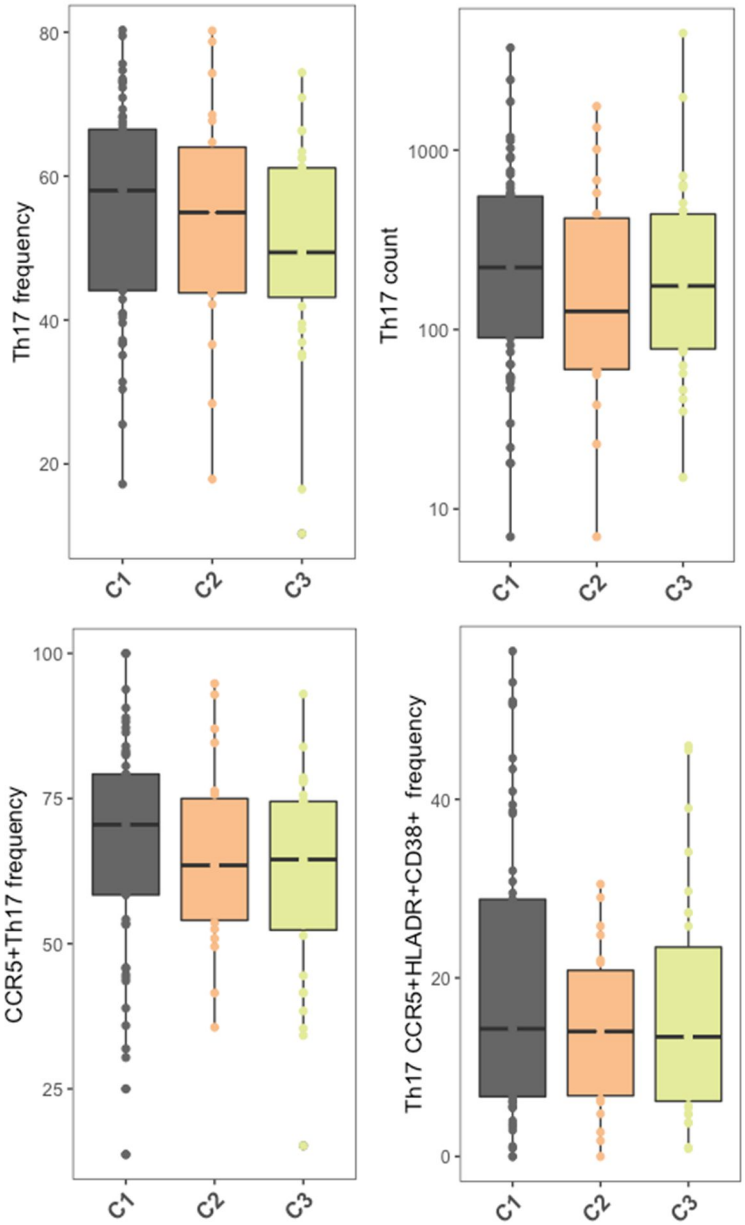


Figure 6.23. Th17-like frequency according to Fuzzy clusters. Boxplot depicting the Th17-like (CD4+CCR6+CCR10-) frequencies (out of the total CD4+ population), Th17-like counts, CCR5+ Th17-like frequencies (out of total Th17-like population) and activated (HLA-DR+CD38+) CCR5+ Th17-like frequencies (out of total Th17-like population) measured by flow cytometry in cervical cytobrushes from 110 participants according to community cluster (C1: n=57; C2: n=22; C3: n=32) defined using Fuzzy clustering with weighted UniFrac distances. P values were calculated using Kruskal Wallis test. Samples that did not meet the minimum probability of ≥ 60% of belonging to any of the three clusters were excluded from the figure (n=5). Y-axis log10 transformed for Th17-like counts.

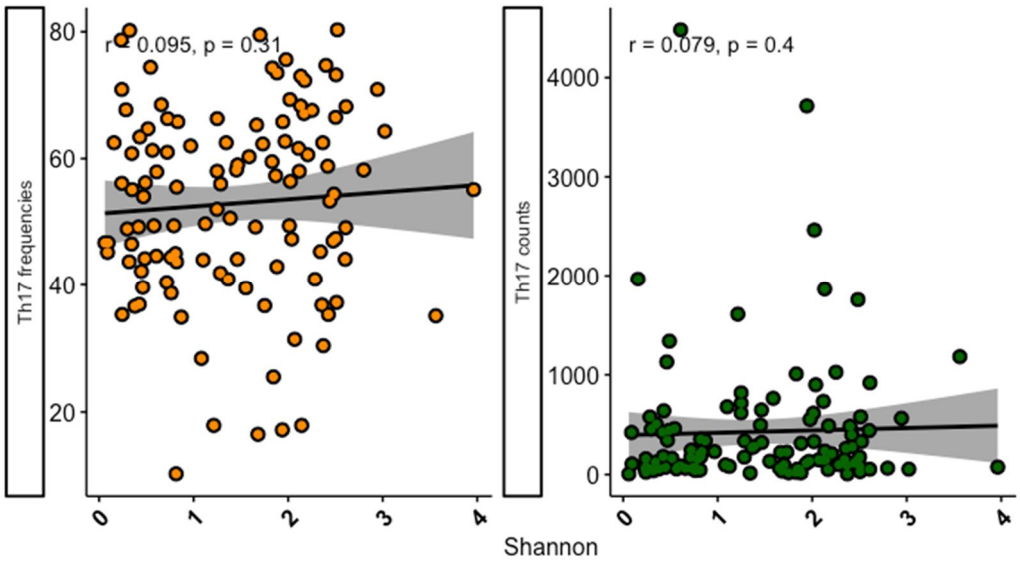


Figure 6.24. Th17-like cell frequencies and alpha diversity. Correlation plots of Th17-like (CD4+CCR6+CCR10-) frequencies (out of total CD4+ T cells), Th17-like counts and alpha diversity measured by Shannon Index. In screening visits from 115 participants The Spearman correlation coefficient (rho, r) and the p values for the different study arms are depicted in the figure.

Additionally, we did not see any significant differences in the frequencies of Th17-like or Th17-like counts between BV positive and negative participants at baseline (**Table 6.24**). Using linear regression, no association between BV status and Th17-like frequency was found after adjusting for the presence of one or more STIs, any individual STI or the presence of yeast cells.

Table 6.24. Th17-like cell frequencies according to BV status at screening.

	BV positive (n=48)	**Intermediate** (n=15)	**BV negative** (n=52)	**P value**
Th17-like %, median (IQR)	58.1 (45.0-66.7)	47.4 (32.6-62.4)	53 (44.2-62.1)	0.281
Th17-like count, median (IQR)	192 (83.5-556)	222 (78.5-575)	190.5 (76.5-445)	0.998
Th17-like CCR5+%, median (IQR)	70.2 (574-82.5)	65.3 (49.0-74.3)	64.4 (54.8-74.9)	0.321
Th17-like CCR5+HLA+CD38+%, median (IQR)	13.5 (6.3-23.3)	20.1 (8.6-27.3)	13.6 (6.0-21.8)	0.317

P values calculated using Kruskal Wallis test.

There were no correlation between Th17-like frequencies (including Th17-like CCR5+ and Th17-like CCR5+HLA-DR+CD38+) or Th17-like counts with IL-17A or IL-17F cytokine concentrations (**Figure 6.25**).

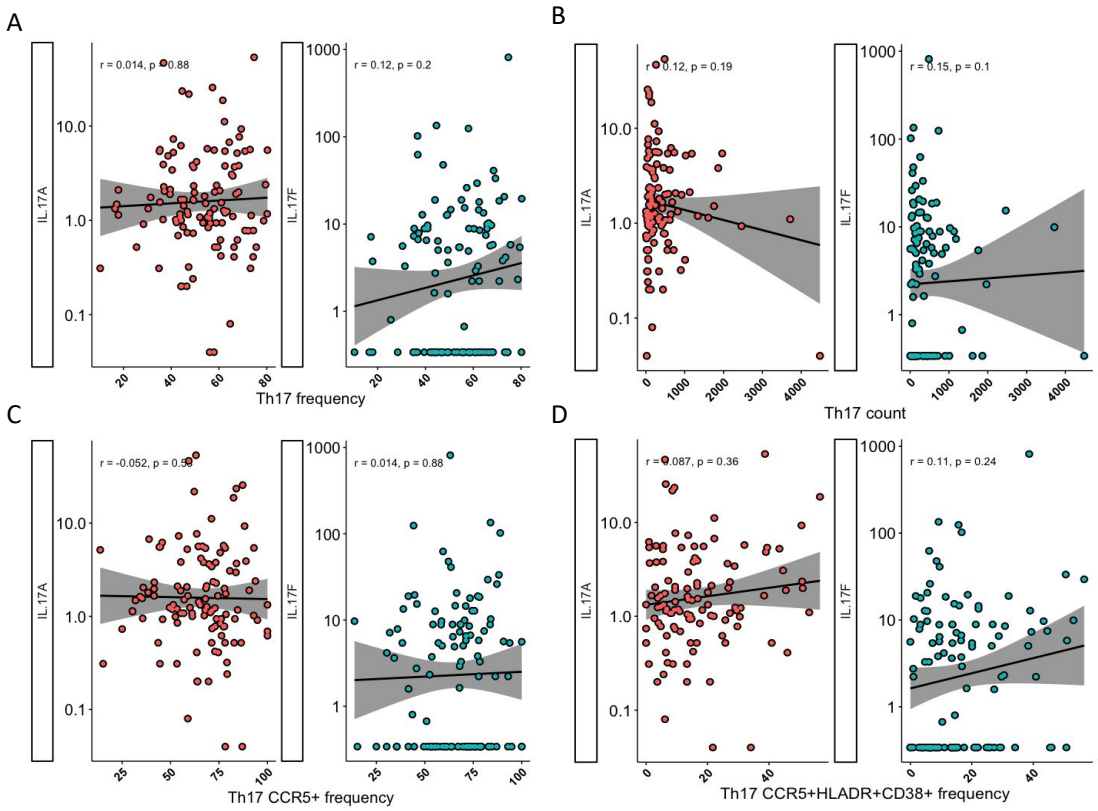


Figure 6.25. Correlation plots of IL-17 cytokines concentrations and Th17-like cell frequencies. Correlation plots showing the levels of IL-17A (pink) and IL-17F (light blue) according to A) Th17-like frequencies, B) Th17-like count, C) Th17-like CCR5+ frequencies and D) Th17-like CCR5+HLA-DR+CD38+ frequencies in cervical cytobrush samples from 115 participants. The Spearman correlation coefficient (rho, r) and the p values for each individual cytokine depicted on the figure. Y-axis is log10 transformed.

After binarization of the Th17-like cell frequencies into high (n=56) and low (n=59) (using the overall median of 55.1%), no differences in the proportion of the three vaginal community types were observed between the two Th17-like groups. Similarly, no differences in alpha diversity, vaginal pH or BV and yeast prevalence were found between the groups (**Table 6.25**). There were no differences in *C. trachomatis* prevalence between the groups, but we observed a trend towards higher *N. gonorrhoea* prevalence in the high Th17-like cell group and there was a significantly higher prevalence of *T. vaginalis* in the low Th17-like group. However, there was no statistically significant difference in the Th17-like cell frequency between *T. vaginalis* positive and negative participants (**Table 6.26**). There was a significantly higher frequency of Th17-like cells in *N. gonorrhoea* positive compared to *N. gonorrhoea* negative participants but not after adjusting for multiple comparisons (**Table 6.26**). Using logistic regression, no association of BV status on Th17-like frequency was found after adjusting for

the presence of one or more STIs, any individual STI and/or presence of yeast cells. No significant differences in HC usage, intravaginal practices and sexual risk behaviour were found between the Th17-like high and low groups (**Table 6.25**). We found no significant differences in beta diversity between the high and low Th17-like group (adonis, p=0.113, R2=0.017, betadisper, p=0.907, F=0.014) (**Figure 6.26**).

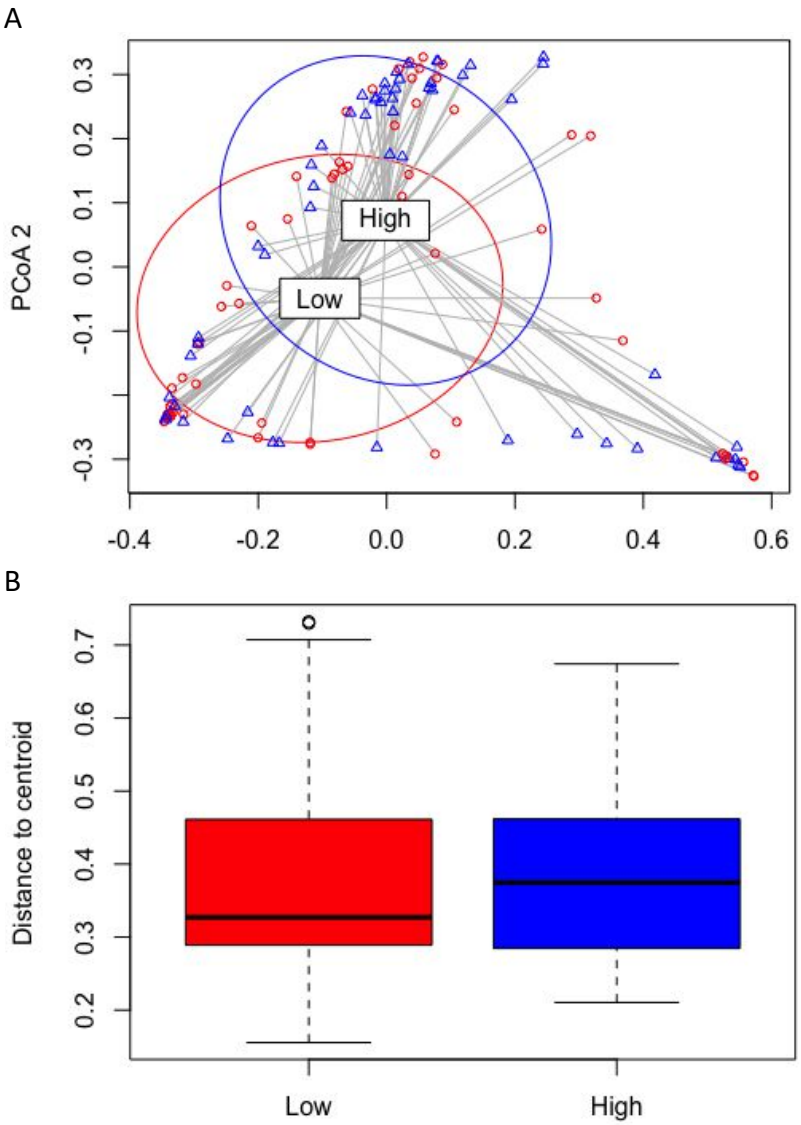


Figure 6.26. Beta diversity within Th17-like clusters. A) Principal Coordinates Analysis (PCoA) of beta diversity calculated using weighted UniFrac according to high and low Th17-like (CD4+CCR6+CCR10-) clusters with standard deviations ellipses around each study arm centroid. B) Barplot depicting the distance to the centroid for each sample in each Th17-like group.

Table 6.25. Participant characteristics according to Th17-like binarization.

	High Th17 (n=56)	Low Th17 (n=59)	P value
Fuzzycluster[1]			0.233
C1	31 (58.5%)	26 (45.6%)	
C2	11 (20.8%)	11 (19.3%)	
C3	11 (20.8%)	20 (35.1%)	
BV prevalence			0.374
BV positive	27 (48.2%)	21 (35.6%)	
BV intermediate	6 (10.7%)	9 (15.3%)	
BV negative	23 (41.1%)	29 (49.2%)	
Vaginal pH, mean (sd)	4.94 (4.36-5.52)	4.95 (4.35-5.55)	0.942
Shannon Index, median (IQR)	1.72 (0.70-2.14)	1.25 (0.53-2.11)	0.318
STI prevalence			
Any STI(s)	21 (37.5%)	25 (42.4%)	0.732
Ct	16 (28.6%)	19 (32.2%)	0.826
Ng	10 (17.9%)	4 (6.8%)	0.089
Tv	1 (1.8%)	8 (13.6%)	**0.032**
Mg	1 (1.8%)	2 (3.4%)	1.000
HSV-2 serology[2]	19 (33.9%)	13 (22.0%)	0.222
Yeast cells present	10 (17.9%)	8 (13.6%)	0.706
Use of hormonal contraception[3]			0.502
None	15 (27.3%)	14 (25.0%)	
Net-En	26 (47.3%)	32 (57.1%)	
COCP	5 (9.1%)	1 (1.8%)	
DMPA	8 (14.5%)	8 (14.3%)	
Implanon	1 (1.8%)	1 (1.8%)	
Intra-vaginal practices[4]			
Douching	1 (1.8%)	0 (0.0%)	0.487
Washing with water	6 (10.9%)	8 (13.6%)	0.858
Washing with soap	6 (10.9%)	6 (10.3%)	1.000
Cloth	1 (1.8%)	1 (%1.7%)	1.000
Drying	1 (1.8%)	0 (0.0%)	0.487
Medication	4 (7.3%)	3 (5.2%)	0.712
Tampon use	3 (5.5%)	2 (3.4%)	0.674
Herbs	1 (1.8%)	0 (0.0%)	0.487
Sexual risk behaviour[5]			
Sexual partners, median (IQR)	1 (1-1)	1 (1-1)	0.364
Multiple sexual partners, n (%)	2 (4.3%)	1 (2.05)	0.610
New partner(s), n (%)			
General condom use			0.523
Never	3 (6.4%)	6 (12.0%)	
Almost never	6 (12.8%)	6 (12.0%)	
Not sure	9 (19.1%)	5 (10.0%)	
Almost always	13 (27.7%)	19 (38.0%)	
Always	16 (34.0%)	14 (28.0%)	
Condom use during last PV intercourse			
Yes	25 (53.2%)	33 (66.0%)	0.338
PV sex acts per week, median (IQR)	1 (1-2)	1 (1-2)	0.633
Intergenerational sex with older partner (≥5 years)	13 (27.7%)	15 (30.0%)	0.826
			0.888
No	19 (40.4%)	17 (34.0%)	
I don't think so	2 (4.3%)	3 (6.0%)	
Not sure	15 (31.9%)	17 (34.0%)	
I think so	2 (4.3%)	1 (2.0%)	
Yes	9 (19.1%)	12 (24.0%)	
Transactional sex	0 (0.0%)	1 (2.05%)	1.000
Penile-anal intercourse	2 (4.3%)	1 (2.05%)	0.610

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups, unpaired Mann-Whitney-Wilcoxon test for comparison of medians and unpaired Student's t test for comparison of means. BV, bacterial vaginosis; Ct, Chlamydia trachomatis*; HSV-2, herpes simplex virus type 2; IQR, interquartile range; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis*.*

1. No Fuzzy cluster assignment for five samples (three in "High Th17" and two in "Low Th17").
2. One equivocal result.
3. Missing data from four samples (one from "High Th17" and three from "Low Th17").
4. Missing data from two participant visits (one from "High Th17" and one from "Low Th17")
5. Missing data from 18 participant visits (nine from "High Th17" and nine from "Low Th17")

Table 6.26. Th17-like cell frequencies levels according to vaginal infections.

	%Th17-like	P	P adj.	Th17-like count	P	P adj.
STI		0.595	0.973		0.973	0.973
Yes	52.7 (44.1-65.4)			203 (86.3-452.3)		
No	55.5 (42.9-62.7)			200 (75-577)		
C. trachomatis		0.431	0.631		0.631	0.631
Yes	49.7 (44.2-67.7)			175 (79.5-448.5)		
No	55.3 (43.5-62.5)			204.5 (75-577.5)		
N. gonorrhoea		**0.040**	0.082		0.945	0.945
Yes	61.5 (54.1-65.9)			159 (75.5-547)		
No	50.6 (42.9-62.7)			222 (77-488)		
T. vaginalis		0.239	0.479		0.843	0.843
Yes	45.3 (44.1-49.1)			261 (151-422)		
No	56.1 (43.8-64.6)			191 (75.0-542)		
Yeast cells		0.779	0.779		0.193	0.387
Yes	55.95 (45.3-60.7)			330 (109-670.5)		
No	53.3 (43.7-64.3)			175 (75-488)		
HSV-2 serology		0.164	0.328		0.676	0.676
Yes	58.6 (46.2-66.4)			178 (85-338)		
No	49.7 (41.9-62.5)			200 (75-553)		

P values calculated using Kruskal-Wallis test and unpaired Mann-Whitney-Wilcoxon test adjusted using Benjamini-Hochberg (BH) method. BV, bacterial vaginosis; HSV-2, herpes simplex virus type 2; STI, sexually transmitted infection.

6.3.9 Differentially abundant taxa between high and low Th17-like groups

Using metagenomeSeq, six taxa (merged at lowest taxonomic level) were differentially abundant between the low and high Th17-like group (**Table 6.27**). *Streptococcus agalactiae* and *Prevotella pallens* had a higher relative abundance in the low Th17-like group while *Coriobacteriaceae*, BVAB1-2 and *Prevotella* had a higher relative abundance in the high Th17-like group.

Table 6.27. Differentially abundant taxa between high and low Th17-like group.

Coeff	P adj.	Family	Genus	Species
-1.731	0.0070	Streptococcaceae	*Streptococcus*	*agalactiae*
-1.465	0.0453	Prevotellaceae	*Prevotella*	*pallens*
1.356	0.0432	Coriobacteriaceae	NA	NA
1.618	2.3e-08	Lachnospiraceae	*Shuttleworthia*	BVAB1
1.700	0.0003	[Paraprevotellaceae]	*[Prevotella]*	NA
2.880	3.5e-05	Clostridiaceae	*Clostridium*	BVAB2

The most predictive taxa in predicting Th17-like group according to random forest analysis were *A. obiensis*, *P. harei_asaccharolyticus_grossensis*, *A. vaginae*, *D. micraerophilus*, *L. crispatus_acidophilus*, *D. invisus*, *F. magna*, *A. prevotii_tetradius*, *H. parainfluenzae* and *L. iners* (Training AUC=0.37, sensitivity=0.61 and specificity=0.56 when using the top five features) (**Figure 6.27** and **Table 6.28**).

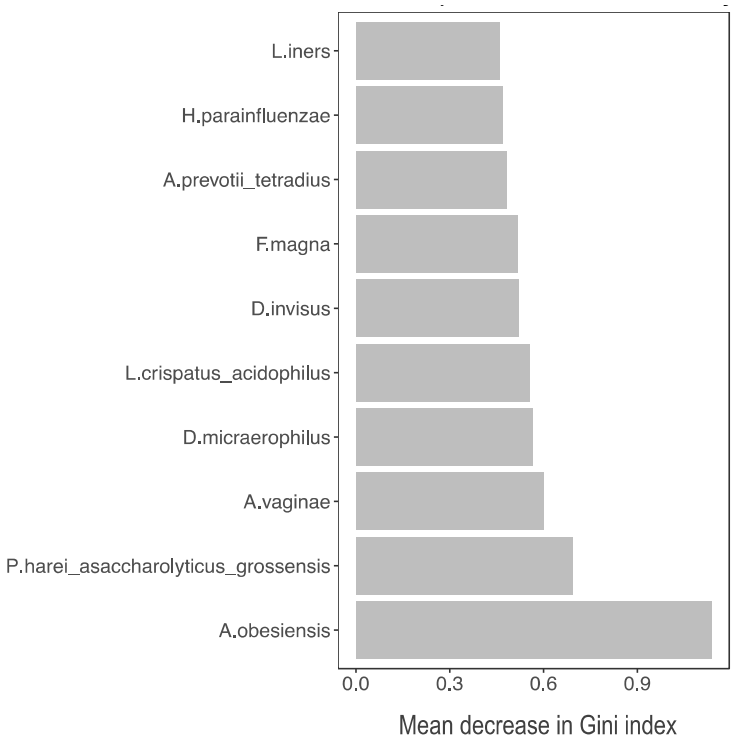


Figure 6.27. Random forest analysis of Th17-like groups. The top 10 most influential taxa by random forest analysis in predicting Th17-like group at baseline. The x-axis indicates the mean decrease in Gini Index (length of bar represents predictive ability of each taxon).

Table 6.28. Top ten taxa predictive of Th17-like group (high versus low) using random forest analysis.

OTU	Mean Decrease Accuracy	Mean Decrease Gini	Taxa
OTU_62	4.624	1.137	*Anaerococcus obesiensis*
OTU_35	2.406	0.692	*Peptoniphilus harei_asaccharolyticus_grossensis*
OTU_8	2.039	0.600	*Atopobium vaginae*
OTU_39	-0.809	0.563	*Dialister micraerophilus*
OTU_2	2.110	0.557	*Lactobacillus crispatus_acidophilus*
OTU_176	0.762	0.520	*Dialister invisus*
OTU_34	0.291	0.517	*Finegoldia magna*
OTU_28	2.154	0.481	*Anaerococcus prevotii_tetradius*
OTU_48	1.609	0.468	*Haemophilus* parainfluenzae
OTU_1	0.341	0.458	*Lactobacillus iners*

Training set size: 77 samples with 38 and 39 samples per class. Test set size: 38 samples with 21 and 17 samples per class. Validation predicted error: 50.0% (using top five features).

Using DeSeq2, seven taxa (merged at lowest taxonomic level) were found to be differentially abundant between the Th17-like groups (alpha 0.05) (**Figure 6.28** and **Table 6.29**). *S. agalactiae*, *Prevotella bivia*, *Dialister invisus* and *M. hominis* were more abundant in low Th17-like group while *Leptotrichiaceae*, *Gardnerella* and BVAB2 were more abundant in the high Th17-like group.

Table 6.29. DESeq2 analysis of high and low Th17-like groups.

Log2FC	P adj.	Family	Genus	Species
-3.017	0.012	Streptococcaceae	*Streptococcus*	*agalactiae*
-1.754	0.012	Prevotellaceae	*Prevotella*	*bivia*
2.164	0.012	Veillonellaceae	*Dialister*	*invisus*
-1.557	0.012	Leptotrichiaceae	NA	NA
-2.095	0.029	Clostridiaceae	*Clostridium*	BVAB2
2.281	0.032	Mycoplasmataceae	*Mycoplasma*	*hominis*
2.6010	0.032	Bifidobacteriaceae	*Gardnerella*	NA

*NOTE: only annotated at order level

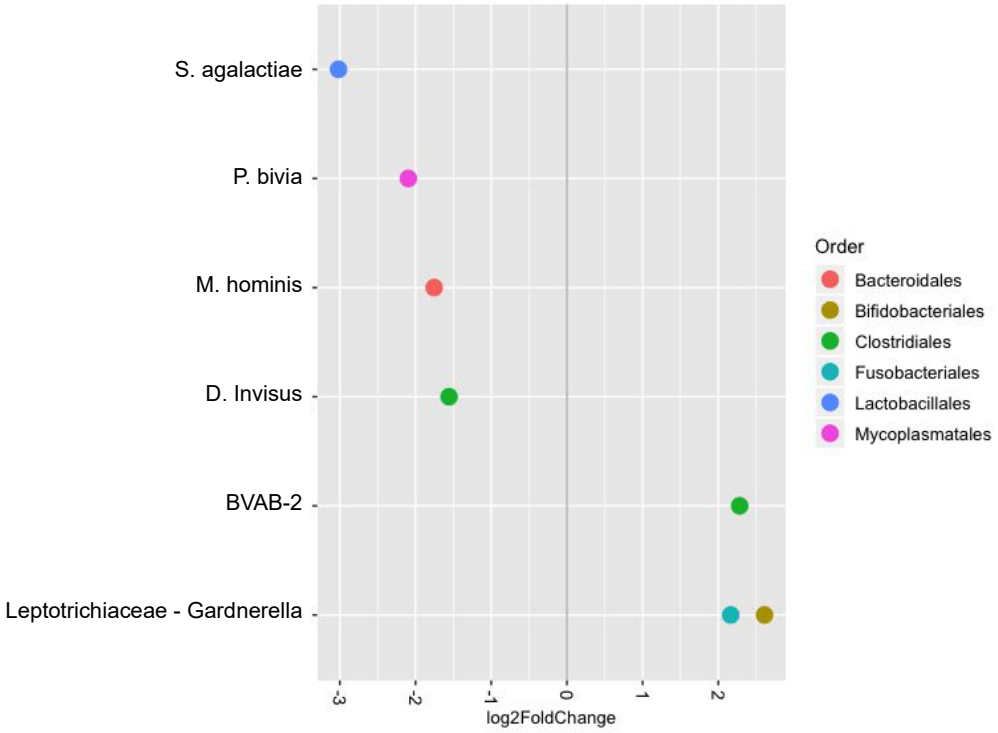


Figure 6.28. DESeq2 analysis of Th17-like groups. Bacterial taxa significantly differentially abundant by Th17-like group using DESeq2 (with an alpha of 0.05). Taxa depicted at species level. Taxa without species level annotation (if >1) are grouped in the first line and order of genera corresponds to order of appearance of dots (from left to right).

6.4 Discussion

6.4.1 Cervical cytokines and the vaginal microbiota

In this study, thirteen cytokines from a Th17 panel were analysed and their concentrations were related to the vaginal microbiota. The concentrations of all but one cytokine, IL-17A, were elevated in the diverse C1 community cluster compared to both of the lactobacilli dominant clusters C2 and C3 and for nine cytokines the concentrations were also significantly higher in the *L. iners* dominant cluster compared to the *L. crispatus* dominant cluster. Furthermore, the concentrations of all thirteen cytokines were positively associated with alpha diversity. Using Kruskal-Wallis test for this analysis is somewhat flawed due to violation of the assumptions of the test as these analyses includes multiple samples from the same adolescents. A lme model was thus generated with visit and participant ID included in the model. Further developent of a statistical model taking subject, time, intervention and other factors such as antibiotic treatment into account will be completed together with a experienced statistician. The results of the lme model gave the same output as the Kruskal-

Wallis test. Furthermore, these results are in line with studies suggesting that high microbial diversity is associated with an inflammatory response, while lactobacilli dominant communities, particularly *L. crispatus* dominant communities, are associated with less inflammation (Lennard et al., 2017; Gautam et al., 2015; Anahtar et al., 2015; Gosmann et al., 2017; Rebbapragada et al., 2008). Furthermore, bacteria associated with BV (e.g. *G. vaginalis*, *Sneathia*, BVAB 1-3*, Megasphaera*, *Dialister*, *A. vaginae, Aerococcus christensenii* and *Mobiluncus mulieris*) and increased HIV risk (e.g. *Prevotella* spp*., Gemella asaccharolytica, Mycoplasma hominis, Sneathia*, *Megasphaera*, and *Parvimonas micra*) were shown to have a significantly higher relative abundance in the high inflammation group compared to the low inflammation group. Accordingly, these bacteria were positively correlated with the concentration of pro-inflammatory cytokines such as IFN-γ and IL-1β. Of interest, these included taxa that were increased in participants on Net-En (e.g. *Sneathia* and *Dialister*) or NuvaRing (e.g. *Prevotella and Parvimonas*) compared to COCP (Chapter 5).

The genus *Arcanobacterium*, which was more abundant in the vaginal microbiota of participants on both Net-En and NuvaRing (Chapter 5), was also associated with high inflammation. In contrast, *Clostridium perfringens* and several *Lactobacillus* species including *L. iners*, which were more abundant in COCP participants (Chapter 5), were associated with the low inflammation group. Correspondingly, the relative abundance of *L. crispatus* and *L. iners* was also negatively correlated with the concentration of pro-inflammatory cytokines. These data are in agreement with prior data from the same setting (WISH study described in Chapter 2.1.3 and Chapter 3 (Lennard et al., 2017)) and other studies conducted in South Africa (Gosmann et al., 2017; Anahtar et al., 2015). Inflammation related to BV-associated bacteria is of major concern due to the high BV prevalence (43.0%) in this cohort. Furthermore, inflammation of the FGT has been shown to recruit HIV target cells to the local mucosa (Arnold et al., 2016) and to correlate with HIV shedding in HIV infected women (Blish et al., 2012; Mauck et al., 2016). Cervicovaginal HIV shedding is associated with increased female-to-male and mother-to-child transmission. Inflammation has also been associated with increased permeability and decreased barrier function

of the vaginal epithelium (Arnold et al., 2016). These results thus underscore the role of BV-associated bacteria as a risk factor for HIV acquisition.

We observed a significantly higher prevalence of the high inflammation group in participants on NuvaRing compared to participants on COCP and significantly elevated levels of individual cytokines in participants on the NuvaRing compared to participants on COCP and Net-En. We also found significant intra-individual differences in cytokine levels depending on contraceptive use with increased cervical cytokine concentrations observed within participants when using NuvaRing compared to when using COCP or Net-En. Since the vaginal microbiota of participants on both Net-En and NuvaRing had higher levels of bacteria associated with inflammation the increased cytokine levels in participants on NuvaRing compared to Net-En could be due to the presence of a foreign object in the FGT. A local inflammatory reaction to the vaginal ring itself could thus be driving the observed differences rather than a microbiota driven response. Since women with elevated genital tract inflammatory markers, including pro-inflammatory cytokines, prior to HIV exposure are at increased risk of acquiring HIV (Masson et al., 2015a), our data suggest that there may be a potential for increased HIV risk in women using NuvaRing. However, more research is needed in order to assess the impact of NuvaRing use on HIV susceptibility.

6.4.2 IL-17 and vaginal infections

In concordance with previous studies, we found that IL-17A and IL-17F concentrations were increased in the genital tracts of participants with one or more STIs, particularly *C. trachomatis* (Masson et al., 2015b). These data suggest that production of IL-17A and IL-17F may be induced in response to bacterial STIs (Masson et al., 2015b), which also has been shown in mouse models (Andrew et al., 2013). HSV-2 serology was not associated with changes in genital IL-17 concentrations in this study. This is in agreement with data from a previous study showing no association of viral STIs (HSV-2 and HIV) with changes in genital IL-17 concentrations suggesting that IL-17 production is not induced in response to viral STIs in the FGT (Masson et al., 2015b). However, we only measured serology and do not have any data on HSV-2 shedding at the time

of sampling which could have added more information on the association between IL-17 and HSV-2.

IL-17 and IL-22 have been described as important cytokines in anti-fungal immunity (De Luca et al., 2010; Bar et al., 2014). In this study, we observed an elevated level of cervical IL-17A, IL-17F and IL-22 in participants with yeast present on microscopy compared to participants with no yeast cells observed. These results are in contrast to previous data showing decreased IL-17 levels in the genital tract in older women with yeast present (Masson et al., 2015b; Morrison et al., 2014). In these studies, it was suggested that yeast may actively suppress IL-17 production or that women with dampened IL-17 responses may be more susceptible to infection (Morrison et al., 2014). As stated in Chapter 5, section 5.3.3, there was a significant higher prevalence of yeast in participants on NuvaRing (26.0%) in this study compared to participants on both Net-En (17.4%) and COCP (8.51%) (p=0.050). Of interest, the IL-17A and IL-17F cytokines were positively correlated with taxa which were found to be more abundant in NuvaRing users compared to COCP and Net-En users (Chapter 5) including *Streptococcus anginosus*, *Gemella asaccharolytica*, *Staphylococcus* and *Corynebacterium striatum*. Together, these data suggest that the presence of the NuvaRing may have had an impact on the cytokine environment in the FGT in this study. Further, due to the age of the participants in this cohort, the *Candida* detected in this study most likely represent new infections and the IL-17 results may reflect acute inflammatory responses. Yet, compared to oropharyngeal candidiasis (or "thrush"), the role of IL-17-mediated immune responses in vaginal candidiasis has been considered controversial (Fidel, 2007). In one study, humans with mutations in the IL-17 axis were not susceptible to vaginal candidiasis (Rosentul et al., 2014) and in knockout mouse and antibody blocking studies, IL-17F did not appear to participate in anti-fungal immunity (Saijo et al., 2010; Iwakura et al., 2011). Thus, additional research on the role of IL-17 in vaginal yeast infections is needed.

We did not find any significant differences in IL-17A levels between *T. vaginalis* positive and negative participants, but we did observe significantly higher levels of other Th17 associated cytokines such as IL-17F, IL-22 and IL-25 in *T. vaginalis* positive versus negative participants. Masson et al (2015)

reported that IL-17 levels did not significantly differ between *T. vaginalis* positive and *T. vaginalis*-negative women (Masson et al., 2015b). In contrast, Makinde et al. reported elevated levels of IL-17 in cervicovaginal lavages from *T. vaginalis* positive women (Makinde et al., 2013). Similarly to our results, the latter group also reported elevated levels of IL-22 in *T. vaginalis* positive women. IL-25 (also known as IL-17E) only shares 16% sequence homology with IL-17A compared to 50% in the case of IL-17F. Correspondingly, IL-25 plays a different role in immunity than IL-17A and IL-17F, including regulating the Th2 response against helminthic parasites (Fort et al., 2001; Fallon et al., 2006). Accordingly, in this study, we observed an increased level of IL-25 in adolescents infected with the protozoan parasite *T. vaginalis*.

6.4.3 CD4+ T cells and the vaginal microbiota

We did not observe any differences in the frequencies of CD4+ T cells expressing CCR5, including activated (HLA-DR+CD38+) CCR5+CD4+ T cells, between the three vaginal community clusters C1-3 or between BV positive and negative participants. These data are in contrast to studies showing increased mucosal levels of activated CD4+ T cells (Gosmann et al., 2017) and CD4+ T cells expressing CCR5 in women with dysbiotic vaginal communities (Thurman et al., 2015) and decreased levels of activated CD4+ T cells in the cervix of women after successful treatment for BV (Rebbapragada et al., 2008). Yet, taxa associated with BV and inflammation such as *Mobiluncus mulieris*, *Prevotella bivia* and *Peptoniphilus* were more abundant in the high CD4+CCR5+ group. These bacteria were also associated with Net-En and/or NuvaRing use. In contrast, species associated with decreased inflammation and COCP use including *Gemella morbillorum_cluster*, *Chryseobacterium*, *Streptococcus* spp. and *Staphylococcus* were more abundant in the low CD4+CCR5 group. Of interest, having BV while being infected with *C. trachomatis* was associated with increased CD4+CCR5+ levels, suggesting that the combined effect of an STI and a dysbiotic microbial environment can lead to increased recruitment of HIV target cells and thus increased risk of HIV.

6.4.4 Th17 cells, the vaginal microbiota and vaginal infections

Despite a strong relationship between genital IL-17 concentrations, vaginal community clusters, bacterial STIs and yeast infections, a similar relationship was not observed with cervical Th17-like cell frequencies. No association was found with community clusters, BV or alpha diversity. Yet, *Streptococcus agalactiae*, which was found to be lower in participants using COCP, had a higher relative abundance in the low Th17-like group while BVAB1-2 and *Prevotella* spp., all species associated with BV, had a higher relative abundance in the high Th17-like group. The discrepancy between the IL-17 and Th17 data may be due to the production of IL-17 by various innate immune cells, which contributes to the total IL-17 concentrations (Cua & Tato, 2010). As such, there was no correlation between Th17 frequency and IL-17 concentrations in our study. Alternatively, we were not measuring true Th17 cells as we only used chemokine receptors to define the population. This thus represents a limitation in our study.

We did not find significant differences in cervical Th17-like frequencies between *C. trachomatis* positive and *C. trachomatis* negative participants or between *T. vaginalis* positive and *T. vaginalis* negative women despite a higher *T. vaginalis* prevalence in low Th17-like group. The prevalence of *T. vaginalis* was however low (8.6%) and this analysis could have been underpowered. We did however see a higher Th17-like cell frequency in *N. gonorrhoea* positive participants. Th17-like cells may play a role in the immune response to this bacterial pathogen. In contrast, these data suggest that the general composition of the vaginal community do not appear to play a strong role in the induction of Th17 cells.

6.4.5 Conclusions

The concentrations of investigated cytokines were positively associated with a diverse vaginal community and with specific bacterial taxa associated with BV and increased risk of HIV including species enriched in participants when using Net-En and NuvaRing. As such, cytokine concentrations were elevated in participants using NuvaRing. In contrast, there were no association of the frequencies of CD4+ T cells expressing CCR5, including activated (HLA-DR+CD38+) CCR5+CD4+ T cells, with the vaginal community or BV status although having BV and an STI simultaneously was associated with increased

frequencies of HIV target cells. There was likewise no significant association with BV or diversity with Th17-like cell frequency, yet BV-associated bacteria were more abundant in participants with higher frequencies of Th17-like cells.

Chapter 7: Summary and discussion

HIV disproportionally affects AGYW in SSA, and sexual risk behaviour does not completely explain this risk. Some of the proposed biological reasons that AGYW are at high risk include endogenous hormonal levels, high use of HC, high STI prevalence, and altered vaginal microbiota. Adolescence is also a time of high risk for STIs and unintended pregnancies. The main aims of this dissertation therefore were to characterize the FGT microbiota in South African adolescents in the context of STI susceptibility and endogenous hormone levels, to determine the impact of HC use on the FGT microbiota and to assess whether HC-induced changes to the vaginal microbiota affect genital immune cell populations and the inflammation status of the genital tract mucosa.

7.1 The genital tract microbiota in South African adolescents

7.1.1 The vaginal microbiota in South African adolescents

Three major FGT bacterial community types were identified in both the WISH (Chapter 3) and the uCHOOSE (Chapter 4) cohorts. Two of these community types were dominated by *Lactobacillus* species (*L. crispatus* (C2) and *L. iners* (C3), respectively) and one consisted of a diverse group of anaerobic bacteria. As seen in other studies conducted on South African women (Anahtar et al., 2015; Gosmann et al., 2017), *L. iners* was the most commonly detected lactobacilli in these adolescent cohorts followed by *L. crispatus* while *L. gasseri* and *L. jensenii* were much less common. Furthermore, the BV prevalence was very high in both cohorts (WISH: 49.0%, uCHOOSE: 43.0%), also common in older women of African descent (Gosmann et al., 2017; Anahtar et al., 2015). The distribution and number of community types that have been described in adult women have been shown to vary depending on ethnicity (Borgdorff et al., 2017; Ravel et al., 2011). The data from our studies in adolescent women suggest that within a given population the vaginal communities of sexually active adolescents and adults are similar in regard to the dominating *Lactobacillus* species, community cluster distribution and BV prevalence. BV has been shown to be associated with having new and multiple sex partners (Schwebke & Desmond, 2005; Jespers et al.,

2015). Yet, despite a low number of sexual partners being reported by the adolescent girls in these studies compared to adult women, these girls are highly affected by BV. This reiterates the need for optimizing the vaginal communities in these highly vulnerable young women.

7.1.2 The genital tract microbiota and STIs

The prevalence of STIs was also high in both adolescent cohorts (WISH: 46%, uCHOOSE: 43%). Since both BV and *C. trachomatis* infection were so prevalent, we hypothesized that these may be related biologically. Using the WISH cohort (Chapter 3), we evaluated the relationship between *C. trachomatis* infection and the FGT microbiota focusing on both the vaginal and endocervical microbiota; as the endocervix is the main site of *C. trachomatis* infection. We observed significant differences in alpha and beta diversity between endocervical and vaginal microbiota and identified multiple taxa that were differentially abundant between the two sites. There is thus a potential of altered functional profiles of the bacterial communities between different anatomical sites, which could alter mucosal susceptibility and should be taken into account when analysing the vaginal microbiota in relation to susceptibility of pathogens such as *C. trachomatis* and *N. gonorrhoea*.

In this study, we identified a group of BV-associated taxa differentially abundant between *C. trachomatis* infected and uninfected women in line with other studies reporting an increased risk of *C. trachomatis* infection among women with BV (Wiesenfeld et al., 2003; Gallo et al., 2012; Brotman et al., 2010). Logistic regression analyses revealed that participants with a community type dominated by *L. iners* (C3) were also tended to more commonly be infected with *C. trachomatis*, compared to those having an *L. crispatus*-dominated community, albeit not significantly so. Microbiota dominated by *L. iners* has previously been described as an independent risk factor for later *C. trachomatis* acquisition (van Houdt et al., 2017). *L. iners* often co-exists with BV (Atashili et al., 2008; Ling et al., 2011) and vaginal colonization of *L. iners* have been associated with a transition state between lactobacilli dominance and BV (Jakobsson & Forsum, 2007; Ferris et al., 2007; Verstraelen et al., 2009). The high prevalence of BV and *L. iners* may thus partly explain why African adolescent women are at high risk

for STIs. One limitation of a cross-sectional study design is that it cannot provide information on the temporal relationship between the exposure and outcome, which is necessary to determine causality. Prospective longitudinal cohort studies allowing comparisons of microbial communities prior to and after infection with *C. trachomatis* will thus provide a deeper understanding of the role of microbiota including *L. iners* in modifying *C. trachomatis* risk in adolescents. Furthermore, the risk of BV and STIs increases with increased condomless sexual activity, thus underlying the importance of longitudinal studies in assessing the risk of STIs including *C. trachomatis* and HIV.

7.1.3 Endogenous hormones and the vaginal microbiota

Throughout a woman's reproductive years, the vaginal microbial community is highly affected by the menstrual cycle. The proportion of *Lactobacillus* species have been shown to decrease during menstruation while *G. vaginalis* levels increase (Eschenbach et al., 2000b; Gajer et al., 2012; Hickey et al., 2013; Srinivasan et al., 2010; Santiago et al., 2012). In contrast, high mid-cycle oestrogen levels are associated with increased lactobacilli proliferation (Eschenbach et al., 2000b; Gajer et al., 2012; Hickey et al., 2013; Srinivasan et al., 2010). During puberty, women undergo dramatic hormonal changes, which could affect the microbiota and hence susceptibility to STIs. In the uCHOOSE cohort (Chapter 4), we investigated the association of the naturally occurring female sex hormones E2, FSH and LH in the circulation, in relation to the adolescent FGT microbiota. In contrast to other studies mainly conducted in non-adolescents (Wilson et al., 2007; Shen et al., 2016), we did not observe any differences in E2, FSH or LH levels between the three vaginal community clusters or between BV positive and negative participants when analysing the data from participants not using HC. This could be due to the irregularity of menstrual cycles during adolescence and the short time passing since menarche (Flug et al., 1984; Widholm & Kantero, 1971; World Health Organization, 1986). A larger sample size of non-contracepting adolescents would have provided more statistical power to evaluate the impact of endogenous hormones on the vaginal microbiota in this age group. Overall, the measured endogenous hormone concentrations were low in comparison to the provided adult reference ranges

irrespective of menstrual phase. Unfortunately, appropriate physiological oestradiol reference ranges derived from adolescent populations are lacking (Stanczyk & Clarke, 2014).

Recent data, primarily from non-human primate studies, have suggested that the susceptibility to HIV/SIV changes over the course of a menstrual cycle with an enhanced risk of infection during the high endogenous progesterone luteal phase compared to the oestrogen-dominant follicular phase (Venkatesh & Cu-Uvin, 2014; Vishwanathan et al., 2011; Kersh et al., 2014; Marx et al., 1996). Similarly, pregnancy has been associated with two-fold increased risk of HIV acquisition (Mugo et al., 2011). Furthermore, HIV infection of explants collected during the luteal phase is more productive than infection of those collected during the follicular phase *in vitro* (Saba et al., 2013). The mechanisms responsible for the enhanced susceptibility are still poorly understood. More research on the endogenous hormone patterns in adolescence and particularly in sub-Saharan African girls is needed to gain a better understanding of the influence of endogenous hormones on the microbiota and STI risk during this life stage.

7.1.4 Behavioural factors influencing vaginal microbiota

In Chapter 4, we evaluated the effect of intravaginal practices and sexual risk behaviour on the FGT microbiota in the uCHOOSE cohort. It has previously been reported that douching correlates with increased BV (Hutchinson et al., 2007; Ness et al., 2002; Schwebke et al., 2004), although the cause and effect relationship is not clear. In this cohort we did not observe any effect of douching or other vaginal cleansing practices on microbial diversity. Yet, the number of participants reporting engaging in intravaginal practices, including douching, was very low, potentially masking any influence of such practices on the vaginal microbiota. Having a new and/or multiple sexual partners have been associated with increased vaginal colonization of BV-associated species and prevalence of BV (Jespers et al., 2015; Schwebke & Desmond, 2005). We did not observe any associations between having a new or multiple sex partners and the vaginal microbiota in this cohort. Again, this result could potentially be due to a low number of participants reporting multiple or new sex partners, or due to the

high proportion of participants with vaginal microbiota dominated by BV-associated organisms. Participants with a diverse vaginal community (C1) were less likely to report condom usage at their last intercourse than participants in the lactobacilli dominated clusters (C2 and C3) and participants in the C1 cluster also reported less condom use in general. As suggested by others, these data indicate that lack of consistent condom use, hence vaginal exposure to semen and potentially penile-associated bacteria, particularly uncircumcised foreskin bacteria (Price et al., 2010; Liu et al., 2013), is associated with increased microbial diversity and decreased vaginal colonization of lactobacilli (Jespers et al., 2017, 2015; Ma et al., 2013; Chen et al., 2015; Zozaya et al., 2016). The study participants were asked to abstain from intercourse 48 hours before sampling, however analysis of the presence of prostate specific antigen (PSA), a marker of recent sex, in cervical secretions could provide further information on the influence of potential recent condomless sex on the vaginal microbiota in adolescents. Overall, our data suggest that sexual risk behaviour, condom use in particular, has an effect on the vaginal microbiota but that the level of high-risk sexual risk behaviour (e.g. multiple and changing partners) and engagement in high-risk sexual networks (e.g. intergenerational and transactional sex) were low in this cohort.

7.2 The impact of hormonal contraception on the vaginal microbiota

7.2.1 The impact of COCP, Net-En and NuvaRing use on the vaginal microbiota

Whether different types of HC affect HIV susceptibility remains a crucial question for the health of AGYW's, particularly in populations where HIV is common. Use of effective HC can be life saving for many AGYW, so evaluating the impact of different HC methods on HIV risk and comparing them to each other is worthwhile. In Chapter 5, we analysed the impact of three different HC options (Net-En, COCPs and NuvaRing) on the FGT microbiota amongst South African adolescent females enrolled in the uCHOOSE cohort. We hypothesized that the progestin-only HC would be harmful to the vaginal microbiota, but that the oestrogen component in NuvaRing and COCP would be protective. Surprisingly, we found that participants assigned to COCP had a significantly less diverse vaginal microbiota and vaginal pH compared to participants assigned to both

Net-En and NuvaRing, in cross-sectional and longitudinal analyses. The beta diversity of the vaginal community also differed between the three HC groups at crossover. In participants on COCP, the most prevalent community cluster was the *L. iners* dominant C3 community while the diverse C1 community type was the most common in both the Net-En and the NuvaRing group. Surprisingly, the alpha diversity of the vaginal microbiota of participants on either Net-En or the NuvaRing was comparable to the alpha diversity of participants not on HC at screening while COCP was significantly lower. Although this comparison is potentially biased due to the behavioural differences between contracepting and non-contracepting participants, it is unlikely that COCP users were less sexually active than non-contracepting participants, and therefore these data could suggest that the difference between the three HCs analysed in this study stems more from a decrease in alpha diversity by COCP rather than an increase in alpha diversity from Net-En or NuvaRing. This is in agreement with previous studies showing that women using COCPs were more likely to be colonized by lactobacilli and less likely to be colonized by BV-associated bacteria compared with women using condoms alone (Brooks et al., 2017; Bradshaw et al., 2013a).

Using differential abundance testing and random forest analyses, we found that species associated with genital inflammation and risk of HIV (Gosmann et al., 2017; McClelland et al., 2018) were significantly more abundant in, and predictive of, participants on Net-En (e.g. *Prevotella*, *Sneathia* and *Dialister*) or NuvaRing (e.g. *Prevotella, Mycoplasma* and *Parvimonas*) compared to COCP while *L. iners* was more common in the COCP group. Taken together, these data suggest that use of COCP may have a protective effect on the composition of the vaginal microbiota by increasing colonization of lactobacilli and decreasing BV-associated bacterial taxa with an accompanying decrease in overall bacterial diversity and vaginal pH. Despite *L. iners* being the predominant taxa among COCP participants, COCP was also associated with a lower risk of vaginal infections (*C. trachomatis*, *N. gonorrhoea* and *Candida*) than Net-En and NuvaRing. These results are concerning for adolescents in SSA as COCPs are rarely used and long-acting reversible contraceptives are preferred (Ross & Agwanda, 2012; Sibeko et al., 2011). COCPs are generally less popular amongst adolescents due to difficulty with adherence to daily-administered medication

(Hooper, 2010; Mansour, 2014). The wide confidence intervals for levels of E2 measured in COCP participants in this study (Chapter 3) suggests the potential for varying degrees of compliance among these participants. We did not have information on adherence to HC at screening, however according to self-reporting by participants, inconsistent use of COCPs was observed among at least a fourth of the participants at crossover (Chapter 5). Yet, a significant suppressive effect on FSH and LH levels was observed compared to participants not using HC indicative of decent combined contraceptive usage.

With COCP and NuvaRing use menstrual bleeding typically occurs every fourth week, during which the user is either taking placebo pills (or no pills) or the ring is removed. If an individual does not remove the ring during the fourth week, or if she starts a new package of COCPs early instead of taking placebo pill, menstruation can be skipped. In contrast, individuals on progestin-only injections often experience irregular periods or spotting initially and amenorrhea (absence of menstruation) after a few months of use. Since menstrual bleeding can alter the vaginal microbiota (Eschenbach et al., 2000b; Gajer et al., 2012; Hickey et al., 2013; Srinivasan et al., 2010; Santiago et al., 2012), we were interested in assessing whether there was any difference in recent bleeding patterns as reported by the participants. We were particularly interested in whether there were any differences between COCP and NuvaRing users, which could partly explain the differences observed in the vaginal microbiota between the two combined contraceptive methods. For both COCP and NuvaRing users the median time past since last menstrual period was around three weeks while this was slightly longer for Net-En (approximately 5 weeks) (Chapter 5). Thus, the differences in vaginal microbiota observed between different HC methods in this study does not seem to be reflective of differences in menstrual bleeding patterns.

Early studies on the impact of CCVR use on the vaginal microbiota using culture and gram staining methods did not show any affect on the vaginal microbiota (Roy et al., 1981; Davies et al., 1992; Schwan et al., 1983; Roumen et al., 1996). In more recent studies, however, an increase in lactobacilli concentrations as measured by qPCR or culture occurred with CCVR use (Huang et al., 2015b; De Seta et al., 2012; Crucitti et al., 2018). In this study, we did not

observe the same pattern with the 16S sequencing data, however we measured the relative abundance of bacteria rather than their absolute quantities. Our data indicate that NuvaRing use is associated with an increased risk of candidiasis and *N. gonorrhoea* infection. These results are consistent with newly published data from a randomised trial on NuvaRing in Rwandan women, in which the percentage of women with vaginal yeast increased from 5 to 22% after NuvaRing initiation (Kestelyn et al., 2018). In vitro data have shown that yeast cells are capable of adhering to the surface of the NuvaRing potentially facilitating the development and recurrence of *Candida* infections (Camacho et al., 2007). In a recent study on women in Rwanda, the density and bacterial composition of CCVR biomass and its association with the vaginal microbiota was analysed and a correlation between vaginal dysbiosis and the density and composition of the ring biomass was observed (Hardy et al., 2017). The study was cross-sectional and therefore did not allow for determination of causality of these associations. These findings could thus either indicate that the composition of the vaginal microbiota influences the formation of biomass on vaginal rings and/or vice versa. Addition of other components to the CCVR, which could be beneficial to the vaginal microbiota, such as probiotics, could be potential a future strategy for ring use in sub-Saharan Africa (Hardy et al., 2017).

7.2.2 Behaviour and contraceptive use

The application of a randomized, crossover study design is crucial in order to tease out the biological impact of various HC methods on the FGT environment and HIV risk, which could otherwise be markedly influenced by differences in behaviour and the reporting of sexual behaviour. The randomized design of this study therefore represents one of its major strengths. Yet, since this study is open-label and, in the case of daily administered COCPs, requires a high level of adherence from the individual participants, we had to take into account that a study participant could potentially alter her sexual behaviour between visits. Furthermore, at crossover participants initially randomized on NuvaRing were given the choice between Net-En and COCP as their second HC method. When we looked at the reported sexual behaviour exclusively from the exit visit, we observed a trend in bias in sexual behaviour with participants who chose COCP

reporting a higher level of condom use since their last visit compared to the participants who chose Net-En. These data could suggest that an impact of increased condom use was partly responsible for the lower alpha diversity and decreased levels of BV-associated bacteria observed in COCP users, yet the differences between contraceptive methods were still significant after adjusting for condom use. More importantly, at crossover, during which time the participants had been on their randomized allocation, there were no difference in reported sexual behaviour between the arms.

7.2.3 Limitations

The longitudinal study design of uCHOOSE provided us with the opportunity to perform both within-subject assessments comparing the time periods before and after randomization to one of the three HCs in addition to the across study arms comparison of the difference between the study interventions. Thus, each participant could function as their own control and paired analyses are possible providing increased statistical power. One limitation to the study, however, was that the screening visit in most cases did not represent a true, contraceptive naïve baseline control as most participants were on HC before initiating the study. We therefore had to take the HC used at screening into account in our analyses. Another limitation of the study was that randomization was only performed for the first follow-up visit, and we could therefore not treat the exit visit as part of a randomized cohort. Furthermore, due to the construction of the study design, the participants were only on two out of three HC options. The uCHOOSE parent study focused on evaluating the feasibility of different contraceptive options. Therefore, compliance to study product was not enforced. However, participants struggling to comply with an assigned study product were encouraged to change to a method of their choice, and therefore we feel comfortable that poor adherence was reported and reporting bias was minimized. For this reason, we performed both ITT and ATP analyses, which were similar. Due to the risk of participants falling pregnant, there were no wash out period between the different HC methods. This could have led to some carryover effect, particularly with Net-En use, use of a specific contraceptive for a 16-week period before sampling should limit the impact of the previously used

HC method. Yet, since low levels of Net-En can be detected in blood for 6-9 months after injection (albeit below levels needed for contraceptive efficacy) this may still have an effect on the microbiota and immune function. An analysis of the potential of hysteriosis should be conducted in the future. Due to the randomization at baseline, any impact of using a specific HC method prior to the study in theory should have been equally distributed among groups at least at crossover. Since some participants discontinued their assigned method before crossover, one way to approach the analyses at crossover in the future, is by excluding participants who changed method early. Future randomized trials with a larger number of participants and a stronger emphasis on compliance are needed to further tease out the biological impact of HC on the FGT microbiota.

7.3 Cytokines, HIV target cells and the vaginal microbiota

7.3.1 Cytokines and the vaginal microbiota

In Chapter 6, we analysed the relationship between the adolescent vaginal microbiota with secreted cytokine levels and the frequency and activation status of cervical CD4+ HIV target cells, including Th17-like cells. The diverse C1 community cluster was associated with elevated cytokine concentrations compared to both of the lactobacilli dominant clusters (C2 and C3) and cytokines concentrations correlated positively with bacterial diversity. In concordance with previous studies, bacterial species associated with BV and increased HIV risk were shown to have a significantly higher relative abundance in participants with high inflammation (Lennard et al., 2017; Anahtar et al., 2015). These bacteria included taxa that were relatively increased in participants on Net-En (e.g. *Sneathia* and *Dialister*) and/or NuvaRing (e.g. *Prevotella and Parvimonas*) compared to COC. In contrast, several *Lactobacillus* species including *L. iners*, which were more abundant in COC participants, were associated with the low inflammation group. Correspondingly, the relative abundance of *L. crispatus* and *L. iners* was also negatively correlated with the concentration of pro-inflammatory cytokines. Since genital inflammation predicts later HIV seroconversion (Masson et al., 2015a), these results emphasize the role of BV and colonization with BV-associated bacteria as risk factors for HIV acquisition (Gosmann et al., 2017; Myer et al., 2005a; Atashili et al., 2008; Low et al., 2011).

This is of major concern due to the high asymptomatic BV prevalence in AGYW in SSA and the high recurrence rates of BV. An increased effort to diagnose, treat and prevent BV, particularly in areas with HIV prevalence, is warranted.

7.3.2 Hormonal contraception and cytokines

Masson et al. (2015) observed a threefold increased risk of HIV-1 infection in South African women who had elevated genital levels of at least five out of nine pro-inflammatory or chemotactic cytokines (MIP-1α, MIP-1β, IP-10, IL-8, MCP-1, IL-1α, IL-1β, IL-6, and TNF-α) (Masson et al., 2015a). The measured cytokines were selected based on results from previous studies showing an association with genital infections and/or HIV disease progression (Masson et al., 2014; Mlisana et al., 2012; Roberts et al., 2012). MIP-1α, MIP- 1β, IP-10, and IL-8 were identified as the most critical of the measured cytokines in predicting HIV risk. We did not measure the concentrations of these four cytokines in the uCHOOSE study, however the concentrations of IL-1β, IL-6, and TNF-α were measured and IL-1β and TNF-α were shown to be associated with a diverse vaginal microbiota and the presence of genital infections (*C. trachomatis* and *T. vaginalis*). For this study, we measured the levels of other cytokines not previously assessed in relation to HIV, including cytokines involved in regulation and differentiation of Th17 cells (e.g. IL-23, IL-25, Il-31, IL-33) and produced by Th17 cells (e.g. IL-17A, IL-17F, IL-21 and IL-22). These cytokines may play a role in HIV susceptibility as Th17 cells have been described as putative HIV target cells (McKinnon et al., 2011; Gosselin et al., 2011; Monteiro et al., 2011; Rodriguez-Garcia et al., 2014). Further research is needed to evaluate any association between the measured Th17-related cytokines and HIV risk.

Elevated levels of cytokines, including cytokines previously associated with HIV risk (i.e. IL-1β, IL-6, TNF-α and sCD40L) (Masson et al., 2015a; Mlisana et al., 2012), was observed in participants on the NuvaRing compared to participants using COCP and Net-En. This is surprising, since NuvaRing is a combination HC and we had hypothesized that the progestin only injectable would be more disruptive to the FGT mucosa. Since both COCP and NuvaRing are combination contraceptives, the differences observed between the two contraceptives could be due to differences in dose and delivery route

(intravaginal versus systemic) or unrelated to hormones, for instance due to the presence of a foreign object in the genital tract or the increased presence of *Candida* with the use of NuvaRing. CCVR use has previously been associated with an increase in leucocytes indicating an inflammatory reaction which was suggested to be due to a local irritant effect (Schwan et al., 1983). Furthermore, women using CCVR report more vaginal complaints, such as irritation and discharge, compared to COCP users (Schwan et al., 1983; Fan et al., 2016; Oddsson et al., 2005; De Seta et al., 2012). Together, these data suggest that the presence of the NuvaRing may increase the cytokine concentrations in the adolescent FGT. Although increased cytokine levels may be protective in the case of some vaginal infections, they are considered counterproductive in the case of HIV exposure. The increased relative abundance and standardized read counts of BV and HIV associated bacteria compared to COCP users and the increase in inflammatory cytokines in NuvaRing users relative to other HC users suggest that the vaginal ring may not be an optimal alternative long-acting reversible contraceptive in women at high risk for HIV. Yet, combined probiotic or microbicide-releasing contraceptive vaginal rings may provide an alternative strategy to prevent pregnancy while decreasing HIV risk.

In this study, COC use was associated with the lowest level of inflammation, thus once again indicating that COC use may have a protective effect on the genital tract mucosal environment in relation to HIV risk. Based on these results, COC use among women at risk of HIV, who are able to comply with daily administration, should be encouraged. For long-term options, combination patches and implants should be explored further. The Levonorgesterol implant, Jadelle, is being assessed in the Evidence for Contraceptive Options and HIV Outcomes (ECHO) study, and may provide valuable information, although it is not a combination implant. Furthermore, a more thorough comparison of Net-En and DMPA injectables would be of great interest, as one type of progestin-only injection could prove safer to use for women at risk of HIV than another while providing the ease of not having to take a pill every day. Our data here suggest that neither the Net-En injectable nor NuvaRing use may be safer alternatives to the DMPA injectable for long-acting reversible contraceptives in young women at high risk for HIV.

7.3.3 Hormonal contraception, vaginal microbiota and HIV target cells

In this study, we found that IL-17A and IL-17F cytokines were positively correlated with bacterial taxa, which were found to be more abundant in NuvaRing users compared to COCP and Net-En users. These cytokines were also strongly associated with vaginal community clusters, bacterial STIs and yeast infections. A similar relationship was, however, not observed between the frequency of cervical Th17 cells and vaginal community clusters, alpha diversity, STIs, BV or *Candida*. Yet, species associated with BV had a higher relative abundance in participants with high frequencies of Th17 cells. Hence, the general composition of the vaginal community does not appear to play a strong role in the induction of Th17 cells although individual bacteria or bacterial metabolites may play a role as have been described in the gut (Ivanov et al., 2008; Shaw et al., 2012; Zaph et al., 2008). More research is needed in order to elucidate whether a similar relationship exits in the genital tract mucosa. The discrepancy between the IL-17 and Th17 data may be due to the production of IL-17 by various innate immune cells, including gamma delta T cells and innate lymphoid cells, which contributes to the total IL-17 concentrations (Cua & Tato, 2010). Alternatively, we were not measuring true Th17 cells as we only used chemokine receptors to define the populations due to difficulty in re-stimulating cervical cells. This thus represents a limitation in our study.

We did not observe any differences in the frequencies of CD4+ T cells expressing CCR5, including activated (HLA-DR+CD38+) CCR5+CD4+ T cells, between the three vaginal community clusters C1-3 nor between BV positive and negative participants. Yet, taxa associated with BV and inflammation were more abundant in the high CD4+CCR5+ group. These bacteria were also associated with Net-En and/or NuvaRing use. In contrast, species associated with decreased inflammation and COCP use were more abundant in the low CD4+CCR5 group. Of interest, having BV while being infected with *C. trachomatis* was associated with increased CD4+CCR5+ frequencies, suggesting that an additive effect of an STI, including asymptomatic STIs, and a dysbiotic microbial environment can lead to increased recruitment of HIV target cells and thus increased risk of HIV. These results underscore the importance of improved strategies for screening of

asymptomatic STI/BV in regions where HIV is common and where STIs are managed syndromically.

7.4 Molecular analyses of bacterial communities

While the 16S rRNA sequencing approach is the most commonly applied in microbiome studies, there are several limitations to the sequencing strategy. One limitation of relative abundance data is that they do not provide absolute concentrations of bacteria. Furthermore, the copy number of the 16S rRNA gene varies among bacterial taxa leading to potential under- or overestimation of relative abundances. In addition, the choice of the targeted 16S rRNA gene hypervariable region can affect the results, as one hypervariable region can result in higher richness (deeper resolution) than other regions while some regions are unable to detect certain species. And PCR analysis can itself bias the results. One example of how relative abundance data and quantitative data, typically generated by targeted qPCR techniques, may differ even within the same cohort was shown by McClelland et al. (2018) in a study examining the association between the concentrations of specific vaginal bacteria and increased risk of HIV acquisition in African women (McClelland et al., 2018). They first performed 16S rRNA gene sequencing on samples from a subset of case and control participants to identify bacterial taxa showing a trend towards an association with HIV risk. Using the sequencing data, they found that a higher relative abundance of *Dialister* genus, *Dialister* species type 2, *D. micraerophilus*, *G. asaccharolytica*, *Eggerthella* species type, *P. micra* and *Leptotrichia amnionii* was associated with significantly higher odds of HIV acquisition. Using real-time PCR of the bacteria of interest, *M. hominis*, *Eggerthella* species type 1, *Leptotrichia/Sneathia*, *G. asaccharolytica*, *Parvimonas* species type 1 and 2 and *Megasphaera* showed significant associations with HIV acquisition. There is thus some degree of consistency between the results (*G. asaccharolytica*, *Eggerthella*, *Parvimonas*, *Leptotrichia/Sneathia* (their primers could not distinguish between these two genera)) but they also contain a few differences in the bacteria showing significant results between the two methods (*Dialister*, *M. hominis, Megasphaera*). The relative abundances of several of these bacteria were associated with increased cytokine levels and Net-En and NuvaRing use in the

uCHOOSE study. Quantitative PCR analyses of bacteria of interest in the uCHOOSE cohort could provide additional relevant information on the relationship between hormonal contraceptive use and bacteria in the adolescent FGT. Marker gene studies, such as 16S rRNA gene sequencing, focus on only one or a few universal genes, and therefore cannot directly identify metabolic or other molecular functions of the microorganisms detected. In contrast, metagenomic sequencing can provide more detail by analysing the total genomic DNA in a sample, potentially allowing strain level resolution and detection of all genes, thus producing detailed information on metabolic and molecular functions. Metagenomic sequencing of a selected number of samples in the uCHOOSE cohort could provide further information on the impact of HC on the functionality and metabolic potential of the FGT microbiota.

7.5 Conclusions

This thesis work represents the first in depth report of the association between different HC methods and the female genital environment in a randomized study. The study included a comparison of the NuvaRing for which little data is available, especially in South African women as it has only been recently available, Net-En, a progestin-only injectable, which may be associated with HIV risk, particularly in young women, and COCPs. In this PhD thesis, the vaginal microbiota of South African adolescents were shown to correspond to previous data on African women with high prevalence of diverse vaginal communities and *L. iners* dominance. Our data suggest that COCP use may exert a positive influence on genital health through an increase in lactobacilli and a decrease in bacterial diversity with an accompanying decrease in vaginal pH and inflammatory cytokine levels. In contrast, the vaginal microbiota of participants on Net-En and NuvaRing had higher diversity and increased relative abundance of bacteria associated HIV risk and increased cytokine levels compared to COCP users. We did not observe any association of the frequencies of CD4+ T cells expressing CCR5 or Th17-like cells with the vaginal community, BV status or HC use. Yet, having BV and an STI simultaneously was associated with increased frequencies of CCR5+ HIV target cells and BV-associated bacteria were more abundant in participants with higher frequencies of Th17-like cells.

Future randomized trials with a larger number of participants are needed to further tease out the biological impact of HC on the FGT microbiota, such as the upcoming ECHO study. Furthermore, our analyses did not explore the mechanisms through which individual bacterial taxa may increase HIV risk. Such mechanistic data will help to further evaluate the likelihood of a causal link between vaginal bacteria and HIV susceptibility, and should be the focus of future studies. Nonetheless, insights gained from studies like this can potentially contribute to the development of therapeutic strategies in modulating the microbiota composition for disease prevention and development of tailored long-acting reversible contraceptive options for young women in HIV prevalent settings. This thesis work could thus have a major impact on health and reproductive management of young South African women, while increasing their protection against STI, including HIV.

References

Aaby, P. et al. (1996) Age of wife as a major determinant of male-to-female transmission of HIV-2 infection: a community study from rural West Africa. *AIDS (London, England)*. 10 (13), 1585–1590.

Abdool Karim, Q. et al. (2010) Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science (New York, N.Y.)*. 329 (5996), 1168–1174. doi:10.1126/science.1193748.

Achilles, S.L. et al. (2018) Impact of contraceptive initiation on vaginal microbiota. *American journal of obstetrics and gynecology*. doi:10.1016/j.ajog.2018.02.017.

Afolabi, B.B. et al. (2016) Bacterial Vaginosis and Pregnancy Outcome in Lagos, Nigeria. *Open Forum Infectious Diseases*. 3 (1), 5. doi:10.1093/ofid/ofw030.

Africander, D. et al. (2011) Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception. *Steroids*. 76 (7), 636–652. doi:10.1016/j.steroids.2011.03.001.

Aitken, R.J. et al. (2008) As the world grows: contraception in the 21st century. *The Journal of clinical investigation*. 118 (4), 1330–1343. doi:10.1172/JCI33873.

Alcaide, M.L. et al. (2016) Bacterial Vaginosis Is Associated with Loss of Gamma Delta T Cells in the Female Reproductive Tract in Women in the Miami Women Interagency HIV Study (WIHS): A Cross Sectional Study. *PloS one*. 11 (4), e0153045. doi:10.1371/journal.pone.0153045.

Alvarez-Olmos, M.I. et al. (2004) Vaginal lactobacilli in adolescents: presence and relationship to local and systemic immunity, and to bacterial vaginosis. *Sexually transmitted diseases*. 31 (7), 393–400.

Amabebe, E. & Anumba, D.O.C. (2018) The Vaginal Microenvironment: The Physiologic Role of Lactobacilli. *Frontiers in medicine*. 5, 181. doi:10.3389/fmed.2018.00181.

Anahtar, M.N. et al. (2015) Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity*. 42 (5), 965–976. doi:10.1016/j.immuni.2015.04.019.

Anderson, M.J. (2005) *Permutational multivariate analysis of variance*. 1–24. http://img2.timg.co.il/forums/1_124959686.pdf.

Andrew, D.W. et al. (2013) The duration of Chlamydia muridarum genital tract infection and associated chronic pathological changes are reduced in IL-17 knockout mice but protection is not increased further by immunization. *PloS one*. 8 (9), e76664. doi:10.1371/journal.pone.0076664.

Arnold, K.B. et al. (2016) Increased levels of inflammatory cytokines in the female reproductive tract are associated with altered expression of proteases, mucosal barrier proteins, and an influx of HIV-susceptible target cells. *Mucosal immunology*. 9 (1), 194–205. doi:10.1038/mi.2015.51.

Atashili, J. et al. (2008) Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. *AIDS (London, England)*. 22 (12), 1493–1501. doi:10.1097/QAD.0b013e3283021a37.

Baeten, J.M. et al. (2007) The influence of hormonal contraceptive use on HIV-1 transmission and disease progression. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 45 (3), 360–369. doi:10.1086/519432.

Baeten, J.M. et al. (2001) Hormonal contraception and risk of sexually transmitted disease acquisition: results from a prospective study. *American journal of obstetrics and gynecology*. 185 (2), 380–385. doi:10.1067/mob.2001.115862.

Bahamondes, M.V. et al. (2014) Human vaginal histology in long-term users of the injectable contraceptive depot-medroxyprogesterone acetate. *Contraception*. 90 (2), 117–122. doi:10.1016/j.contraception.2014.01.024.

Balle, C. et al. (2018) Endocervical and vaginal microbiota in South African adolescents with asymptomatic Chlamydia trachomatis infection. *Scientific Reports*. 8 (1), 11109. doi:10.1038/s41598-018-29320-x.

Bar, E. et al. (2014) IL-17 regulates systemic fungal immunity by controlling the functional competence of NK cells. *Immunity*. 40 (1), 117–127. doi:10.1016/j.immuni.2013.12.002.

Barnabas, S.L. et al. (2017) *Converging epidemics of sexually transmitted infections and bacterial vaginosis in Southern African female adolescents at risk of HIV.* doi:10.1177/0956462417740487.

Bates, D. et al. (2015) Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*. 67(1), 1–48. doi:10.18637/jss.v067.i01.

Bemis, D.A. et al. (2008) Isolation of Arcanobacterium hippocoleae from a case of placentitis and stillbirth in a mare. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*. 20 (5), 688–691. doi:10.1177/104063870802000532.

Biddlecom, A.E. et al. (2007) Protecting the Next Generation in Sub-Saharan Africa: Learning from Adolescents to Prevent HIV and Unintended Pregnancy. *Guttmacher Institute*. 1–60.

Birse, K. et al. (2015) Molecular Signatures of Immune Activation and Epithelial Barrier Remodeling Are Enhanced during the Luteal Phase of the Menstrual Cycle: Implications for HIV Susceptibility. *Journal of virology*. 89 (17), 8793–8805. doi:10.1128/JVI.00756-15.

Birse, K.D. et al. (2017) Genital Injury Signatures and Microbiome Alterations Associated With Depot Medroxyprogesterone Acetate Usage and Intravaginal Drying Practices. *The Journal of infectious diseases*. 215 (4), 590–598. doi:10.1093/infdis/jiw590.

Blish, C.A. et al. (2012) Genital Inflammation Predicts HIV-1 Shedding Independent of Plasma Viral Load and Systemic Inflammation. *Journal of acquired immune deficiency syndromes (1999)*. 61 (4), 436–440. doi:10.1097/QAI.0b013e31826c2edd.

Bojanowski, M. & Edwards, R. (2016) *alluvial: R Package for Creating Alluvial Diagrams: R package*

version: 0.1-2. https://github.com/mbojan/alluvial.

Borgdorff, H. et al. (2014) Lactobacillus-dominated cervicovaginal microbiota associated with reduced HIV/STI prevalence and genital HIV viral load in African women. *The ISME journal*. 8 (9), 1781–1793. doi:10.1038/ismej.2014.26.

Borgdorff, H. et al. (2017) The association between ethnicity and vaginal microbiota composition in Amsterdam, the Netherlands. *PloS one*. 12 (7), e0181135. doi:10.1371/journal.pone.0181135.

Borgdorff, H. et al. (2015) The impact of hormonal contraception and pregnancy on sexually transmitted infections and on cervicovaginal microbiota in african sex workers. *Sexually transmitted diseases*. 42 (3), 143–152. doi:10.1097/OLQ.0000000000000245.

Bradshaw, C.S. et al. (2006) High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *The Journal of infectious diseases*. 193 (11), 1478–1486. doi:10.1086/503780.

Bradshaw, C.S. et al. (2013a) Recurrence of bacterial vaginosis is significantly associated with posttreatment sexual activities and hormonal contraceptive use. *Clinical Infectious Diseases*. 56 (6), 777–786. doi:10.1093/cid/cis1030.

Bradshaw, C.S. et al. (2013b) Prevalent and incident bacterial vaginosis are associated with sexual and contraceptive behaviours in young Australian women. *PloS one*. 8 (3), e57688. doi:10.1371/journal.pone.0057688.

Bray, R.. & Curtis, T.. (1957) An ordination of upland forest communities of southern Wisconsin. *Ecological Monographs*. 27 (4), 325–349.

Brenchley, J.M. et al. (2006) Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nature medicine*. 12 (12), 1365–1371. doi:10.1038/nm1511.

Breshears, L.M. et al. (2015) Lactobacillus crispatus inhibits growth of Gardnerella vaginalis and Neisseria gonorrhoeae on a porcine vaginal mucosa model. *BMC microbiology*. 15, 276. doi:10.1186/s12866-015-0608-0.

Brooks, J.P. et al. (2017) Effects of combined oral contraceptives, depot medroxyprogesterone acetate and the levonorgestrel-releasing intrauterine system on the vaginal microbiome. *Contraception*. 95 (4), 405–413. doi:10.1016/j.contraception.2016.11.006.

Brotman, R.M. et al. (2010) Bacterial vaginosis assessed by gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection. *The Journal of infectious diseases*. 202 (12), 1907–1915. doi:10.1086/657320.

Buchmann, E. et al. (2002) Legal Termination of Preganncy among Teenagers and older Women in Soweto, 1999-2001. *SAMJ: South African Medical Journal*. 23 (2), 27–54. doi:10.1080/0749446042000204545.

Butler, A.R. et al. (2013) Modelling the global competing risks of a potential interaction between injectable hormonal contraception and HIV risk. *Aids*. 27 (1), 105–113.

doi:10.1097/QAD.0b013e32835a5a52.

Buve A, Jespers V, Crucitti T, F.R. (2014) The vaginal microbiota and susceptibility to HIV. *Aids*. 28 (16), 2333–2344. doi:10.1097/QAD.0000000000000432.

Byrne, E.H. et al. (2016) Association between injectable progestin-only contraceptives and HIV acquisition and HIV target cell frequency in the female genital tract in South African women: a prospective cohort study. *The Lancet. Infectious diseases*. 16 (4), 441–448. doi:10.1016/S1473-3099(15)00429-6.

Byrne, E.H. et al. (2015) Association between injectable progestin-only contraceptives and HIV acquisition and HIV target cell frequency in the female genital tract in South African women: A prospective cohort study. *The Lancet Infectious Diseases*. 3099 (15), 7–10. doi:10.1016/S1473-3099(15)00429-6.

Camacho, D.P. et al. (2007) Vaginal yeast adherence to the combined contraceptive vaginal ring (CCVR). *Contraception*. 76 (6), 439–443. doi:10.1016/j.contraception.2007.07.012.

Carias, A.M. et al. (2013) Defining the interaction of HIV-1 with the mucosal barriers of the female reproductive tract. *Journal of virology*. 87 (21), 11388–11400. doi:10.1128/JVI.01377-13.

Cecchinato, V. et al. (2008) Altered balance between Th17 and Th1 cells at mucosal sites predicts AIDS progression in simian immunodeficiency virus-infected macaques. *Mucosal immunology*. 1 (4), 279–288. doi:10.1038/mi.2008.14.

Chandra, N. et al. (2013) Depot medroxyprogesterone acetate increases immune cell numbers and activation markers in human vaginal mucosal tissues. *AIDS research and human retroviruses*. 29 (3), 592–601. doi:10.1089/aid.2012.0271.

Chatterji, M. et al. (2004) The Factors Influencing Transactional Sex Among Young Men and Women in 12 Sub-Saharan African Countries. *Social Biology*. 52 (1/2), 56–72. doi:10.1080/19485565.2002.9989099.

Chen, C. et al. (2017) The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nature communications*. 8 (1), 875. doi:10.1038/s41467-017-00901-0.

Chen, R.-Y. et al. (2015) Estradiol inhibits Th17 cell differentiation through inhibition of RORgammaT transcription by recruiting the ERalpha/REA complex to estrogen response elements of the RORgammaT promoter. *Journal of immunology (Baltimore, Md. : 1950)*. 194 (8), 4019–4028. doi:10.4049/jimmunol.1400806.

Chesson, H.W. et al. (2012) Disparities in sexually transmitted disease rates across the 'eight Americas'. *Sexually transmitted diseases*. 39 (6), 458–464. doi:10.1097/OLQ.0b013e318248e3eb.

Chesson, H.W. & Pinkerton, S.D. (2000) Sexually transmitted diseases and the increased risk for HIV transmission: Implications for cost-effectiveness analyses of sexually transmitted disease prevention interventions. *J Acquir Immune Defic Syndr*. 24 (1), 48–56. http://www.ncbi.nlm.nih.gov/pubmed/10877495.

Christensen-Quick, A. et al. (2016) Human T(h)17 Cells Lack HIV-Inhibitory RNases and Are Highly Permissive to Productive HIV Infection G Silvestri (ed.). *Journal of Virology*. 90 (17), 7833–7847. doi:10.1128/JVI.02869-15.

Cleland, J. et al. (2006) Family planning: the unfinished agenda. *Lancet (London, England)*. 368 (9549), 1810–1827. doi:10.1016/S0140-6736(06)69480-4.

Clifton, D. et al. (2008) Family Planning Worldwide, 2008 Data Sheet. *Population Reference Bureau*. http://www.prb.org/pdf08/fpds08.pdf.

Cowan, F. & Pettifor, A. (2009) HIV in adolescents in sub-Saharan Africa. *Current opinion in HIV and AIDS*. 4 (4), 288–293. doi:10.1097/COH.0b013e32832c7d10.

Creinin, M.D. et al. (2008) Multicenter comparison of the contraceptive ring and patch: a randomized controlled trial. *Obstetrics and gynecology*. 111 (2 Pt 1), 267–277. doi:10.1097/01.AOG.0000298338.58511.d1.

Critchlow, C.W. et al. (1995) Determinants of cervical ectopia and of cervicitis: age, oral contraception, specific cervical infection, smoking, and douching. *American journal of obstetrics and gynecology*. 173 (2), 534–543.

Crook, A.M. et al. (2014) Injectable and oral contraceptives and risk of HIV acquisition in women: an analysis of data from the MDP301 trial. *Human reproduction (Oxford, England)*. 29 (8), 1810–1817. doi:10.1093/humrep/deu113.

Crucitti, T. et al. (2018) Contraceptive rings promote vaginal lactobacilli in a high bacterial vaginosis prevalence population: A randomised, open-label longitudinal study in Rwandan women. *PloS one*. 13 (7), e0201003. doi:10.1371/journal.pone.0201003.

Cua, D.J. & Tato, C.M. (2010) Innate IL-17-producing cells: The sentinels of the immune system. *Nature Reviews Immunology*. 10 (7), 479–489. doi:10.1038/nri2800.

D'Arpe, S. et al. (2016) Ovarian function during hormonal contraception assessed by endocrine and sonographic markers: a systematic review. *Reproductive biomedicine online*. 33 (4), 436–448. doi:10.1016/j.rbmo.2016.07.010.

Davies, G.C. et al. (1992) The effects of a combined contraceptive vaginal ring releasing ethinyloestradiol and 3-ketodesogestrel on vaginal flora. *Contraception*. 45 (5), 511–518.

Donders, G. et al. (2017) Influence of contraceptive choice on vaginal bacterial and fungal microflora. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*. 36 (1), 43–48. doi:10.1007/s10096-016-2768-8.

Dorgan, J.F. et al. (2003) Diet and sex hormones in girls: findings from a randomized controlled clinical trial. *Journal of the National Cancer Institute*. 95 (2), 132–141.

Doyle, R. et al. (2018) A Lactobacillus-Deficient Vaginal Microbiota Dominates Postpartum Women in Rural Malawi. *Applied and Environmental Microbiology*. 84 (6). doi:10.1128/AEM.02150-17.

Eade, C.R. et al. (2015) HIV-Enhancing Factors Are Secreted by Reproductive Epithelia upon

Inoculation with Bacterial Vaginosis-Associated Bacteria. *Protein and peptide letters*. 22 (8), 672–680. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4516132/.

Edelman, S.M. et al. (2012) Identification of a high-molecular-mass Lactobacillus epithelium adhesin (LEA) of Lactobacillus crispatus ST1 that binds to stratified squamous epithelium. *Microbiology (Reading, England)*. 158 (Pt 7), 1713–1722. doi:10.1099/mic.0.057216-0.

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics (Oxford, England)*. 26 (19), 2460–2461. doi:10.1093/bioinformatics/btq461.

Edgar, R.C. et al. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics (Oxford, England)*. 27 (16), 2194–2200. doi:10.1093/bioinformatics/btr381.

Eschenbach, D.A. et al. (2000a) Effects of oral contraceptive pill use on vaginal flora and vaginal epithelium. *Contraception*. 62 (3), 107–112.

Eschenbach, D.A. et al. (2000b) Influence of the normal menstrual cycle on vaginal tissue, discharge, and microflora. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 30 (6), 901–907. doi:10.1086/313818.

European Study Group on Heterosexual Transmission of HIV. (1992) Comparison of female to male and male to female transmission of HIV in 563 stable couples. *BMJ (Clinical research ed.)*. 304 (6830), 809–813.

Faith, D.P. et al. (1987) Compositional dissimilarity as a robust measure of ecological distance. *Vegetatio*. 69 (1–3), 57–68. doi:10.1007/BF00038687.

Fallon, P.G. et al. (2006) Identification of an interleukin (IL)-25–dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *The Journal of Experimental Medicine*. 203 (4), 1105–1116. doi:10.1084/jem.20051615.

Fan, G.S. et al. (2016) Efficacy and safety of the contraceptive vaginal ring (NuvaRing) compared with a combined oral contraceptive in Chinese women: a 1-year randomised trial. *The European journal of contraception & reproductive health care : the official journal of the European Society of Contraception*. 21 (4), 303–309. doi:10.1080/13625187.2016.1186269.

Farage, M. & Maibach, H. (2006) Lifetime changes in the vulva and vagina. *Archives of gynecology and obstetrics*. 273 (4), 195–202. doi:10.1007/s00404-005-0079-x.

Farquhar, C. et al. (2010) Illness during pregnancy and bacterial vaginosis are associated with in-utero HIV-1 transmission. *AIDS (London, England)*. 24 (1), 153–155. doi:10.1097/QAD.0b013e32832326d8.

Fashemi, B. et al. (2013) Effects of feminine hygiene products on the vaginal mucosal biome. *Microbial ecology in health and disease*. 24. doi:10.3402/mehd.v24i0.19703.

Favre, D. et al. (2009) Critical loss of the balance between Th17 and T regulatory cell populations in pathogenic SIV infection. *PLoS pathogens*. 5 (2), e1000295. doi:10.1371/journal.ppat.1000295.

Favre, D. et al. (2010) Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of

TH17 to regulatory T cells in HIV disease. *Science translational medicine*. 2 (32), 32ra36. doi:10.1126/scitranslmed.3000632.

Feinen, B. et al. (2010) Critical role of Th17 responses in a murine model of Neisseria gonorrhoeae genital infection. *Mucosal immunology*. 3 (3), 312–321. doi:10.1038/mi.2009.139.

Ferris, M.J. et al. (2007) Cultivation-independent analysis of changes in bacterial vaginosis flora following metronidazole treatment. *Journal of Clinical Microbiology*. 45 (3), 1016–1018. doi:10.1128/JCM.02085-06.

Fethers, K.A. et al. (2009) *Early Sexual Experiences and Risk Factors for Bacterial Vaginosis*. 200. doi:10.1086/648092.

Fettweis, J.M. et al. (2012) Species-level classification of the vaginal microbiome. *BMC genomics*. 13 Suppl 8, S17. doi:10.1186/1471-2164-13-S8-S17.

Fidel, P.L.J. (2007) History and update on host defense against vaginal candidiasis. *American journal of reproductive immunology (New York, N.Y. : 1989)*. 57 (1), 2–12. doi:10.1111/j.1600-0897.2006.00450.x.

Filardo, S. et al. (2017) Diversity of Cervical Microbiota in Asymptomatic Chlamydia trachomatis Genital Infection: A Pilot Study. *Frontiers in cellular and infection microbiology*. 7, 321. doi:10.3389/fcimb.2017.00321.

Fleming, D.T. et al. (1999) From epidemiological synergy to public health policy and practice : the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. *Sexually Transmitted Infections*. (January 2006), 3–17. doi:10.1136/sti.75.1.3.

Flug, D. et al. (1984) Menstrual patterns in adolescent Swiss girls: a longitudinal study. *Annals of human biology*. 11 (6), 495–508.

Fort, M.M. et al. (2001) IL-25 Induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity*. 15 (6), 985–995. doi:10.1016/S1074-7613(01)00243-6.

France, M.T. et al. (2016) Genomic Comparisons of Lactobacillus crispatus and Lactobacillus iners Reveal Potential Ecological Drivers of Community Composition in the Vagina. *Applied and environmental microbiology*. 82 (24), 7063–7073. doi:10.1128/AEM.02385-16.

Francis, S.C. et al. (2016) Bacterial vaginosis among women at high risk for HIV in Uganda: high rate of recurrent diagnosis despite treatment. *Sexually transmitted infections*. 92 (2), 142–148. doi:10.1136/sextrans-2015-052160.

Gajer, P. et al. (2012) Temporal dynamics of the human vaginal microbiota. *Science translational medicine*. 4 (132), 132ra52. doi:10.1126/scitranslmed.3003605.

Galhardo, C.L. et al. (2006) Estrogen effects on the vaginal pH, flora and cytology in late postmenopause after a long period without hormone therapy. *Clinical and experimental obstetrics & gynecology*. 33 (2), 85–89.

Gallo, M.F. et al. (2012) Bacterial vaginosis, gonorrhea, and chlamydial infection among women

attending a sexually transmitted disease clinic: a longitudinal analysis of possible causal links. *Annals of epidemiology*. 22 (3), 213–220. doi:10.1016/j.annepidem.2011.11.005.

Galvin, S.R. & Cohen, M.S. (2004) The role of sexually transmitted diseases in HIV transmission. *Nature Reviews Microbiology*. 2 (1), 33–42. http://www.wisdomofwhores.com/wp-content/uploads/2010/12/galvin-role-of-stis.pdf.

Gaujoux, R. (2014) *Generating heatmaps for Nonnegative Matrix Factorization.*

Gautam, R. et al. (2015) Correlates of the molecular vaginal microbiota composition of African women. *BMC infectious diseases*. 15, 86. doi:10.1186/s12879-015-0831-1.

van Gemert, W.A.M. et al. (2015) Effect of weight loss, with or without exercise, on body composition and sex hormones in postmenopausal women: the SHAPE-2 trial. *Breast cancer research : BCR*. 17, 120. doi:10.1186/s13058-015-0633-9.

Ghanem, K.G. et al. (2005) Influence of sex hormones, HIV status, and concomitant sexually transmitted infection on cervicovaginal inflammation. *The Journal of infectious diseases*. 191 (3), 358–366. doi:10.1086/427190.

Glynn, E.F. (2005) *Hierarchical Clustering*. 1–9.

Gong, Z. et al. (2014) Lactobacilli inactivate Chlamydia trachomatis through lactic acid but not H2O2. *PloS one*. 9 (9), e107758. doi:10.1371/journal.pone.0107758.

Goode, D. et al. (2014) Sex hormones selectively impact the endocervical mucosal microenvironment: implications for HIV transmission. *PloS one*. 9 (5), e97767. doi:10.1371/journal.pone.0097767.

Gosmann, C. et al. (2017) Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. *Immunity*. 46 (1), 29–37. doi:10.1016/j.immuni.2016.12.013.

Gosselin, A. et al. (2011) Peripheral blood CCR4+CCR6+ and CXCR3+CCR6+CD4+ T cells are highly permissive to HIV-1 infection. *Journal of immunology (Baltimore, Md. : 1950)*. 187 (8), 4618–4630. doi:10.4049/jimmunol.1101836.

Grant, M.J. & Hallman, K.K. (2008) Pregnancy-related school dropout and prior school performance in KwaZulu-Natal, South Africa. *Studies in Family Planning*. 39 (4), 369–382. doi:10.1111/j.1728-4465.2008.00181.x.

Gregson, S. et al. (2002) Sexual mixing patterns and sex-differentials in teenage exposure to HIV infection in rural Zimbabwe. *Lancet*. 359 (9321), 1896–1903. doi:10.1016/S0140-6736(02)08780-9.

Grossman, Z. et al. (2006) Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nature medicine*. 12 (3), 289–295. doi:10.1038/nm1380.

Gupta, K. et al. (2000) Effects of contraceptive method on the vaginal microbial flora: a prospective evaluation. *The Journal of infectious diseases*. 181 (2), 595–601. doi:10.1086/315267.

Haggerty, C.L. et al. (2004) Bacterial Vaginosis and Anaerobic Bacteria Are Associated with

Endometritis. *Clinical Infectious Diseases*. 39 (7), 990–995. doi:10.1086/423963.

Hardy, L. et al. (2017) Association of vaginal dysbiosis and biofilm with contraceptive vaginal ring biomass in African women. *PloS one*. 12 (6), e0178324. doi:10.1371/journal.pone.0178324.

Harmanli, O.H. et al. (2000) Urinary tract infections in women with bacterial vaginosis. *Obstetrics and gynecology*. 95 (5), 710–712.

Hearps, A.C. et al. (2017) Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition. *Mucosal immunology*. 10 (6), 1480–1490. doi:10.1038/mi.2017.27.

Hed, A. El et al. (2010) Human Th17 cells are susceptible to HIV and are perturbed during infection. *The Journal of infectious diseases*. 201 (6), 843–854. doi:10.1086/651021.

Heffron, R. et al. (2018) Pharmacokinetic, biologic and epidemiologic differences in MPA- and NET-based progestin-only injectable contraceptives relative to the potential impact on HIV acquisition in women. *Contraception*. doi:10.1016/j.contraception.2018.12.001.

Heffron, R. et al. (2012) Use of hormonal contraceptives and risk of HIV-1 transmission: a prospective cohort study. *The Lancet. Infectious diseases*. 12 (1), 19–26. doi:10.1016/S1473-3099(11)70247-X.

Heffron, R. et al. (2017) Objective Measurement of Inaccurate Condom Use Reporting Among Women Using Depot Medroxyprogesterone Acetate for Contraception. *AIDS and behavior*. 21 (7), 2173–2179. doi:10.1007/s10461-016-1563-y.

Hickey, R.J. et al. (2013) Effects of tampons and menses on the composition and diversity of vaginal microbial communities over time. *BJOG : an international journal of obstetrics and gynaecology*. 120 (6), 695–696. doi:10.1111/1471-0528.12151.

Hickey, R.J. et al. (2015) Vaginal microbiota of adolescent girls prior to the onset of menarche resemble those of reproductive-age women. *mBio*. 6 (2). doi:10.1128/mBio.00097-15.

Hill, G.B. et al. (1995) Anaerobes predominate among the vaginal microflora of prepubertal girls. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 20 Suppl 2, S269-70.

Hill, J.A. & Anderson, D.J. (1992) Human vaginal leukocytes and the effects of vaginal fluid on lymphocyte and macrophage defense functions. *American journal of obstetrics and gynecology*. 166 (2), 720–726.

Hillier, S.L. (1998) The vaginal microbial ecosystem and resistance to HIV. *AIDS research and human retroviruses*. 14 Suppl 1, S17-21.

Hladik, F. et al. (2007) Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1. *Immunity*. 26 (2), 257–270. doi:10.1016/j.immuni.2007.01.007.

Hooper, D.J. (2010) Attitudes, awareness, compliance and preferences among hormonal contraception users: a global, cross-sectional, self-administered, online survey. *Clinical drug investigation*. 30

(11), 749–763. doi:10.2165/11538900-000000000-00000.

van Houdt, R. et al. (2017) Lactobacillus iners-dominated vaginal microbiota is associated with increased susceptibility to Chlamydia trachomatis infection in Dutch women: a case-control study. *Sexually transmitted infections*. doi:10.1136/sextrans-2017-053133.

Hoyles, L. et al. (2002) Arcanobacterium hippocoleae sp. nov., from the vagina of a horse. *International journal of systematic and evolutionary microbiology*. 52 (Pt 2), 617–619. doi:10.1099/00207713-52-2-617.

Huang, Y.-E. et al. (2015a) Homogeneity of the vaginal microbiome at the cervix, posterior fornix, and vaginal canal in pregnant Chinese women. *Microbial ecology*. 69 (2), 407–414. doi:10.1007/s00248-014-0487-1.

Huang, Y. et al. (2015b) Effects of a One Year Reusable Contraceptive Vaginal Ring on Vaginal Microflora and the Risk of Vaginal Infection: An Open-Label Prospective Evaluation. *PloS one*. 10 (8), e0134460. doi:10.1371/journal.pone.0134460.

Hutchinson, K.B. et al. (2007) Vaginal douching and development of bacterial vaginosis among women with normal and abnormal vaginal microflora. *Sexually transmitted diseases*. 34 (9), 671–675. doi:10.1097/01.olq.0000258435.34879.da.

Ildgruben, A.K. et al. (2003) Influence of hormonal contraceptives on the immune cells and thickness of human vaginal epithelium. *Obstetrics and gynecology*. 102 (3), 571–582.

Ishigame, H. et al. (2009) Differential Roles of Interleukin-17A and -17F in Host Defense against Mucoepithelial Bacterial Infection and Allergic Responses. *Immunity*. 30 (1), 108–119. doi:10.1016/j.immuni.2008.11.009.

Ivanov, I.I. et al. (2008) Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell host & microbe*. 4 (4), 337–349. doi:10.1016/j.chom.2008.09.009.

Iwakura, Y. et al. (2011) Functional specialization of interleukin-17 family members. *Immunity*. 34 (2), 149–162. doi:10.1016/j.immuni.2011.02.012.

Jackson, C.H. (2011) Multi-State Models for Panel Data: The msm Package for R. *Journal of Statistical Software*. 38 (8), 1–28. doi:10.18637/jss.v038.i08.

Jain, A.K. (2012) Hormonal contraception and HIV acquisition risk: Implications for individual users and public policies. *Contraception*. 86 (6), 645–652. doi:10.1016/j.contraception.2012.03.008.

Jakobsson, T. & Forsum, U. (2007) Lactobacillus iners: A marker of changes in the vaginal flora? [2]. *Journal of Clinical Microbiology*. 45 (9), 3145. doi:10.1128/JCM.00558-07.

Jaspan, H. (2011) The wrong place at the wrong time: geographic disparities in young people's HIV Risk. *The Journal of adolescent health : official publication of the Society for Adolescent Medicine*.49 (3) pp.227–229. doi:10.1016/j.jadohealth.2011.07.004.

Jespers, V. et al. (2016) Association of sexual debut in adolescents with microbiota and inflammatory

markers. *Obstetrics and Gynecology*. 128 (1), 22–31. doi:10.1097/AOG.0000000000001468.

Jespers, V. et al. (2017) A longitudinal analysis of the vaginal microbiota and vaginal immune mediators in women from sub-Saharan Africa. *Scientific Reports*. 7 (1). doi:10.1038/s41598-017-12198-6.

Jespers, V. et al. (2015) The significance of Lactobacillus crispatus and L. vaginalis for vaginal health and the negative effect of recent sex: a cross-sectional descriptive study across groups of African women. *BMC infectious diseases*. 15, 115. doi:10.1186/s12879-015-0825-z.

Joint United Nations Programme on HIV/AIDS (2010) Unaids Report on the Global Aids Epidemic | 2010. *WHO Library Cataloguing-in-Publication Data*. 6–8. http://www.unaids.org/documents/20101123_globalreport_em.pdf.

Kao, C.-Y. et al. (2004) IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. *Journal of immunology (Baltimore, Md. : 1950)*. 173 (5), 3482–3491.

Kapiga, S.H. et al. (1998) The incidence of HIV infection among women using family planning methods in Dar es Salaam, Tanzania. *AIDS (London, England)*. 12 (1), 75–84.

Katz, I. & Low-Beer, D. (2008) Why has HIV stabilized in South Africa, yet not declined further? Age and sexual behavior patterns among youth. *Sexually transmitted diseases*. 35 (10), 837–842. doi:10.1097/OLQ.0b013e31817c0be5.

Kaufman, L. & Rousseeuw, P.J. (1987) *Clustering by means of Medoids, in Statistical Data Analysis Based on the L1Norm and Related Methods*.

Kaufman, R. (1990) *Partitioning Around Medoids (Program PAM)*. doi:10.1002/9780470316801.ch2.

Kazi, Y.F. et al. (2012) Investigation of vaginal microbiota in sexually active women using hormonal contraceptives in Pakistan. *BMC Urol*. 12.

Kersh, E.N. et al. (2014) SHIV susceptibility changes during the menstrual cycle of pigtail macaques. *Journal of medical primatology*. 43 (5), 310–316. doi:10.1111/jmp.12124.

Kestelyn, E. et al. (2018) A randomised trial of a contraceptive vaginal ring in women at risk of HIV infection in Rwanda: Safety of intermittent and continuous use. *PloS one*. 13 (6), e0197572. doi:10.1371/journal.pone.0197572.

Khader, S.A. et al. (2009) Th17 cells at the cross roads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal immunology*. 2 (5), 403–411. doi:10.1038/mi.2009.100.

Khashan, A.S. et al. (2010) Preterm birth and reduced birthweight in first and second teenage pregnancies: A register-based cohort study. *BMC Pregnancy and Childbirth*. 10. doi:10.1186/1471-2393-10-36.

Kim, T.K. et al. (2009) Heterogeneity of vaginal microbial communities within individuals. *Journal of clinical microbiology*. 47 (4), 1181–1189. doi:10.1128/JCM.00854-08.

Kinugasa, T. et al. (2000) Claudins regulate the intestinal barrier in response to immune mediators. *Gastroenterology*. 118 (6), 1001–1011.

Klatt, N.R. et al. (2013) Immune activation and HIV persistence: Implications for curative approaches to HIV infection. *Immunological reviews*. 254 (1), 326–342. doi:10.1111/imr.12065.

Kleinschmidt, I. et al. (2007) Injectable progestin contraceptive use and risk of HIV infection in a South African family planning cohort. *Contraception*. 75 (6), 461–467. doi:10.1016/j.contraception.2007.02.002.

Kolde, R. (2015) *Pretty Heatmaps. R package version 1.0.8.*

Korn, T. et al. (2009) IL-17 and Th17 Cells. *Annual review of immunology*. 27, 485–517. doi:10.1146/annurev.immunol.021908.132710.

Koumans, E.H. et al. (2007) The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. *Sexually Transmitted Diseases*. 34 (11), 864–869. doi:10.1097/OLQ.0b013e318074e565.

Krishnan, S. et al. (2008) Poverty, gender inequities, and women's risk of human immunodeficiency virus/AIDS. *Annals of the New York Academy of Sciences*. 1136, 101–110. doi:10.1196/annals.1425.013.

Kuhl, H. (2005) Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric : the journal of the International Menopause Society*. 8 Suppl 1, 3–63. doi:10.1080/13697130500148875.

Laan, M. et al. (1999) Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *Journal of immunology (Baltimore, Md. : 1950)*. 162 (4), 2347–2352.

Lai, S.K. et al. (2009) Human Immunodeficiency Virus Type 1 Is Trapped by Acidic but Not by Neutralized Human Cervicovaginal Mucus. *Journal of Virology*. 83 (21), 11196–11200. doi:10.1128/JVI.01899-08.

Lavreys, L. et al. (2004) Hormonal contraception and risk of cervical infections among HIV-1-seropositive Kenyan women. *AIDS (London, England)*. 18 (16), 2179–2184.

Lennard, K. et al. (2017) Microbial composition predicts genital tract inflammation and persistent bacterial vaginosis in adolescent South African women. *Infection and immunity*. doi:10.1128/IAI.00410-17.

Lewis, D.A. et al. (2012) Prevalence and associations of genital ulcer and urethral pathogens in men presenting with genital ulcer syndrome to primary health care clinics in South Africa. *Sexually transmitted diseases*. 39 (11), 880–885. doi:10.1097/OLQ.0b013e318269cf90.

Liang, S.C. et al. (2006) Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *The Journal of experimental medicine*. 203 (10), 2271–2279. doi:10.1084/jem.20061308.

Liaw, a & Wiener, M. (2002) Classification and Regression by randomForest. *R news*. 2 (December),

18–22. doi:10.1177/154405910408300516.

Ling, Z. et al. (2011) Diversity of cervicovaginal microbiota associated with female lower genital tract infections. *Microbial ecology*. 61 (3), 704–714. doi:10.1007/s00248-011-9813-z.

Liu, C.M. et al. (2013) Male circumcision significantly reduces prevalence and load of genital anaerobic bacteria. *mBio*. 4 (2), e00076. doi:10.1128/mBio.00076-13.

Longfield, K. et al. (2004) Relationships between older men and younger women: Implications for STIs/HIV in Kenya. *Studies in Family Planning*. 35 (2), 125–134. doi:10.1111/j.1728-4465.2004.00014.x.

Losikoff, P. et al. (2007) Genital tract interleukin-8 but not interleukin-1beta or interleukin-6 concentration is associated with bacterial vaginosis and its clearance in HIV-infected and HIV-uninfected women. *Infectious diseases in obstetrics and gynecology*. 2007, 92307. doi:10.1155/2007/92307.

Love, M.I. et al. (2014) Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2 Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. *Genome biology*. 15 (12), 521–550. doi:10.1101/002832.

Low, N. et al. (2011) Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. *PLoS medicine*. 8 (2), e1000416. doi:10.1371/journal.pmed.1000416.

Lozupone, C. & Knight, R. (2005) UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*. 71 (12), 8228–8235. doi:10.1128/AEM.71.12.8228-8235.2005.

De Luca, A. et al. (2010) IL-22 defines a novel immune pathway of antifungal resistance. *Mucosal immunology*. 3 (4), 361–373. doi:10.1038/mi.2010.22.

Ma, L. et al. (2013) Consistent condom use increases the colonization of Lactobacillus crispatus in the vagina. *PloS one*. 8 (7), e70716. doi:10.1371/journal.pone.0070716.

Machado, A. et al. (2013) Interactions between Lactobacillus crispatus and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. *International journal of molecular sciences*. 14 (6), 12004–12012. doi:10.3390/ijms140612004.

MacIntyre, D.A. et al. (2015) The vaginal microbiome during pregnancy and the postpartum period in a European population. *Scientific Reports*. 5. doi:10.1038/srep08988.

Mackenzie, A. et al. (1995) Incidence and pathogenicity of Arcanobacterium haemolyticum during a 2-year study in Ottawa. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 21 (1), 177–181.

Macklaim, J.M. et al. (2013) Comparative meta-RNA-seq of the vaginal microbiota and differential expression by Lactobacillus iners in health and dysbiosis. *Microbiome*. 1 (1), 12. doi:10.1186/2049-2618-1-12.

MACLEAN, P.D. et al. (1946) A hemolytic corynebacterium resembling Corynebacterium ovis and Corynebacterium pyogenes in man. *The Journal of infectious diseases*. 79, 69–90.

Maechler, M. et al. (2017) Cluster Analysis Basics and Extensions. R package version 2.0.6. *Cran*. http://cran.r-project.org/web/packages/cluster/index.html.

Makinde, H.M. et al. (2013) IL-22 levels are associated with trichomonas vaginalis infection in the lower genital tract. *American Journal of Reproductive Immunology*. 70 (1), 38–44. doi:10.1111/aji.12100.

Mansour, D. (2014) International survey to assess women's attitudes regarding choice of daily versus nondaily female hormonal contraception. *International journal of women's health*. 6, 367–375. doi:10.2147/IJWH.S59059.

Martin, H.L.J. et al. (1998) Hormonal contraception, sexually transmitted diseases, and risk of heterosexual transmission of human immunodeficiency virus type 1. *The Journal of infectious diseases*. 178 (4), 1053–1059.

Marx, P.A. et al. (1996) Progesterone implants enhance SIV vaginal transmission and early virus load. *Nature medicine*. 2 (10), 1084–1089.

Masese, L. et al. (2015) Changes in the contribution of genital tract infections to HIV acquisition among Kenyan high-risk women from 1993 to 2012. *Aids*. 29 (9), 1077–1085. doi:10.1097/QAD.0000000000000646.

Masson, L. et al. (2018) Inflammatory cytokine biomarkers of asymptomatic sexually transmitted infections and vaginal dysbiosis: a multicentre validation study. *Sexually transmitted infections*. doi:10.1136/sextrans-2017-053506.

Masson, L. et al. (2014) Defining genital tract cytokine signatures of sexually transmitted infections and bacterial vaginosis in women at high risk of HIV infection: a cross-sectional study. *Sexually transmitted infections*. 90 (8), 580–587. doi:10.1136/sextrans-2014-051601.

Masson, L. et al. (2015a) Genital inflammation and the risk of HIV acquisition in women. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 61 (2), 260–269. doi:10.1093/cid/civ298.

Masson, L. et al. (2015b) Relationship between female genital tract infections, mucosal interleukin-17 production and local T helper type 17 cells. *Immunology*. 146 (4), 557–567. doi:10.1111/imm.12527.

Mati, J.K. et al. (1995) Contraceptive use and the risk of HIV infection in Nairobi, Kenya. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics*. 48 (1), 61–67.

Mauck, C. et al. (2016) Biomarkers of Cervical Inflammation and Immunity Associated with Cervical Shedding of HIV-1. *AIDS Research and Human Retroviruses*. 32 (5), 443–451. doi:10.1089/aid.2015.0088.

Mauck, C.K. et al. (1999) The effect of one injection of Depo-Provera on the human vaginal epithelium and cervical ectopy. *Contraception*. 60 (1), 15–24.

Mayer, B.T. et al. (2015) Rapid and Profound Shifts in the Vaginal Microbiota Following Antibiotic Treatment for Bacterial Vaginosis. *The Journal of infectious diseases*. doi:10.1093/infdis/jiv079.

Mbulawa, Z.Z.A. et al. (2015) Human papillomavirus prevalence in South African women and men according to age and human immunodeficiency virus status. *BMC infectious diseases*. 15, 459. doi:10.1186/s12879-015-1181-8.

McClelland, R.S. et al. (2018) Evaluation of the association between the concentrations of key vaginal bacteria and the increased risk of HIV acquisition in African women from five cohorts: a nested case-control study. *The Lancet. Infectious diseases*. doi:10.1016/S1473-3099(18)30058-6.

McClelland, R.S. et al. (2008) A prospective study of risk factors for bacterial vaginosis in HIV-1-seronegative African women. *Sexually transmitted diseases*. 35 (6), 617–623. doi:10.1097/OLQ.0b013e31816907fa.

McCoy, S.I. et al. (2014) Are hormonal contraceptive users more likely to misreport unprotected sex? Evidence from a biomarker validation study in Zimbabwe. *AIDS and behavior*. 18 (12), 2259–2264. doi:10.1007/s10461-014-0741-z.

McCoy, S.I. et al. (2013) Oral and injectable contraception use and risk of HIV acquisition among women in sub-Saharan Africa. *AIDS (London, England)*. 27 (6), 1001–1009. doi:10.1097/QAD.0b013e32835da401.

McKinnon, L.R. et al. (2011) Characterization of a human cervical CD4+ T cell subset coexpressing multiple markers of HIV susceptibility. *Journal of immunology (Baltimore, Md. : 1950)*. 187 (11), 6032–6042. doi:10.4049/jimmunol.1101836.

McMurdie, P.J. & Holmes, S. (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one*. 8 (4), e61217. doi:10.1371/journal.pone.0061217.

Methe, B.A. et al. (2012) A framework for human microbiome research. *Nature*. 486 (7402), 215–221. doi:10.1038/nature11209.A.

Miller, L. et al. (2000a) Depomedroxyprogesterone-induced hypoestrogenism and changes in vaginal flora and epithelium. *Obstetrics and gynecology*. 96 (3), 431–439.

Miller, L. et al. (2000b) Depomedroxyprogesterone-induced Hypoestrogenism and Changes in Vaginal Flora and Epithelium. *Obstetrics and Gynecology*. 96 (3), 431–439. doi:10.1097/00006250-200009000-00020.

Mirmonsef, P. et al. (2014) Free glycogen in vaginal fluids is associated with Lactobacillus colonization and low vaginal pH. *PloS one*. 9 (7), e102467. doi:10.1371/journal.pone.0102467.

Mirmonsef, P. et al. (2016) Glycogen Levels in Undiluted Genital Fluid and Their Relationship to Vaginal pH, Estrogen, and Progesterone. *PloS one*. 11 (4), e0153553. doi:10.1371/journal.pone.0153553.

Mirmonsef, P. et al. (2015) Exploratory comparison of vaginal glycogen and Lactobacillus levels in premenopausal and postmenopausal women. *Menopause (New York, N.Y.)*. 22 (7), 702–709. doi:10.1097/GME.0000000000000397.

Mitchell, C. et al. (2013) Interaction between lactobacilli, bacterial vaginosis-associated bacteria, and HIV Type 1 RNA and DNA Genital shedding in U.S. and Kenyan women. *AIDS research and human retroviruses*. 29 (1), 13–19. doi:10.1089/AID.2012.0187.

Mitchell, C. et al. (2015) Hydrogen peroxide-producing lactobacilli are associated with lower levels of vaginal interleukin-1β, independent of bacterial vaginosis. *Sexually Transmitted Diseases*. 42 (7), 358–363. doi:10.1097/OLQ.0000000000000298.

Mitchell, C.M. et al. (2014) Long-term effect of depot medroxyprogesterone acetate on vaginal microbiota, epithelial thickness and HIV target cells. *The Journal of infectious diseases*. 210 (4), 651–655. doi:10.1093/infdis/jiu176.

Mitchell, C.M. et al. (2018) Associations between improvement in genitourinary symptoms of menopause and changes in the vaginal ecosystem. *Menopause (New York, N.Y.)*. 25 (5), 500–507. doi:10.1097/GME.0000000000001037.

Mitchell, C.M. et al. (2017) Vaginal microbiota and genitourinary menopausal symptoms: a cross-sectional analysis. *Menopause (New York, N.Y.)*. 24 (10), 1160–1166. doi:10.1097/GME.0000000000000904.

Miyamoto, M. et al. (2003) Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways. *Journal of immunology (Baltimore, Md. : 1950)*. 170 (9), 4665–4672.

Mlisana, K. et al. (2012) Symptomatic vaginal discharge is a poor predictor of sexually transmitted infections and genital tract inflammation in high-risk women in South Africa. *The Journal of infectious diseases*. 206 (1), 6–14. doi:10.1093/infdis/jis298.

Monteiro, P. et al. (2011) Memory CCR6+CD4+ T cells are preferential targets for productive HIV type 1 infection regardless of their expression of integrin beta7. *Journal of immunology (Baltimore, Md. : 1950)*. 186 (8), 4618–4630. doi:10.4049/jimmunol.1004151.

Morrison, C. et al. (2014) Cervical inflammation and immunity associated with hormonal contraception, pregnancy, and HIV-1 seroconversion. *Journal of acquired immune deficiency syndromes (1999)*. 66 (2), 109–117. doi:10.1097/QAI.0000000000000103.

Morrison, C.S. et al. (2015) Hormonal contraception and the risk of HIV acquisition: an individual participant data meta-analysis. *PLoS medicine*. 12 (1), e1001778. doi:10.1371/journal.pmed.1001778.

Morrison, C.S. et al. (2010) Hormonal contraception and HIV acquisition: reanalysis using marginal structural modeling. *AIDS (London, England)*. 24 (11), 1778–1781. doi:10.1097/QAD.0b013e32833a2537.

Morrison, C.S. et al. (2007) Hormonal contraception and the risk of HIV acquisition. *AIDS (London, England)*. 21 (1), 85–95. doi:10.1097/QAD.0b013e3280117c8b.

Morrison, C.S. et al. (2012) Hormonal contraception and the risk of HIV acquisition among women in South Africa. *AIDS (London, England)*. 26 (4), 497–504. doi:10.1097/QAD.0b013e32834fa13d.

Morrison, C.S. et al. (2009) Highly effective contraception and acquisition of HIV and other sexually transmitted infections. *Best practice & research. Clinical obstetrics & gynaecology*. 23 (2), 263–284. doi:10.1016/j.bpobgyn.2008.11.004.

Mostad, S.B. et al. (1997) Hormonal contraception, vitamin A deficiency, and other risk factors for shedding of HIV-1 infected cells from the cervix and vagina. *Lancet*. 350 (9082), 922–927. doi:10.1016/S0140-6736(97)04240-2.

Mugo, N.R. et al. (2011) Increased risk of HIV-1 transmission in pregnancy: a prospective study among African HIV-1-serodiscordant couples. *AIDS (London, England)*. 25 (15), 1887–1895. doi:10.1097/QAD.0b013e32834a9338.

Myer, L. et al. (2005a) Bacterial vaginosis and susceptibility to HIV infection in South African women: a nested case-control study. *The Journal of infectious diseases*. 192 (8), 1372–1380. doi:10.1086/462427.

Myer, L. et al. (2007) Prospective study of hormonal contraception and women's risk of HIV infection in South Africa. *International journal of epidemiology*. 36 (1), 166–174. doi:10.1093/ije/dyl251.

Myer, L. et al. (2005b) Intravaginal practices, bacterial vaginosis, and women's susceptibility to HIV infection: epidemiological evidence and biological mechanisms. *The Lancet. Infectious diseases*. 5 (12), 786–794. doi:10.1016/S1473-3099(05)70298-X.

Nardini, P. et al. (2016) Lactobacillus crispatus inhibits the infectivity of Chlamydia trachomatis elementary bodies, in vitro study. *Scientific reports*. 6, 29024. doi:10.1038/srep29024.

Nasioudis, D. et al. (2015) α-Amylase in Vaginal Fluid: Association with Conditions Favorable to Dominance of Lactobacillus. *Reproductive Sciences*. 22 (11), 1393–1398. doi:10.1177/1933719115581000.

Ness, R.B. et al. (2002) Douching in relation to bacterial vaginosis, lactobacilli, and facultative bacteria in the vagina. *Obstetrics and gynecology*. 100 (4), 765.

Newman, L. et al. (2015) Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. *PloS one*. 10 (12), e0143304. doi:10.1371/journal.pone.0143304.

Noguchi, L.M. et al. (2015a) Risk of HIV-1 acquisition among women who use different types of injectable progestin contraception in South Africa: a prospective cohort study. *The lancet. HIV*. 2 (7), e279-87. doi:10.1016/S2352-3018(15)00058-2.

Noguchi, L.M. et al. (2015b) Risk of HIV-1 acquisition among women who use diff erent types of injectable progestin contraception in South Africa: A prospective cohort study. *The Lancet HIV*. 2

(7), e279–e287. doi:10.1016/S2352-3018(15)00058-2.

Nove, A. et al. (2014) Maternal mortality in adolescents compared with women of other ages: Evidence from 144 countries. *The Lancet Global Health*. 2 (3), 155–164. doi:10.1016/S2214-109X(13)70179-7.

Nugent, R.P. et al. (1991) Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of clinical microbiology*. 29 (2), 297–301.

Nunn, K.L. et al. (2015) Enhanced Trapping of HIV-1 by Human Cervicovaginal Mucus Is Associated with Lactobacillus crispatus-Dominant Microbiota. *mBio*. 6 (5). doi:10.1128/mBio.01084-15.

Oddsson, K. et al. (2005) Efficacy and safety of a contraceptive vaginal ring (NuvaRing) compared with a combined oral contraceptive: a 1-year randomized trial. *Contraception*. 71 (3), 176–182. doi:10.1016/j.contraception.2004.09.001.

Oksanen, J. et al. (2016) *vegan: Community Ecology Package. R. package version 2.4-1.* ttps://cran.r-project.org/package=vegan.

Olmsted, S.S. et al. (2005) Low pH immobilizes and kills human leukocytes and prevents transmission of cell-associated HIV in a mouse model. *BMC infectious diseases*. 5, 79. doi:10.1186/1471-2334-5-79.

OpenStax (2018) *Anatomy & Physiology*. OpenStax CNX. http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@10.1.

Ortiz, A.M. et al. (2016) IL-21 and Probiotic Therapy Improve T(H)17 Frequencies, Microbial Translocation, and Microbiome in ARV-Treated, SIV-Infected Macaques. *Mucosal immunology*. 9 (2), 458–467. doi:10.1038/mi.2015.75.

Ouyang, W. et al. (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity*. 28 (4), 454–467. doi:10.1016/j.immuni.2008.03.004.

Pabich, W.L. et al. (2003) Prevalence and determinants of vaginal flora alterations in postmenopausal women. *The Journal of infectious diseases*. 188 (7), 1054–1058. doi:10.1086/378203.

Pandiyan, P. et al. (2011) CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17 cell infection model. *Immunity*. 34 (3), 422–434. doi:10.1016/j.immuni.2011.03.002.

Parolin, C. et al. (2018) Insights Into Vaginal Bacterial Communities and Metabolic Profiles of Chlamydia trachomatis Infection: Positioning Between Eubiosis and Dysbiosis. *Frontiers in microbiology*. 9, 600. doi:10.3389/fmicb.2018.00600.

Paulson, J.N. et al. (2013) Differential abundance analysis for microbial marker-gene surveys. *Nature methods*. 10 (12), 1200–1202. doi:10.1038/nmeth.2658.

Pearce, M.M. et al. (2014) The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. *mBio*. 5 (4), e01283-14. doi:10.1128/mBio.01283-14.

Petrova, M.I. et al. (2013) Vaginal microbiota and its role in HIV transmission and infection.

Microbiology Rev. 37 (1), 762–792. doi:10.1111/1574-6976.12029.

Pettifor, A. et al. (2009) Use of injectable progestin contraception and risk of STI among South African women. *Contraception*. 80 (6), 555–560. doi:10.1016/j.contraception.2009.06.007.

Pettifor, A.E. et al. (2011) A tale of two countries: rethinking sexual risk for HIV among young people in South Africa and the United States. *The Journal of adolescent health : official publication of the Society for Adolescent Medicine*. 49 (3), 237–243.e1. doi:10.1016/j.jadohealth.2010.10.002.

Pietrella, D. et al. (2011) Th17 cells and IL-17 in protective immunity to vaginal candidiasis. *PloS one*. 6 (7), e22770. doi:10.1371/journal.pone.0022770.

Polis, C.B. et al. (2016) An updated systematic review of epidemiological evidence on hormonal contraceptive methods and HIV acquisition in women. *Aids*. 30 (17), 2665–2683. doi:http://dx.doi.org/10.1097/QAD.0000000000001228.

Polis, C.B. et al. (2014) Hormonal contraceptive methods and risk of HIV acquisition in women: a systematic review of epidemiological evidence. *Contraception*. 90 (4), 360–390. doi:10.1016/j.contraception.2014.07.009.

Prakash, M. et al. (2002) Oral contraceptive use induces upregulation of the CCR5 chemokine receptor on CD4(+) T cells in the cervical epithelium of healthy women. *Journal of reproductive immunology*. 54 (1–2), 117–131.

Prakash, M. et al. (2005) Hormonal upregulation of CCR5 expression on T lymphocytes as a possible mechanism for increased HIV-1 risk. *Journal of acquired immune deficiency syndromes (1999)*. 38 Suppl 1, S14-6.

Price, L.B. et al. (2010) The effects of circumcision on the penis microbiome. *PloS one*. 5 (1), e8422. doi:10.1371/journal.pone.0008422.

R Core Team (2016) *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.* https://www.r-project.org/.

Ralph, L.J. et al. (2015) Hormonal contraceptive use and women's risk of HIV acquisition: a meta-analysis of observational studies. *The Lancet. Infectious diseases*. 15 (2), 181–189. doi:10.1016/S1473-3099(14)71052-7.

Ravel, J. et al. (2011) Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences of the United States of America*. 108 Suppl, 4680–4687. doi:10.1073/pnas.1002611107.

Rebbapragada, A. et al. (2008) Bacterial vaginosis in HIV-infected women induces reversible alterations in the cervical immune environment. *Journal of acquired immune deficiency syndromes (1999)*. 49 (5), 520–522. doi:10.1097/QAI.0b013e318189a7ca.

Richardson, B.A. et al. (2007) Hormonal contraception and HIV-1 disease progression among postpartum Kenyan women. *AIDS (London, England)*. 21 (6), 749–753. doi:10.1097/QAD.0b013e328032790f.

Riggs, M. et al. (2007) Longitudinal Association Between Hormonal Contraceptives and Bacterial Vaginosis in Women of Reproductive Age. *Sex. Transm. Dis.* 34 (12), 954–959. doi:10.1097/OLQ.0b013e31811ed0e4.

Rivera, R. et al. (1999) The mechanism of action of hormonal contraceptives and intrauterine contraceptive devices. *American journal of obstetrics and gynecology*. 181 (5 Pt 1), 1263–1269.

Roberts, L. et al. (2012) Genital tract inflammation during early HIV-1 infection predicts higher plasma viral load set point in women. *The Journal of infectious diseases*. 205 (2), 194–203. doi:10.1093/infdis/jir715.

Rodriguez-Garcia, M. et al. (2014) Phenotype and Susceptibility to HIV-infection of CD4(+) Th17 Cells in the Human Female Reproductive Tract. *Mucosal immunology*. 7 (6), 1375–1385. doi:10.1038/mi.2014.26.

Rodriguez, M.I. et al. (2012) Evaluating the competing risks of HIV acquisition and maternal mortality in Africa: a decision analysis. *BJOG : an international journal of obstetrics and gynaecology*. 119 (9), 1067–1073. doi:10.1111/j.1471-0528.2012.03402.x.

Rognes, T. et al. (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ*. 4, e2584. doi:10.7717/peerj.2584.

Romero, R. et al. (2014) The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome*. 2 (1), 4. doi:10.1186/2049-2618-2-4.

de Roon, M. et al. (2018) Effect of exercise and/or reduced calorie dietary interventions on breast cancer-related endogenous sex hormones in healthy postmenopausal women. *Breast cancer research : BCR*. 20 (1), 81. doi:10.1186/s13058-018-1009-8.

Rosentul, D.C. et al. (2014) Gene polymorphisms in pattern recognition receptors and susceptibility to idiopathic recurrent vulvovaginal candidiasis. *Frontiers in microbiology*. 5, 483. doi:10.3389/fmicb.2014.00483.

Ross, J. a & Agwanda, A.T. (2012) Increased use of injectable contraception in sub-Saharan Africa. *African journal of reproductive health*. 16 (4), 68–80. doi:10.2307/23485777.

Roumen, F.J. et al. (1996) The cervico-vaginal epithelium during 20 cycles' use of a combined contraceptive vaginal ring. *Human reproduction (Oxford, England)*. 11 (11), 2443–2448.

Roxby, A.C. et al. (2016) Changes in Vaginal Microbiota and Immune Mediators in HIV-1-Seronegative Kenyan Women Initiating Depot Medroxyprogesterone Acetate. *Journal of acquired immune deficiency syndromes (1999)*. 71 (4), 359–366. doi:10.1097/QAI.0000000000000866.

Roy, S. et al. (1981) The effect of a contraceptive vaginal ring and oral contraceptives on the vaginal flora. *Contraception*. 24 (4), 481–491.

Ryan, E.S. et al. (2016) Loss of Function of Intestinal IL-17 and IL-22 Producing Cells Contributes to Inflammation and Viral Persistence in SIV-Infected Rhesus Macaques Daniel C Douek (ed.). *PLoS*

Pathogens. 12 (2), e1005412. doi:10.1371/journal.ppat.1005412.

Saba, E. et al. (2010) HIV-1 sexual transmission: early events of HIV-1 infection of human cervico-vaginal tissue in an optimized ex vivo model. *Mucosal immunology*. 3 (3), 280–290. doi:10.1038/mi.2010.2.

Saba, E. et al. (2013) Productive HIV-1 infection of human cervical tissue ex vivo is associated with the secretory phase of the menstrual cycle. *Mucosal immunology*. 6 (6), 1081–1090. doi:10.1038/mi.2013.2.

Sagar, M. et al. (2004) Identification of modifiable factors that affect the genetic diversity of the transmitted HIV-1 population. *AIDS (London, England)*. 18 (4), 615–619.

Sagar, M. et al. (2003) Infection with multiple human immunodeficiency virus type 1 variants is associated with faster disease progression. *Journal of virology*. 77 (23), 12921–12926.

Saijo, S. et al. (2010) Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. *Immunity*. 32 (5), 681–691. doi:10.1016/j.immuni.2010.05.001.

Santiago, G.L. dos S. et al. (2012) Longitudinal qPCR study of the dynamics of L. crispatus, L. iners, A. vaginae, (sialidase positive) G. vaginalis, and P. bivia in the vagina. *PloS one*. 7 (9), e45281. doi:10.1371/journal.pone.0045281.

Sawa, S. et al. (2011) RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nature immunology*. 12 (4), 320–326. doi:10.1038/ni.2002.

Schacker, T. et al. (2001) Productive infection of T cells in lymphoid tissues during primary and early human immunodeficiency virus infection. *The Journal of infectious diseases*. 183 (4), 555–562. doi:10.1086/318524.

Schwan, A. et al. (1983) Effects of contraceptive vaginal ring treatment on vaginal bacteriology and cytology. *Contraception*. 28 (4), 341–347.

Schwebke, J.R. & Desmond, R. (2005) Risk Factors for Bacterial Vaginosis in Women at High Risk for Sexually Transmitted Diseases. *Sex. Transm. Dis.* 32 (11), 654–658. doi:10.1097/01.olq.0000175396.10304.62.

Schwebke, J.R. et al. (2004) Predictors of bacterial vaginosis in adolescent women who douche. *Sexually transmitted diseases*. 31 (7), 433–436.

Schwebke, J.R. et al. (1999) Correlation of behaviors with microbiological changes in vaginal flora. *The Journal of infectious diseases*. 180 (5), 1632–1636. doi:10.1086/315065.

Scurlock, A.M. et al. (2011) Interleukin-17 contributes to generation of Th1 immunity and neutrophil recruitment during Chlamydia muridarum genital tract infection but is not required for macrophage influx or normal resolution of infection. *Infection and immunity*. 79 (3), 1349–1362. doi:10.1128/IAI.00984-10.

Sehrawat, S. & Rouse, B.T. (2017) Interplay of Regulatory T Cell and Th17 Cells during Infectious Diseases in Humans and Animals. *Frontiers in Immunology*. 8, 341. doi:10.3389/fimmu.2017.00341.

De Seta, F. et al. (2014) Effects of estroprogestins containing natural estrogen on vaginal flora. *Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology*. 30 (11), 830–835. doi:10.3109/09513590.2014.936847.

De Seta, F. et al. (2012) Effects of hormonal contraception on vaginal flora. *Contraception*. 86 (5), 526–529. doi:10.1016/j.contraception.2012.02.012.

Shattock, R. & Moore, J. (2003) Inhibiting sexual transmission of HIV-1 infection. *Nature Reviews Microbiology*. 1 (1), 25–34. doi:10.1038/nrmicro729.

Shaw, M.H. et al. (2012) Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. *The Journal of experimental medicine*. 209 (2), 251–258. doi:10.1084/jem.20111703.

Shaw, N.D. et al. (2010) Estrogen negative feedback on gonadotropin secretion: evidence for a direct pituitary effect in women. *The Journal of clinical endocrinology and metabolism*. 95 (4), 1955–1961. doi:10.1210/jc.2009-2108.

Shen, J. et al. (2016) Effects of low dose estrogen therapy on the vaginal microbiomes of women with atrophic vaginitis. *Scientific reports*. 6, 24380. doi:10.1038/srep24380.

Sibeko, S. et al. (2011) Contraceptive Choices, Pregnancy Rates, and Outcomes in a Microbicide Trial. *Obstetrics and gynecology*. 118 (4), 895–904. doi:10.1097/AOG.0b013e31822be512.

Smith, J.A. et al. (2017) Could misreporting of condom use explain the observed association between injectable hormonal contraceptives and HIV acquisition risk?()()(). *Contraception*. 95 (4), 424–430. doi:10.1016/j.contraception.2016.12.003.

Smith, S.M. et al. (2004) Topical estrogen protects against SIV vaginal transmission without evidence of systemic effect. *AIDS (London, England)*. 18 (12), 1637–1643.

Smith, W.L. et al. (2014) Cervical and Vaginal Flora Specimens Are Highly Concordant with Respect to Bacterial Vaginosis-Associated Organisms and Commensal Lactobacillus Species in Women of Reproductive Age. *Journal of Clinical Microbiology*. 52 (8), 3078–3081. doi:10.1128/JCM.00795-14.

Sobel, J.D. et al. (1993) Long-term follow-up of patients with bacterial vaginosis treated with oral metronidazole and topical clindamycin. *The Journal of infectious diseases*.167 (3) pp.783–784.

Spear, G.T. et al. (2014) Human α-amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by Lactobacillus. *Journal of Infectious Diseases*. 210 (7), 1019–1028. doi:10.1093/infdis/jiu231.

Srinivasan, S. et al. (2010) Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PloS one*. 5 (4), e10197. doi:10.1371/journal.pone.0010197.

Stanczyk, F.Z. & Clarke, N.J. (2014) Measurement of estradiol-challenges ahead. *Journal of Clinical Endocrinology and Metabolism*. 99 (1), 56–58. doi:10.1210/jc.2013-2905.

Stanczyk, F.Z. et al. (2013) Progestogens used in postmenopausal hormone therapy: differences in their pharmacological properties, intracellular actions, and clinical effects. *Endocrine reviews*. 34 (2), 171–208. doi:10.1210/er.2012-1008.

Stieh, D.J. et al. (2016) Th17 Cells Are Preferentially Infected Very Early after Vaginal Transmission of SIV in Macaques. *Cell host & microbe*. 19 (4), 529–540. doi:10.1016/j.chom.2016.03.005.

Stringer, E.M. et al. (2007) A randomized trial of the intrauterine contraceptive device vs hormonal contraception in women who are infected with the human immunodeficiency virus. *American journal of obstetrics and gynecology*. 197 (2), 144.e1-8. doi:10.1016/j.ajog.2007.03.031.

Swidsinski, A. et al. (2014) Infection through structured polymicrobial Gardnerella biofilms (StPM-GB). *Histology and Histopathology*. 29 (5), 567–587. doi:10.14670/HH-29.10.567.

Taha, T.E. et al. (1998) Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. *AIDS (London, England)*. 12 (13), 1699–1706.

Tamarelle, J. et al. (2018) Vaginal microbiota composition and association with prevalent Chlamydia trachomatis infection: a cross-sectional study of young women attending a STI clinic in France. *Sexually transmitted infections*. doi:10.1136/sextrans-2017-053346.

Thoma, M.E. et al. (2011) Longitudinal changes in vaginal microbiota composition assessed by gram stain among never sexually active pre- and postmenarcheal adolescents in Rakai, Uganda. *Journal of pediatric and adolescent gynecology*. 24 (1), 42–47. doi:10.1016/j.jpag.2010.07.002.

Thorsen, P. et al. (2006) Bacterial vaginosis in early pregnancy is associated with low birth weight and small for gestational age, but not with spontaneous preterm birth: A population-based study on Danish women. *Journal of Maternal-Fetal & Neonatal Medicine*. 19 (1), 1–7. doi:10.1080/14767050500361604.

Thurman, A.R. et al. (2015) Bacterial Vaginosis and Subclinical Markers of Genital Tract Inflammation and Mucosal Immunity. *AIDS research and human retroviruses*. 31 (11), 1139–1152. doi:10.1089/aid.2015.0006.

UNAIDS (2017) *Ending AIDS: Progress towards 90-90-90 targets*.

UNAIDS (2016) *Guidance: HIV Prevention among adolescent girls and young women*.

van der Veer, C. et al. (2017) The Cervicovaginal Microbiota in Women Notified for Chlamydia trachomatis Infection: A Case-Control Study at the Sexually Transmitted Infection Outpatient Clinic in Amsterdam, The Netherlands. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 64 (1), 24–31. doi:10.1093/cid/ciw586.

Venkatesh, K.K. & Cu-Uvin, S. (2014) Anatomic and hormonal changes in the female reproductive tract immune environment during the life cycle: implications for HIV/STI prevention research. *American journal of reproductive immunology (New York, N.Y. : 1989)*. 71 (6), 495–504.

doi:10.1111/aji.12247.

Veres, S. et al. (2004) A comparison between the vaginal ring and oral contraceptives. *Obstetrics and gynecology*. 104 (3), 555–563. doi:10.1097/01.AOG.0000136082.59644.13.

Verstraelen, H. et al. (2009) Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora. *BMC microbiology*. 9, 116. doi:10.1186/1471-2180-9-116.

Vicetti Miguel, R.D. et al. (2016) Intravaginal Chlamydia trachomatis Challenge Infection Elicits TH1 and TH17 Immune Responses in Mice That Promote Pathogen Clearance and Genital Tract Damage. *PloS one*. 11 (9), e0162445. doi:10.1371/journal.pone.0162445.

Vishwanathan, S.A. et al. (2015) Cataloguing of Potential HIV Susceptibility Factors during the Menstrual Cycle of Pig-Tailed Macaques by Using a Systems Biology Approach. *Journal of virology*. 89 (18), 9167–9177. doi:10.1128/JVI.00263-15.

Vishwanathan, S.A. et al. (2011) High susceptibility to repeated, low-dose, vaginal SHIV exposure late in the luteal phase of the menstrual cycle of pigtail macaques. *Journal of acquired immune deficiency syndromes (1999)*. 57 (4), 261–264. doi:10.1097/QAI.0b013e318220ebd3.

Vodstrcil, L.A. et al. (2013) Hormonal contraception is associated with a reduced risk of bacterial vaginosis: a systematic review and meta-analysis. *PloS one*. 8 (9), e73055. doi:10.1371/journal.pone.0073055.

Vujkovic-Cvijin, I. et al. (2015) Gut-resident Lactobacillus abundance associates with IDO1 inhibition and Th17 dynamics in SIV-infected macaques. *Cell reports*. 13 (8), 1589–1597. doi:10.1016/j.celrep.2015.10.026.

Walther-Antonio, M.R.S. et al. (2014) Pregnancy's stronghold on the vaginal microbiome. *PloS one*. 9 (6), e98514. doi:10.1371/journal.pone.0098514.

Wand, H. & Ramjee, G. (2012) The effects of injectable hormonal contraceptives on HIV seroconversion and on sexually transmitted infections. *AIDS (London, England)*. 26 (3), 375–380. doi:10.1097/QAD.0b013e32834f990f.

Weiss, S. et al. (2017) Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*. 5, 27. doi:10.1186/s40168-017-0237-y.

WHO (2017) Global Health Estimates 2015: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2015. *Global Health Estimates Technical Paper*. 1–81. doi:10.1016/j.mpmed.2016.06.006.

Widholm, O. & Kantero, R.L. (1971) A statistical analysis of the menstrual patterns of 8,000 Finnish girls and their mothers. *Acta obstetricia et gynecologica Scandinavica. Supplement*. 14, Suppl 14:1-36.

Wiesenfeld, H.C. et al. (2003) Bacterial vaginosis is a strong predictor of Neisseria gonorrhoeae and Chlamydia trachomatis infection. *Clinical infectious diseases : an official publication of the*

Infectious Diseases Society of America. 36 (5), 663–668. doi:10.1086/367658.

Van De Wijgert, J.H.H.M. et al. (2009) Disentangling contributions of reproductive tract infections to hiv acquisition in african women. *Sexually transmitted diseases*. 36 (6), 357–364. doi:http://dx.doi.org/10.1097/OLQ.0b013e3181a4f695.

Van De Wijgert, J.H.H.M. et al. (2008) Bacterial vaginosis and vaginal yeast, but not vaginal cleansing, increase HIV-1 acquisition in African women. *Journal of Acquired Immune Deficiency Syndromes*. 48 (2), 203–210. doi:10.1097/QAI.0b013e3181743936.

van de Wijgert, J.H.H.M. et al. (2013) Hormonal contraception decreases bacterial vaginosis but oral contraception may increase candidiasis: implications for HIV transmission. *AIDS (London, England)*. 27 (13), 2141–2153. doi:10.1097/QAD.0b013e32836290b6.

Wilson, J.D. et al. (2007) Bacterial vaginal flora in relation to changing oestrogen levels. *International Journal of STD and AIDS*. 18 (5), 308–311. doi:10.1258/095646207780749583.

Woo, P.C.Y. et al. (2003) Actinomyces hongkongensis sp. nov. a novel Actinomyces species isolated from a patient with pelvic actinomycosis. *Systematic and applied microbiology*. 26 (4), 518–522. doi:10.1078/072320203770865819.

World Health Organization (2017) *Hormonal contraceptive methods for women at high risk of HIV and living with HIV 2016 guidance statement*.

World Health Organization (1986) World Health Organization multicenter study on menstrual and ovulatory patterns in adolescent girls. II. Longitudinal study of menstrual patterns in the early postmenarcheal period, duration of bleeding episodes and menstrual cycles. World Health Organiza. *Journal of adolescent health care : official publication of the Society for Adolescent Medicine*. 7 (4), 236–244.

Yamamoto, T. et al. (2009) Bacterial populations in the vaginas of healthy adolescent women. *Journal of pediatric and adolescent gynecology*. 22 (1), 11–18. doi:10.1016/j.jpag.2008.01.073.

Yang, B. et al. (2016) Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinformatics*. 17, 135. doi:10.1186/s12859-016-0992-y.

Yudin, M.H. et al. (2003) Clinical and cervical cytokine response to treatment with oral or vaginal metronidazole for bacterial vaginosis during pregnancy: a randomized trial. *Obstetrics and gynecology*. 102 (3), 527–534.

Zang, Y.C.Q. et al. (2002) Regulatory effects of estriol on T cell migration and cytokine profile: inhibition of transcription factor NF-kappa B. *Journal of neuroimmunology*. 124 (1–2), 106–114.

Zaph, C. et al. (2008) Commensal-dependent expression of IL-25 regulates the IL-23-IL-17 axis in the intestine. *The Journal of experimental medicine*. 205 (10), 2191–2198. doi:10.1084/jem.20080720.

Zhang, Z. et al. (1999) Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science (New York, N.Y.)*. 286 (5443), 1353–1357.

Zhou, X. et al. (2007) Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME Journal*. 1 (2), 121–133. doi:10.1038/ismej.2007.12.

Zozaya, M. et al. (2016) Bacterial communities in penile skin, male urethra, and vaginas of heterosexual couples with and without bacterial vaginosis. *Microbiome*. 4, 16. doi:10.1186/s40168-016-0161-6.

Appendices

Appendix I: 16S rRNA V4 Amplicon Library Primers

Table A1. Primer sequences for 16S rRNA amplicon sequencing of the V4 region

Primer	Primer sequence (5' to 3')
Primary PCR primers	
Modified 515F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA
Modified 806R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT
Secondary PCR primers	
501	AATGATACGGCGACCACCGAGATCTACAC**TAGATCGC**TCGTCGGCAGCGTC
502	AATGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**TCGTCGGCAGCGTC
503	AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**TCGTCGGCAGCGTC
504	AATGATACGGCGACCACCGAGATCTACAC**AGAGTAGA**TCGTCGGCAGCGTC
505	AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**TCGTCGGCAGCGTC
506	AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**TCGTCGGCAGCGTC
507	AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**TCGTCGGCAGCGTC
508	AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**TCGTCGGCAGCGTC
701	CAAGCAGAAGACGGCATACGAGAT**TCGCCTTA**GTCTCGTGGGCTCGG
702	CAAGCAGAAGACGGCATACGAGAT**CTAGTACG**GTCTCGTGGGCTCGG
703	CAAGCAGAAGACGGCATACGAGAT**TTCTGCCT**GTCTCGTGGGCTCGG
704	CAAGCAGAAGACGGCATACGAGAT**GCTCAGGA**GTCTCGTGGGCTCGG
705	CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTCTCGTGGGCTCGG
706	CAAGCAGAAGACGGCATACGAGAT**CATGCCTA**GTCTCGTGGGCTCGG
707	CAAGCAGAAGACGGCATACGAGAT**GTAGAGAG**GTCTCGTGGGCTCGG
708	CAAGCAGAAGACGGCATACGAGAT**CCTCTCTG**GTCTCGTGGGCTCGG
709	CAAGCAGAAGACGGCATACGAGAT**AGCGTAGC**GTCTCGTGGGCTCGG
710	CAAGCAGAAGACGGCATACGAGAT**CAGCCTCG**GTCTCGTGGGCTCGG
711	CAAGCAGAAGACGGCATACGAGAT**TGCCTCTT**GTCTCGTGGGCTCGG
712	CAAGCAGAAGACGGCATACGAGAT**TCCTCTAC**GTCTCGTGGGCTCGG

The adapter sequences are underlined in the 501 and 701 secondary primer sequences. The same sequences are found in each of the 500 and 700 primer series. The 8-nucleotide sample indices are bolded. The secondary primers were premixed in plates with a unique combination of forward and reverse primers as depicted below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	701/501	702/501	703/501	704/501	705/501	706/501	707/501	708/501	709/501	710/501	711/501	712/501
B	701/502	702/502	703/502	704/502	705/502	706/502	707/502	708/502	709/502	710/502	711/502	712/502
C	701/503	702/503	703/503	704/503	705/503	706/503	707/503	708/503	709/503	710/503	711/503	712/503
D	701/504	702/504	703/504	704/504	705/504	706/504	707/504	708/504	709/504	710/504	711/504	712/504
E	701/505	702/505	703/505	704/505	705/505	706/505	707/505	708/505	709/505	710/505	711/505	712/505
F	701/506	702/506	703/506	704/506	705/506	706/506	707/506	708/506	709/506	710/506	711/506	712/506
G	701/507	702/507	703/507	704/507	705/507	706/507	707/507	708/507	709/507	710/507	711/507	712/507
H	701/508	702/508	703/508	704/508	705/508	706/508	707/508	708/508	709/508	710/508	711/508	712/508

Figure A1. Overview of secondary primer combination used for multiplexing of 96 samples.

Appendix II: Flow cytometry gating strategy

Data generated and analysed in FlowJo by Iyaloo Konstantinus, PhD candidate, Division of Virology, University of Cape Town and shared with me for use in this dissertation.

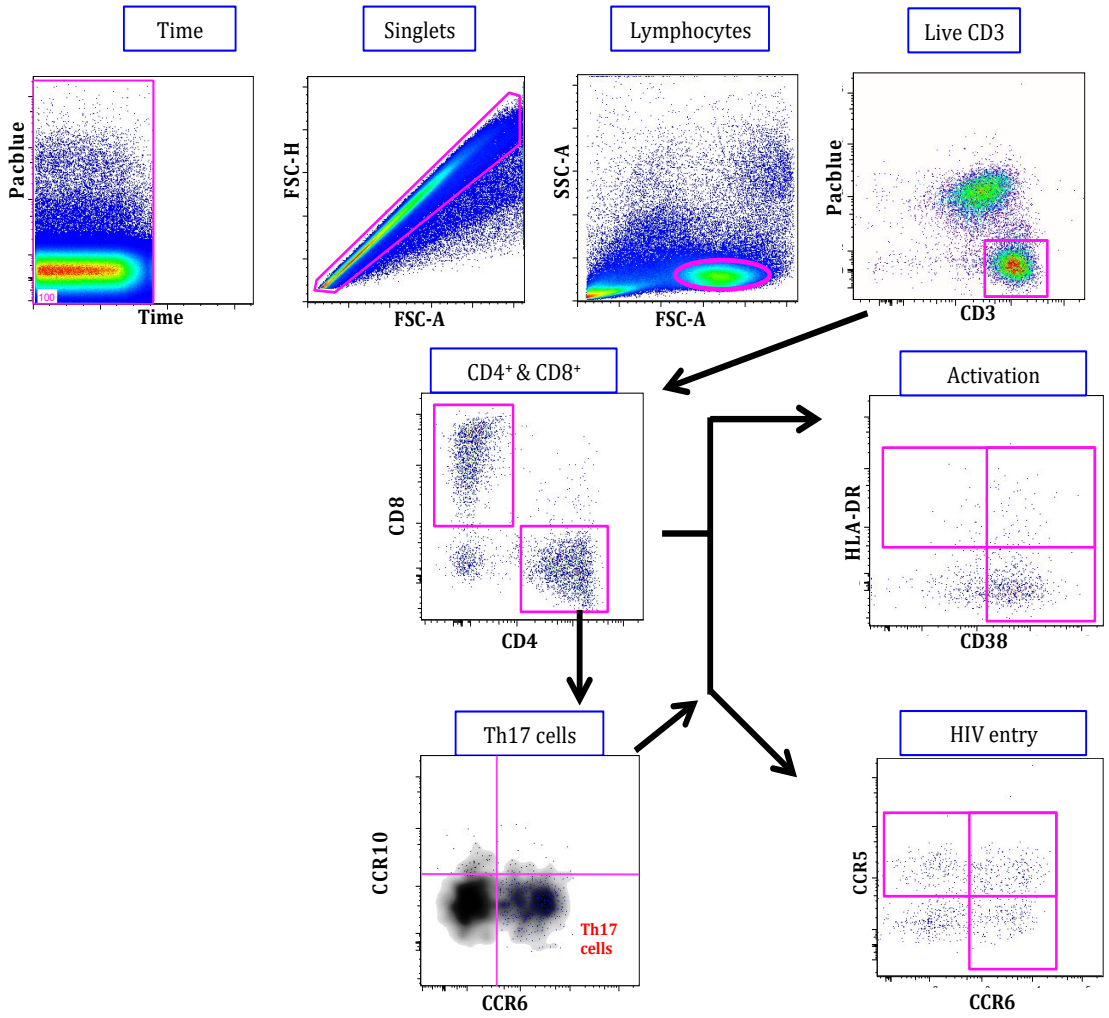


Figure A2. Gating strategy for flow cytometry analyses of HIV target cells.

Appendix III: Baseline characteristics of uCHOOSE participants

Table A2. Baseline characteristics of uCHOOSE participants.

	Screened (n=151)	Randomized (n=131)	P value
Age at screening, median yrs (IQR)	17 (16-18)	17 (16-18)	0.590
BMI, median (IQR)	25.1 (21.9-29.0)	25.0 (22.0-28.3)	0.998
STI prevalence			
Any STI(s)	65 (43.0%)	56 (42.7%)	1.000
Ct	50 (33.1%)	44 (33.6%)	1.000
Ng	18 (11.9%)	14 (10.7%)	0.891
Tv	13 (8.6%)	12 (9.2%)	1.000
Mg	4 (2.6%)	3 (2.3%)	1.000
BV prevalence			0.845
BV positive	65 (43.0%)	58 (44.2%)	
BV intermediate	17 (11.3%)	12 (9.2%)	
BV negative	69 (45.7%)	61 (46.6%)	
HSV-2 serology[1]	41 (27.2%)	37 (34.6%)	0.800
Yeast cells present	23 (15.2%)	21 (16.0%)	0.984
Age menarche, median (IQR)	13 (12-14)	13 (12-14)	0.743
Tanner, median (IQR)	4.0 (4.0-4.0)	4.0 (4.0-4.0)	1.000
Allergies, n	18 (12.3%)	16 (12.6%)	1.000
Leucocyte count (x10^9/L)	6.86 (5.1-8.3)	7.2 (5.8-8.4)	0.214
Haemoglobin (g/dL)	13.1 (12.3-13.7)	13.2 (12.3-13.7)	0.935
Biochemistry			
Na (mmol/L)	139 (138-140)	139 (137-140)	0.663
K (mmol/L)	4.2 (4.0-4.6)	4.2 (4.0-4.6)	0.896
Creatinine (umol/L)	54 (50.0-60.0)	54 (49.8-60.0)	0.964
Glucose (mmol/L)	4.5 (4.0-4.9)	4.5 (4.0-4.9)	0.821
Endocrinology[2]			
E2	100.5 (75.3-140.8)	102.5 (76-143.3)	0.992
LH	4.3 (2.0-6.0)	4.4 (2.0-6.0)	0.999
FSH	4.9 (3.6-6.0)	5.0 (3.6-6.0)	0.888
Parity			
Previously pregnant	17 (11.5%)	17 (13.1%)	0.826
Vaginal delievery (VD)	10 (6.8%)	10 (7.7%)	
Caesarean section (CS)	5 (3.4%)	5 (3.8%)	
Abortion (AB)	2 (1.4%)	2 (1.5%)	
Use of hormonal contraception[3]			0.988
Naive	7 (4.8%)	5 (3.9%)	
Not currently	29 (19.9%)	26 (20.2%)	
Net-EN	78 (53.4%)	69 (53.5%)	
COCP	9 (6.2%)	6 (4.7%)	
DMPA	20 (13.7%)	19 (14.7%)	
Implanon	3 (2.1%)	3 (2.3%)	
Intra-vaginal practices[4]			
Douching	1 (0.7%)	1 (0.8%)	1.000
Washng with water	19 (12.8%)	16 (12.4%)	1.000
Washing with soap	13 (8.8%)	12 (9.3%)	1.000
Cloth	4 (2.7%)	4 (3.1%)	1.000
Drying	1 (0.7%)	1 (0.8%)	1.000
Medication	7 (4.7%)	5 (3.9%)	0.958
Tampon use	8 (5.4%)	8 (6.2%)	0.980
Herbs	1 (0.7%)	1 (0.8%)	1.000

Sexual risk behaviour[5]			
Median age of sexual debut (IQR)[6]	15 (14-16)	15 (14-16)	1.000
Sexual partners past year, median (IQR)[6]	1 (1-1)	1 (1-1)	1.000
Multiple sexual partners	1 (0.7%)	1 (0.8%)	1.000
New partner	0 (0-1)	0 (0-1)	1.000
General condom use[7]			
Never	12 (9.4%)	12 (9.4%)	1.000
Almost never	13 (10.2%)	13 (10.2%)	
Not sure	15 (11.7%)	15 (11.7%)	
Almost always	46 (35.9%)	46 (35.9%)	
Always	42 (32.8%)	42 (32.8%)	
Condom use during last PV intercourse [7]			
Yes	78 (61.4%)	78 (61.4%)	1.000
Sex acts per week, median (IQR)	1 (1-2)	1 (1-2)	1.000
Intergenerational sex with older partner			1.000
(≥5 years)[6]	50 (39.1%)	50 (39.1%)	
No	8 (6.3%)	8 (6.3%)	
I don't think so	40 (31.3%)	40 (31.3%)	
Not sure	4 (3.1%)	4 (3.1%)	
I think so	26 (20.3)	26 (20.3)	
Yes	1 (0.8%)	1 (0.8%)	1.000
Transactional sex [6]	4 (3.1%)	4 (3.1%)	1.000
Penile-anal intercourse[6]			
Education[6]			
School attendance	114 (85.7%)	112 (86.8%)	0.936
Highest grade, median (IQR)	10 (8-11)	10 (8-11)	0.922
Tertiary attendance	6 (4.5%)	4 (3.1%)	0.749

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups, unpaired Wilcoxon Rank Sum test for comparison of medians and unpaired Student's t test for comparison of means. BMI, body mass index; BV; bacterial vaginosis; Ct, Chlamydia trachomatis*; IQR, interquartile range; Mg,* Mycoplasma genitalium*; Ng,* Neisseria gonorrhoea*; PV, penile-vaginal; sd, standard deviation; STI, sexually transmitted infection; Tv,* Trichomonas vaginalis; yrs, years*.*

1. One equivocal result.
2. Missing values from two participants.
3. Missing data from five participants.
4. Missing data from four participants.
5. Missing data from six participants.
6. Missing data from 23 participants.
7. Missing data from 24 participants.

Appendix IV: Sequencing depth expressed as read counts of 16S rRNA gene amplicon sequencing dataset of uCHOOSE samples

Extraction and PCR controls in green. Positive mock communities in red.
Cut-off for analyses: 5000 reads.
UC135_LW_V1: sample missing.
UC131_LW_V3: de-multiplexing failed.

Sample ID: read count

PCR-C1-run1: 0
PCR-C2-run1: 0
ExC-run1: 6
PCR-C1-run5: 36
PCR-C2-run5: 37
PCR-C2-run4: 56
PCR-C1-run3: 69
PCR-C1-run6: 72
PCR-C1-run4: 83
ExC-run4: 85
ExC-run5: 87
PCR-C2-run3: 150
ExC-run3: 151
PCR-C1-run2: 152
ExC-run2: 193
ExC-run6: 261
PCR-C2-run2: 275
PCR-C2-run6: 431
UC092_LW_V2: 816
UC171_LW_V3: 825
UC159_LW_V2: 1097
UC132_LW_V1: 1990
UC164_LW_V1: 3087
UC132_LW_V3: 3950
UC159_LW_V1: 4185
UC141_LW_V2: 4499
UC172_LW_V3: 6220
UC177_LW_V2: 6571
UC018_LW_V2: 6848
UC051_LW_V2: 7227
UC040_LW_V1: 7757
UC131_LW_V2: 8146
UC092_LW_V1: 8267
UC047_LW_V1: 8408
UC131_LW_V1: 8969
UC145_LW_V1: 9063
UC149_LW_V2: 9279
UC087_LW_V1: 9325
UC159_LW_V3: 9654
UC056_LW_V2: 9661
UC084_LW_V3: 9889
UC124_LW_V1: 10763
UC141_LW_V1: 11002
UC053_LW_V3: 11464
UC172_LW_V2: 11520
UC147_LW_V3: 11785
UC160_LW_V2: 12079
UC044_LW_V1: 12168
UC055_LW_V3: 12529
UC057_LW_V2: 13096
UC009_LW_V1: 13155
UC022_LW_V2: 13331
UC065_LW_V1: 13489
UC022_LW_V3: 13707
UC160_LW_V1: 14812
UC028_LW_V1: 15031
UC170_LW_V3: 15323
UC177_LW_V1: 15343
UC179_LW_V1: 15439
UC056_LW_V1: 15566
UC162_LW_V1: 15598
UC094_LW_V1: 15878
UC147_LW_V2: 15906
UC172_LW_V1: 16375
UC024_LW_V1: 16453
UC058_LW_V1: 16739
UC155_LW_V1: 16786
UC147_LW_V1: 17884
UC113_LW_V3: 17930
UC132_LW_V2: 18045
UC015_LW_V1: 18130
UC157_LW_V3: 18178
UC125_LW_V3: 18217
UC089_LW_V1: 18994
UC113_LW_V2: 19305
UC113_LW_V1: 19432
UC139_LW_V1: 19510
UC041_LW_V1: 20042
UC109_LW_V1: 20245
UC057_LW_V1: 20817
UC167_LW_V2: 20890
UC017_LW_V1: 21078
UC066_LW_V1: 21318
UC143_LW_V3: 21367
UC115_LW_V3: 21953
UC114_LW_V2: 22005
UC164_LW_V2: 22533
UC140_LW_V2: 23126
UC087_LW_V2: 23204
UC096_LW_V1: 23272
UC158_LW_V3: 23289
UC141_LW_V3: 23405
UC003_LW_V1: 23690
Mock-run1: 23928
UC111_LW_V1: 24067
UC164_LW_V3: 24070
UC119_LW_V2: 24228
UC083_LW_V2: 24536
UC087_LW_V3: 24847
UC059_LW_V1: 24973
UC057_LW_V3: 25024
UC063_LW_V1: 25631
UC165_LW_V1: 26082
UC091_LW_V1: 26100
UC120_LW_V2: 26585
UC155_LW_V2: 26762
UC086_LW_V3: 27149
UC059_LW_V3: 27251
UC133_LW_V1: 27785
UC058_LW_V2: 28439
UC117_LW_V1: 28594
UC037_LW_V1: 28680
UC075_LW_V1: 28737
UC103_LW_V1: 29222
UC037_LW_V2: 29883
UC127_LW_V1: 31054
UC152_LW_V1: 31232
UC059_LW_V2: 31438
UC038_LW_V3: 31595
UC114_LW_V1: 31743
UC122_LW_V3: 32775
UC048_LW_V2: 33252
UC143_LW_V1: 33473
UC123_LW_V1: 34068
UC145_LW_V2: 34423
UC107_LW_V2: 34818
UC127_LW_V2: 35400
UC070_LW_V2: 35487
UC116_LW_V1: 35915
UC054_LW_V1: 36493
UC018_LW_V3: 36697
UC155_LW_V3: 37057
UC018_LW_V1: 37552
UC069_LW_V1: 37644
UC095_LW_V1: 38146
UC089_LW_V2: 38565
UC134_LW_V1: 38587
UC139_LW_V2: 38602
UC128_LW_V1: 38689
UC009_LW_V2: 39066
UC115_LW_V2: 39146
UC130_LW_V3: 39390
UC086_LW_V1: 39665
UC104_LW_V1: 39926
UC085_LW_V1: 40276
UC114_LW_V3: 40566
UC047_LW_V3: 41396
UC121_LW_V1: 41470
UC107_LW_V1: 41924
UC144_LW_V1: 42048
UC125_LW_V2: 42654
UC149_LW_V1: 42758
UC038_LW_V1: 42809
UC039_LW_V2: 42972
UC025_LW_V1: 43042
UC166_LW_V1: 43277
UC042_LW_V1: 43506
UC138_LW_V1: 43530
UC099_LW_V3: 43673

UC019_LW_V2: 44092
UC028_LW_V2: 44156
UC033_LW_V1: 44200
UC156_LW_V1: 44245
UC137_LW_V1: 44274
UC066_LW_V2: 44477
UC073_LW_V1: 44681
UC170_LW_V2: 44885
UC144_LW_V2: 45025
UC068_LW_V2: 45407
UC029_LW_V2: 45449
UC163_LW_V1: 45569
UC102_LW_V1: 45649
UC119_LW_V3: 46089
UC156_LW_V2: 46172
UC076_LW_V2: 46561
UC161_LW_V1: 47027
UC062_LW_V1: 47036
UC098_LW_V1: 47155
UC093_LW_V1: 47337
UC120_LW_V1: 47353
UC127_LW_V3: 47542
UC056_LW_V3: 47561
UC055_LW_V2: 47649
UC062_LW_V2: 47766
UC046_LW_V2: 47856
UC149_LW_V3: 48504
UC158_LW_V2: 48718
UC062_LW_V3: 48755
UC095_LW_V2: 48904
UC053_LW_V2: 49466
UC055_LW_V1: 50065
UC139_LW_V3: 50367
UC063_LW_V3: 51116
UC084_LW_V2: 51516
UC094_LW_V3: 51766
UC035_LW_V1: 51999
UC006_LW_V1: 52295
UC163_LW_V2: 52777
UC104_LW_V2: 53046
UC071_LW_V1: 53345
UC023_LW_V3: 54392
UC170_LW_V1: 54526
UC091_LW_V2: 54853
UC125_LW_V1: 55024
UC045_LW_V1: 55033
UC130_LW_V1: 55509
UC129_LW_V1: 55834
UC099_LW_V2: 55880
UC024_LW_V3: 55946
UC036_LW_V3: 56096
UC089_LW_V3: 56671
UC112_LW_V1: 56680
UC072_LW_V1: 56935
UC083_LW_V1: 57276
UC063_LW_V2: 57602
UC038_LW_V2: 58043
UC016_LW_V3: 59102
UC067_LW_V3: 59267
UC129_LW_V2: 59316
UC160_LW_V3: 59666
UC161_LW_V2: 59844
UC099_LW_V1: 60033
UC137_LW_V2: 60067
UC122_LW_V2: 61052
UC130_LW_V2: 61276
UC064_LW_V1: 61295
UC140_LW_V3: 61815
UC020_LW_V1: 61890
UC043_LW_V1: 62134
UC104_LW_V3: 62207
UC106_LW_V1: 62913
UC174_LW_V1: 63403
UC031_LW_V1: 63685
UC042_LW_V2: 63980
UC071_LW_V2: 64247
UC074_LW_V1: 64897
Mock-run4: 65400
UC118_LW_V1: 65517
UC051_LW_V3: 65893
UC084_LW_V1: 66024
UC140_LW_V1: 66814
UC091_LW_V3: 67977
UC003_LW_V2: 68142
UC030_LW_V1: 68215
UC101_LW_V1: 69301
UC101_LW_V2: 70262
UC074_LW_V2: 70687
UC067_LW_V1: 71461
UC040_LW_V2: 72345
UC008_LW_V1: 72636
UC120_LW_V3: 73032
UC025_LW_V2: 73785
Mock-run6: 74008
UC007_LW_V3: 74282
UC115_LW_V1: 75078
UC008_LW_V2: 75139
UC045_LW_V3: 75501
UC092_LW_V3: 76075
UC028_LW_V3: 76892
UC033_LW_V3: 76944
UC029_LW_V1: 78847
UC012_LW_V1: 80297
UC058_LW_V3: 80454
UC002_LW_V1: 81059
UC095_LW_V3: 81152
UC088_LW_V1: 81404
UC076_LW_V3: 82160
UC013_LW_V2: 82324
UC094_LW_V2: 83462
UC001_LW_V1: 84356
UC076_LW_V1: 84418
UC042_LW_V3: 84516
UC023_LW_V2: 85996
UC011_LW_V1: 86303
UC036_LW_V2: 86385
UC065_LW_V2: 86493
UC066_LW_V3: 86547
UC041_LW_V2: 86681
UC021_LW_V3: 86779
UC051_LW_V1: 87044
UC067_LW_V2: 88145
UC077_LW_V2: 89162
UC040_LW_V3: 89392
UC048_LW_V1: 90022
UC046_LW_V3: 90910
UC033_LW_V2: 91301
UC176_LW_V1: 92572
UC007_LW_V1: 92897
UC167_LW_V3: 92928
UC021_LW_V1: 93578
UC122_LW_V1: 95009
UC009_LW_V3: 95164
UC022_LW_V1: 96026
UC004_LW_V1: 96761
UC090_LW_V1: 97402
Mock-run3: 99172
UC044_LW_V3: 99218
UC019_LW_V1: 100694
UC079_LW_V2: 100798
UC158_LW_V1: 101462
UC024_LW_V2: 102379
UC082_LW_V3: 102526
UC023_LW_V1: 104429
UC106_LW_V2: 104621
UC044_LW_V2: 104659
UC129_LW_V3: 106152
UC119_LW_V1: 106152
UC167_LW_V1: 108247
UC171_LW_V2: 108333
UC039_LW_V1: 108622
UC136_LW_V1: 109028
UC005_LW_V1: 110726
UC075_LW_V2: 110918
UC156_LW_V3: 111159
UC048_LW_V3: 111377
UC073_LW_V3: 112078
UC068_LW_V1: 112084
UC016_LW_V2: 113722
UC109_LW_V2: 115416
UC070_LW_V1: 119698
UC079_LW_V1: 119797
Mock-run5: 120037
UC065_LW_V3: 120193
UC015_LW_V2: 124613
UC096_LW_V3: 124777
UC041_LW_V3: 124784
UC173_LW_V1: 125354
UC021_LW_V2: 130633
UC053_LW_V1: 131188
UC047_LW_V2: 131510
UC126_LW_V1: 132087
UC082_LW_V2: 132612
UC036_LW_V1: 132768
UC013_LW_V1: 134332
UC106_LW_V3: 135432
UC096_LW_V2: 135917
UC093_LW_V2: 136589
UC138_LW_V2: 140225
UC068_LW_V3: 141726
UC109_LW_V3: 142411
UC093_LW_V3: 142573
UC007_LW_V2: 142812
UC077_LW_V3: 146525
UC013_LW_V3: 147430
UC082_LW_V1: 147749
UC180_LW_V1: 150062
UC086_LW_V2: 150825
UC171_LW_V1: 151315
UC015_LW_V3: 153287
UC010_LW_V1: 156841
UC008_LW_V3: 157136
UC070_LW_V3: 159023
UC110_LW_V1: 161728
UC074_LW_V3: 161885

UC079_LW_V3: 162206
UC019_LW_V3: 162562
UC016_LW_V1: 167850
UC045_LW_V2: 170218
UC046_LW_V1: 174229
Mock-run2: 180944
UC072_LW_V2: 181374
UC077_LW_V1: 185628
UC073_LW_V2: 212143
UC072_LW_V3: 212489
UC049_LW_V1: 214754
UC146_LW_V1: 226263
UC081_LW_V1: 262353
UC143_LW_V2: 279267
UC157_LW_V2: 281174
UC003_LW_V3: 284696
UC157_LW_V1: 315222
UC165_LW_V3: 394989
UC165_LW_V2: 413337
UC101_LW_V3: 655080
UC128_LW_V2: 743090
UC128_LW_V3: 96178

Appendix V: Endogenous hormones and vaginal microbiota at screening and exit

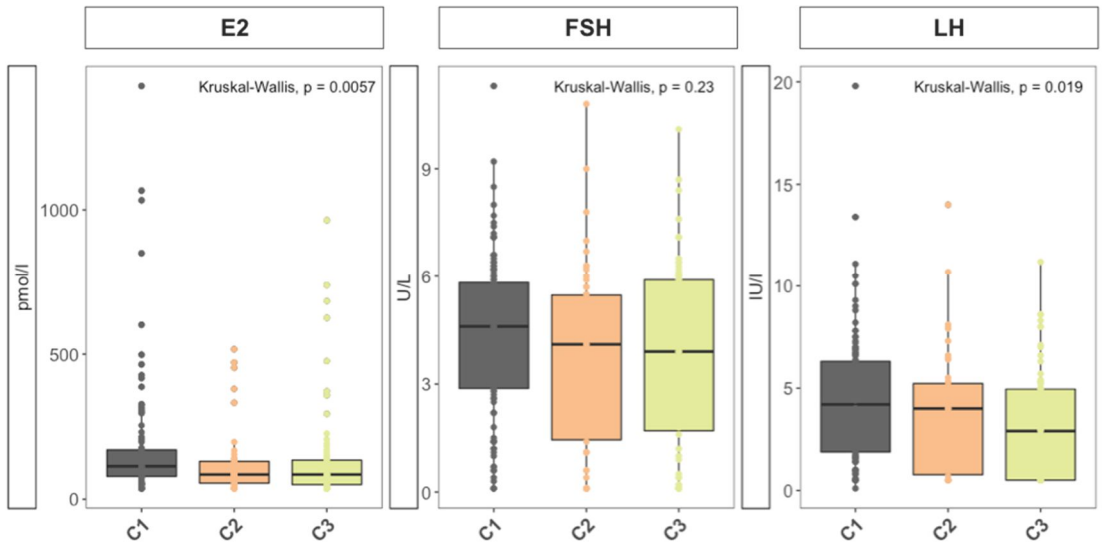


Figure A3. Endogenous hormones according to vaginal community cluster at screening and exit visits. Boxplots of endogenous oestradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels in screening and exit samples from 228 participants stratified by community cluster generated using Fuzzy clustering with weighted UniFrac distances. P values were generated using the Kruskal-Wallis test. C1, n=108; C2=50; C3, n=70.

Table A5. Endogenous hormones according to vaginal community cluster at screening and exit visits.

	E2		**FSH**		**LH**	
	P	P adj.	P	P adj.	P	P adj.
C1 vs. C2	**0.032**	**0.048**	0.438	0.438	0.442	0.442
C1 vs. C3	**0.013**	**0.039**	0.383	0.383	**0.002**	**0.007**
C2 vs. C3	0.427	0.427	0.320	0.320	**0.007**	**0.011**

P values calculated using Kruskal-Wallis with Dunn's post hoc test using Benjamini-Hochberg method for multiple testing correction (MTC). E2: oestradiol; FSH: follicle-stimulating hormone; LH: luteinizing hormone. C1, n=108; C2=50; C3, n=70.

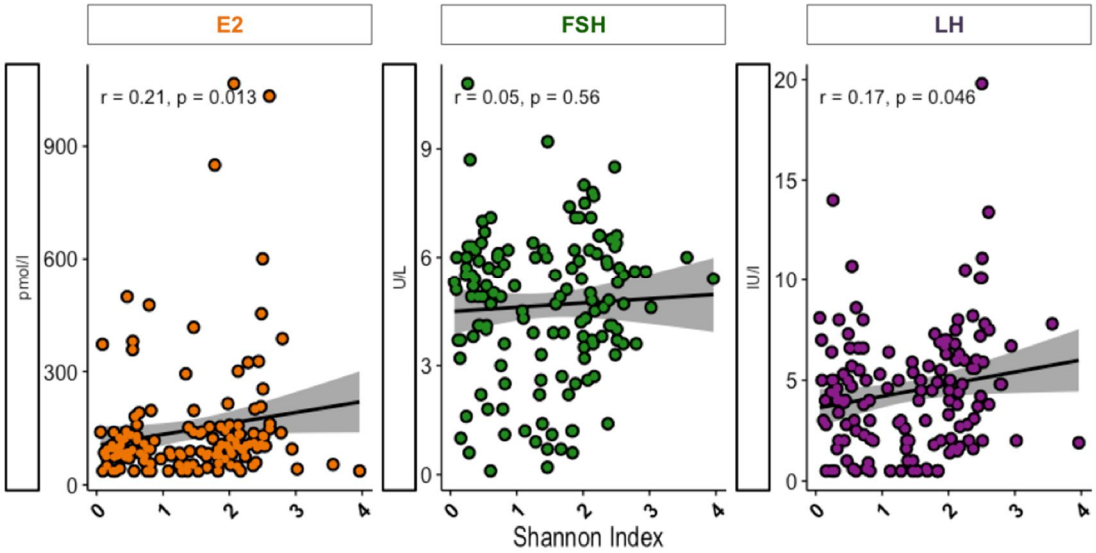


Figure A4. Endogenous hormones according to alpha diversity at screening and exit visits. Correlation plots of endogenous oestradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels according to alpha diversity measured with Shannon Index in baseline samples from 236 participants at the screening or exit visits. The Spearman correlation coefficient is depicted on the figure for each individual hormone.

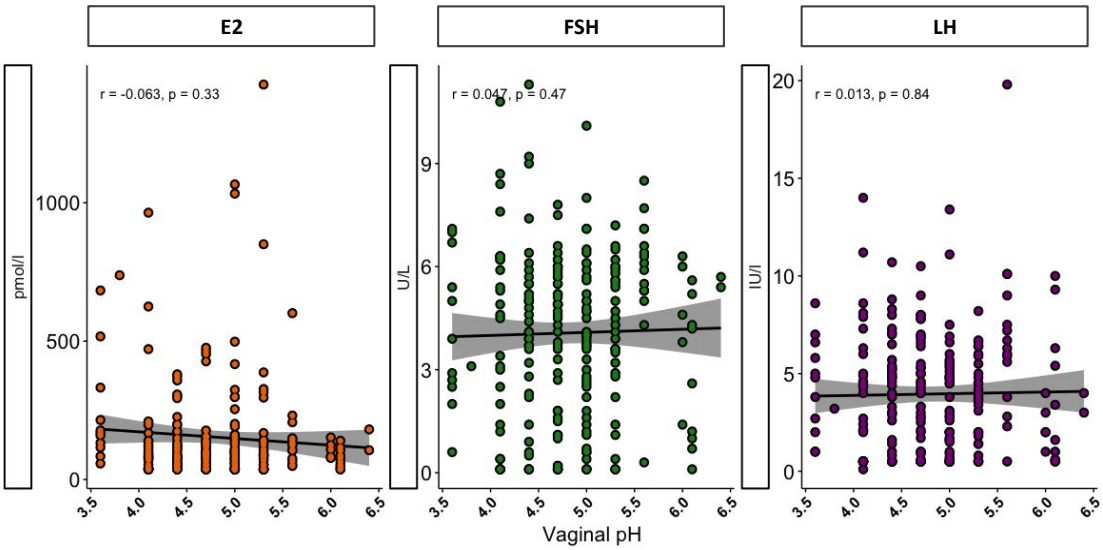


Figure A5. Endogenous hormones and vaginal pH at screening and exit visits. Correlation plots of endogenous oestradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels (measured in pmol/l, U/L and IU/L, respectively) according to vaginal pH in samples from 236 participants at the screening and exit visits. The Spearman correlation coefficient (rho, r) and the p values for each individual hormone depicted on the figure.

Appendix VI: Endogenous hormones and BV status at screening

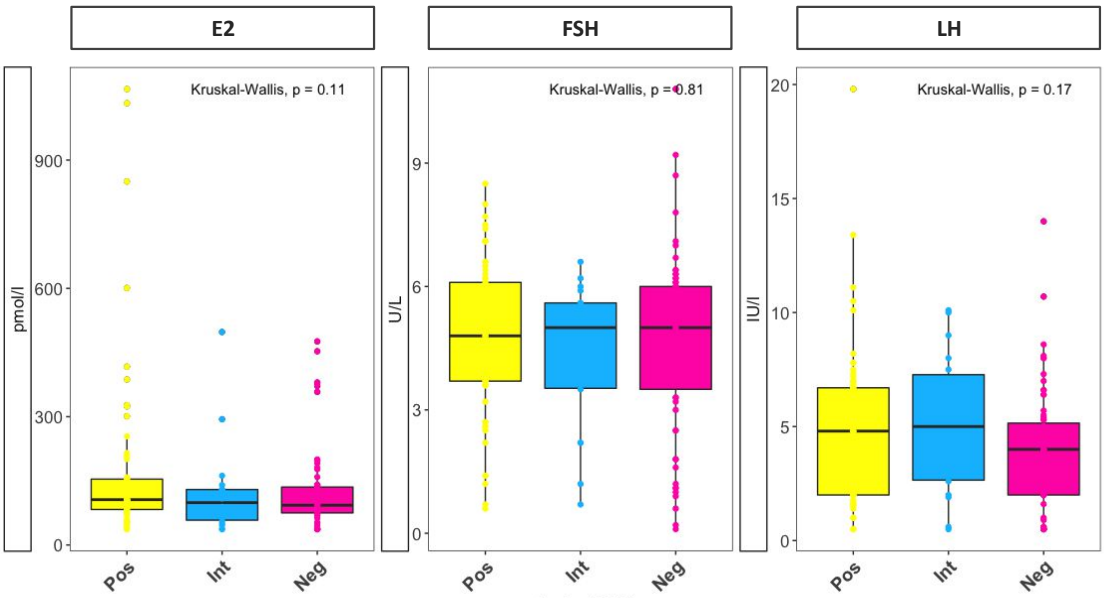


Figure A6. Endogenous hormones by BV status at screening visit. Boxplots of endogenous oestradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels (measured in pmol/l, U/L and IU/L, respectively) in baseline samples from 146 participants at the screening visit stratified by BV status according to Nugent score (BV positive (Pos): n=61, BV intermediate (Int): n=18, BV negative (Neg): n=67). P values were generated using the Kruskal-Wallis test.

Table A6. Endogenous hormones according to BV status at baseline.

	BV positive (n=61)	**Intermediate** (n=18)	**BV negative** (n=67)	**P**	**BV- vs. BV+**		**BV- vs. Int**		**BV+ vs. Int**	
					P	P adj.	P	P adj.	P	P adj.
E2 (pmol/l)	106 (83.0-154)	99.0 (58.0-130)	93.0 (75.0-136)	0.11	**0.044**	0.398	0.910	0.910	0.220	0.494
FSH (U/L)	4.80 (2.00-6.70)	5.00 (2.65-7.28)	4.00 (2.00-5.15)	0.81	0.717	0.897	0.776	0.897	0.487	0.876
LH (IU/L)	4.80 (3.70-6.10)	5.00 (3.53-5.60)	5.00 (3.50-6.00)	0.17	0.096	0.432	0.176	0.494	0.797	0.897

P values generated with Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests using Benjamini-Hochberg (BH) method for multiple testing correction (MTC). BV, bacterial vaginosis; E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone. BV+, BV positive; Int, BV intermediate, BV-, BV negative. BV status according to Nugent scoring.

Appendix VII: Diversity and community clusters at exit visit

Table A10. Characteristics of participants at exit according to hormonal contraceptive method.

	COCP (n=12)	**Net-En** (n=32)	**NuvaRing** (n=45)	**P value**
Fuzzy cluster distribution [1]				0.599
C1	4 (33.3%)	12 (37.5%)	19 (42.2%)	
C2	5 (41.7%)	7 (21.9%)	8 (17. 8%)	
C3	3 (25.0%)	12 (37.5%)	17 (37.8%)	
Vaginal pH, mean (sd)	4.29 (3.82-4.77)	4.66 (4.09-5.24)	4.77 (4.18-5.36)	**0.040**
Shannon Index, median (IQR)	0.65 (0.27-1.82)	1.44 (0.74-1.91)	1.32 (0.59-2.06)	0.319
HSV-2 serology[2]	3 (25.0%)	13 (40.6%)	17 (37.8%)	0.686
Yeast cells present	1 (8.33%)	5 (15.6%)	14 (31.1%)	0.171
BV prevalence				1.000
BV positive	5 (41.7%)	14 (43. 8%)	19 (42.2%)	
BV intermediate	0 (0.0%)	1 (3.13%)	1 (2.22%)	
BV negative	7 (58.3%)	17 (53.1%)	25 (55.6%)	
STI prevalence				
Any STI(s)	6 (50%)	4 (12.5%)	17 (37.8%)	**0.013**
Ct	4 (33.3%)	3 (9.4%)	10 (22.2%)	0.149
Ng	2 (16.7%)	2 (6.3%)	2 (4.4%)	0.278
Tv	1 (8.3%)	1 (3.1%)	1 (2.2%)	0.525
Mg	0 (0.0%)	0 (0.0%)	2 (4.4%)	0.632
Sexual risk behavior since last visit[3]				
Sexual partner(s), median (IQR)	1 (1-1)	1 (0-1)	1 (1-1)	0.082
Multiple sexual partners, n	0 (0.0%)	1 (3.1%)	2 (4.5%)	0.563
New partner(s), n	0 (0.0%)	1 (3.1%)	1 (2.3%)	1.000
Condom use				0.083
Never	1 (8.3%)	13 (40.6%)	14 (31.8%)	
Less than half the time	1 (8.3%)	3 (9.4%)	11 (25.0%)	
Half the time	3 (25.0%)	7 (21.9%)	8 (18.2%)	
More than half the time	2 (16.7%)	6 (18.8%)	3 (6.8%)	
Always	5 (41.7)	3 (9.4%)	8 (18.2%)	
Condom use during last PV intercourse				
Yes	11 (91.7%)	22 (68.8%)	27 (61.4%)	0.145
PV sex acts per week, median (IQR)	2 (1-2)	2 (1-2)	2 (1-2)	0.907
Intergenerational sex with older partner (≥5 years)				
Yes	0 (0.0%)	0 (0.0%)	3 (6.8%)	0.371
No	12 (100%)	32 (100%)	41 (93.2%)	
Transactional sex	1 (8.3%)	0 (0.0%)	0 (0.0%)	0.136
Penile-anal intercourse	0 (0.0%)	0 (0.0%)	0 (0.0%)	NA

Chi-squared test (Fisher's exact test when expected values < 5) for the assessment of association of frequency among groups, Kruskal-Wallis test for comparison of medians and ANOVA test for comparison of means. BV; bacterial vaginosis; HSV-2, herpes simplex virus type 2.

1. Two participants with "no.cluster" assignment (COCP: n=0; Net-En: n=1; NuvaRing: n=1).

2. One equivocal result.

3. Data missing from one participant (on NuvaRing).

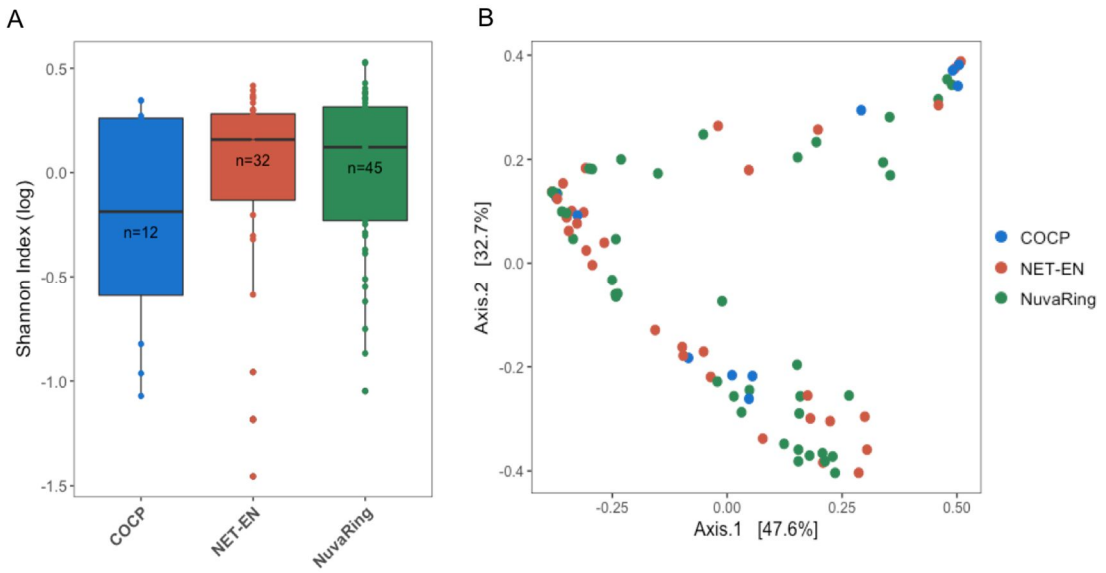


Figure A10. Alpha and beta diversity according to hormonal contraceptive method at exit. A) Boxplot depicting the alpha diversity measured using Shannon Index (log transformed values) of the microbiota from 89 participants at exit visits according to the hormonal contraceptive used at time of sampling (COCP: n=12; Net-En: n=32; NuvaRing: n=45). P values were calculated using Kruskal-Wallis and unpaired Mann-Whitney-Wilcoxon tests and adjusted for multiple comparisons using Benjamini-Hochberg (BH) method. B) Principle Coordinate Analysis (PCoA) of samples from 89 participants at the exit visit using weighted UniFrac distances. Samples are coloured by the hormonal contraceptive method that the participant was on at the time of sampling.

Table A11. Alpha diversity according to hormonal contraceptive method at exit.

	Shannon Index	
	P	P adj.
All hormonal contraceptive methods	0.319	0.319
COCP vs. Net-En	0.298	0.298
COCP vs. NuvaRing	0.115	0.115
Net-En vs. NuvaRing	0.714	0.714

P values calculated using Kruskal-Wallis with Dunn's post hoc test using Benjamini-Hochberg method for multiple testing correction (MTC). E2: oestradiol; FSH: follicle-stimulating hormone; LH: luteinizing hormone. COCP: n=12; Net-En: n=32; NuvaRing: n=45

Appendix VIII: Luminex results and quality control measures

Table A19. Percentage detection of cytokines (n=74/plate).

	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Overall
IL-1β	100	92	100	100	100	100	99
IL-4	30	22	27	68	35	53	39
IL-6	88	100	100	100	100	99	98
IL-10	43	70	47	53	14	30	43
IL-17A	97	93	100	100	100	97	98
IL-17F	66	35	51	88	51	61	59
IL-21	47	74	97	100	66	88	79
IL-22	96	97	97	100	92	100	97
IL-23	42	22	31	73	99	78	58
IL-25	57	89	62	96	39	46	65
IL-31	82	100	100	99	91	96	95
IL-33	77	50	86	99	93	86	82
IFN-γ	92	97	96	97	82	86	92
sCD40L	39	46	100	73	43	59	60
TNF-α	88	78	92	100	72	97	88
Average	**70**	**71**	**79**	**90**	**72**	**78**	**77**

Table A20. Luminex intra-plate controls.

Plate	**1** (n=5)		**2** (n=5)		**3** (n=6)		**4** (n=6)		**5** (n=6)		**6** (n=6)		**Overall** (n=34)	
	Rho	**p**	**Rho**	**p**	**Rho**	p	**Rho**	p	**Rho**	p	**Rho**	p	**Rho**	p
IL-1β	1.00	*	1.00	*	0.94	0.005	1.00	*	0.89	0.02	1.00	*	0.96	*
IL-4	0.79	0.11	1.00	*	-	-	0.84	0.04	0.47	0.35	-	-	0.82	*
IL-6	0.90	0.04	0.82	0.09	0.95	0.005	0.99	0.0003	0.46	0.35	0.74	0.09	0.97	*
IL-10	-0.25	0.69	0.45	0.45	0.28	0.59	0.90	0.02	-	-	-	-	0.37	0.03
IL-17A	0.92	0.03	0.97	0.005	0.37	0.47	0.97	0.0003	0.60	0.21	0.30	0.57	0.85	*
IL-17F	0.92	0.03	0.79	0.11	-0.28	0.59	0.72	0.11	0.75	0.09	-	-	0.73	*
IL-21	0.34	0.57	0.80	0.10	0.23	0.66	0.84	0.04	0.65	0.16	0.59	0.22	0.77	*
IL-22	0.41	0.49	0.70	0.19	-0.31	0.54	0.84	0.04	0.09	0.87	0.17	0.74	0.27	0.12
IL-23	0.88	0.05	0.79	0.11	-	-	0.91	0.01	0.74	0.10	0.17	0.75	0.59	0.0002
IL-25	1.00	*	1.00	*	0.27	0.60	0.66	0.16	0.69	0.13	-	-	0.65	*
IL-31	0.83	0.09	0.92	0.03	0.81	0.05	0.65	0.16	0.84	0.04	-0.32	0.54	0.60	0.0002
IL-33	0.90	0.04	1.00	*	0.18	0.73	0.75	0.08	0.84	0.04	0.26	0.62	0.71	*
IFN-γ	0.71	0.18	0.60	0.28	0.09	0.87	0.52	0.29	0.90	0.01	0.20	0.71	0.50	0.003
sCD40L	0.92	0.03	1.00	*	0.64	0.17	0.95	0.003	0.86	0.03	-	-	0.84	*
TNF-α	0.98	0.005	0.98	0.005	0.77	0.07	0.99	0.0003	0.93	0.008	0.23	0.67	0.82	*
SCORE	**11/15**		**12/15**		**6/15**		**12.5/15**		**9.5/15**		**2/15**		**10/15**	

Scoring for quality control: Rho 1.00-0.80 = 1, rho 0.80-0.60 = 0.5, rho 0.60-0.00 = 0. Total score out of 15.

Table A21. Luminex inter-plate control.

Plate	**1 vs. 2** (n=7)		**1 vs. 3** (n=6)		**1 vs. 4** (n=7)		**1 vs. 5** (n=7)		**1 vs. 6** (n=7)		**2 vs. 3** (n=6)		**2 vs. 4** (n=7)	
	Rho	**p**	**Rho**	**p**	**Rho**	p	**Rho**	p	**Rho**	p	**Rho**	p	**Rho**	p
IL-1β	1.00	*	1.00	*	0.94	0.005	1.00	*	0.89	0.02	1.00	*	0.96	*
IL-4	0.83	0.11	1.00	*	-	-	0.84	0.04	0.47	0.35	-	-	0.82	*
IL-6	1.00	0.04	0.82	0.09	0.95	0.005	0.99	0.0003	0.46	0.35	0.74	0.09	0.97	*
IL-10	0.66	0.69	0.45	0.45	0.28	0.59	0.90	0.02	-	-	-	-	0.37	0.03
IL-17A	1.00	0.03	0.97	0.005	0.37	0.47	0.97	0.0003	0.60	0.21	0.30	0.57	0.85	*
IL-17F	0.96	0.03	0.79	0.11	-0.28	0.59	0.72	0.11	0.75	0.09	-	-	0.73	*
IL-21	0.89	0.57	0.80	0.10	0.23	0.66	0.84	0.04	0.65	0.16	0.59	0.22	0.77	*
IL-22	0.94	0.49	0.70	0.19	-0.31	0.54	0.84	0.04	0.09	0.87	0.17	0.74	0.27	0.12
IL-23	0.77	0.05	0.79	0.11	-	-	0.91	0.01	0.74	0.10	0.17	0.75	0.59	0.0002
IL-25	1.00	*	1.00	*	0.27	0.60	0.66	0.16	0.69	0.13	-	-	0.65	*
IL-31	0.94	0.09	0.92	0.03	0.81	0.05	0.65	0.16	0.84	0.04	-0.32	0.54	0.60	0.0002
IL-33	0.96	0.04	1.00	*	0.18	0.73	0.75	0.08	0.84	0.04	0.26	0.62	0.71	*
IFN-γ	0.91	0.18	0.60	0.28	0.09	0.87	0.52	0.29	0.90	0.01	0.20	0.71	0.50	0.003
sCD40L	1.00	0.03	1.00	*	0.64	0.17	0.95	0.003	0.86	0.03	-	-	0.84	*
TNF-α	0.99	0.005	0.98	0.005	0.77	0.07	0.99	0.0003	0.93	0.008	0.23	0.67	0.82	*
SCORE	**14/15**		**13.5/15**		**15/15**		**14.5/15**		**14.5/15**		**14.5/15**		**14.5/15**	

Cont.	**2 vs. 5** (n=7)		**2 vs. 6** (n=7)		**3 vs. 4** (n=6)		**3 vs. 5** (n=6)		**4 vs. 5** (n=6)		**4 vs. 6** (n=7)		**5 vs. 6** (n=7)	
	Rho	p	Rho	p	Rho	p	Rho	p	Rho	p	Rho	p	Rho	p
IL-1β	1.00	*	1.00	*	1.00	*	1.00	*	1.00	*	1.00	*	1.00	*
IL-4	0.81	0.03	0.81	0.03	0.95	0.003	0.94	0.005	0.94	0.005	0.98	0.0001	0.98	0.0001
IL-6	1.00	*	1.00	*	0.99	0.0003	1.00	*	1.00	*	0.99	*	0.99	*
IL-10	0.80	0.03	0.80	0.03	0.59	0.21	0.80	0.06	0.80	0.06	0.88	0.008	0.88	0.008
IL-17A	0.99	*	1.00	*	1.00	*	0.99	0.0003	1.00	*	0.99	*	1.00	*
IL-17F	0.96	0.0005	0.93	0.002	0.94	0.005	0.94	0.005	0.99	0.0003	1.00	*	0.96	0.0008
IL-21	0.63	0.13	0.82	0.02	0.75	0.08	0.74	0.10	0.81	0.05	0.67	0.10	0.93	0.003
IL-22	0.94	0.002	0.93	0.003	1.00	*	0.99	0.0003	0.94	0.005	0.99	*	0.96	0.0005
IL-23	0.81	0.03	0.88	0.008	0.75	0.09	0.93	0.008	0.84	0.03	0.88	0.009	0.87	0.01
IL-25	0.95	0.0008	0.86	0.01	0.84	0.04	0.90	0.02	0.94	0.005	0.84	0.02	0.89	0.007
IL-31	0.99	*	0.96	0.0005	0.94	0.005	1.00	*	0.99	0.0003	0.96	0.0005	0.96	0.0008
IL-33	0.97	0.0002	0.98	0.0001	0.99	0.0003	0.96	0.003	0.99	0.0003	0.99	*	0.99	*
IFN-γ	0.99	*	0.99	*	0.82	0.05	0.82	0.04	0.87	0.03	0.99	*	0.94	0.002
sCD40L	0.97	0.0002	0.97	0.0002	0.94	0.005	1.00	*	1.00	*	0.94	0.002	0.94	0.002
TNF-α	1.00	*	1.00	*	1.00	*	1.00	*	1.00	*	1.00	*	1.00	*
SCORE	**14.4/15**		**15/15**		**13/15**		**14.5/15**		**15/15**		**14.5/15**		**15/15**	

Scoring for quality control: Rho 1.00-0.80 = 1, rho 0.80-0.60 = 0.5, rho 0.60-0.00 = 0. Total score out of 15.

Table A22. Cytokine concentrations according to vaginal infections.

	IL-1β	P	P adj.	IL-6	P	P adj.	IL-21	P	P adj.	IL-22	P	P adj.
BV		**< 2e-16**	**< 2e-16**		**4.9e-12**	**1.1e-11**		**1.5e-11**	**2.7e-11**		**1.4e-06**	**1.8e-06**
Positive	57.1 (14.3-214)			2.62 (0.80-13.2)			10.7 (5.50-24.9)			8.99 (6.05-16.3)		
Intermediate	38.6 (9.74-391)			6.17 (0.92-22.5)			7.94 (0.01-17.7)			6.73 (3.58-16.5)		
Negative	1.55 (0.28-1.76)			0.48 (0.24-1.76)			3.30 (0.01-7.94)			5.70 (3.87-8.65)		
STI(s)		**0.003**	**0.019**		0.031	0.081		0.374	0.374		0.058	0.125
Yes	19.6 (2.02-203)			1.54 (0.46-12.2)			6.43 (0.53-20.4)			7.16 (4.64-15.5)		
No	9.58 (0.77-59.3)			0.92 (0.36-4.85)			6.15 (0.87-12.1)			6.70 (4.08-10.9)		
C. trachomatis		**0.038**	0.163		0.226	0.383		0.247	0.383		0.453	0.476
Yes	21.3 (1.61-197)			1.39 (0.43-12.0)			7.11 (1.17-19.56)			6.85 (4.51-15.8)		
No	11.0 (1.04-71.3)			1.01 (0.36-6.34)			6.15 (0.53-12.7)			6.80 (4.49-11.7)		
N. gonorrhaea		0.151	0.408		0.064	0.408		0.860	0.860		0.157	0.408
Yes	25.6 (1.70-05)			2.4 (0.47-20.7)			6.15 (0.17-19.3)			8.21 (4.59-17.3)		
No	11.0 (1.09 83.1)			1.01 (0.37-6.34)			6.36 (0.70-13.5)			6.73 (4.49-11.7)		
T. vaginalis		0.072	0.116		0.139	0.164		0.128	0.164		**0.039**	0.116
Yes	32.3 (6.19-453)			3.25 (0.54-18.7)			13.5 (2.18-29.1)			11.8 (5.30-67.2)		
No	11.1 (1.13-83.7)			1.01 (0.37-6.89)			6.15 (0.53-12.8)			6.73 (4.49-11.7)		
Yeast cells		0.976	0.976		0.700	0.976		0.740	0.976		**1.0e-05**	**6.5e-05**
Yes	10.8 (0.73-121)			0.78 (0.29-11.8)			6.32 (0.01-19.2)			14.8 (5.62-29.4)		
No	12.4 (1.32-83.1)			1.09 (0.42-6.79)			6.36 (1.05-13.2)			6.38 (4.49-10.4)		
	IL-23	**P**	**P adj.**	**IL-25**	**P**	**P adj.**	**IL-31**	**P**	**P adj.**	**IL-33**	**P**	**P adj.**
BV		**2.4e-08**	**3.5e-08**		**8.4e-13**	**2.7e-12**		**3.7e-06**	**4.4e-06**		**3.1e-13**	**1.3e-12**
Positive	7.11 (0.12-15.7)			0.37 (0.08-1.34)			45.0 (26.1-97.2)			9.50 (4.24-20.9)		
Intermediate	4.50 (0.36-13.8)			0.24 (0.01-0.83)			43.9 (13.4-57.1)			4.08 (1.29-14.6)		
Negative	0.12 (0.12-5.26)			0.01 (0.01-0.24)			26.4 (17.1-52.1)			3.04 (0.73-5.85)		
STI(s)		0.128	0.128		0.104	0.185		0.314	0.354		0.238	0.309
Yes	1.06 (0.12-13.0)			0.18 (0.01-0.90)			35.3 (18.7-86.5)			5.25 (1.65-16.5)		
No	1.06 (0.12-9.08)			0.12 (0.01-0.43)			35.4 (21.0-58.9)			4.67 (1.68-10.1)		
C. trachomatis		0.265	0.383		0.358	0.435		0.476	0.476		0.368	0.435
Yes	1.24 (0.12-12.2)			0.13 (0.01-0.75)			35.3 (18.0-86.5)			4.67 (1.59-14.9)		
No	1.06 (0.12-9.14)			0.12 (0.01-0.49)			35.4 (21.0-58.9)			5.01 (1.68-10.7)		
N. gonorrhaea		0.326	0.498		0.335	0.498		0.345	0.498		0.248	0.498
Yes	1.06 (0.12-11.0)			0.13 (0.01-0.95)			36.7 (21.4-83.3)			5.25 (2.95-13.5)		
No	1.06 (0.12-9.41)			0.13 (0.01-0.49)			35.0 (20.0-63.3)			4.69 (1.56-11.3)		
T. vaginalis		0.056	0.116		**0.005**	**0.046**		0.361	0.365		0.097	0.140
Yes	7.22 (0.12-30.2)			0.49 (0.20-1.58)			53.8 (19.4-92.8)			10.1 (4.35-21.0)		
No	1.06 (0.12-9.41)			0.12 (0.01-0.46)			35.0 (20.0-62.4)			4.66 (1.68-10.7)		
Yeast cells		0.099	0.321		0.842	0.976		0.931	0.976		0.700	0.976
Yes	2.29 (0.12-12.9)			0.12 (0.01-0.60)			31.2 (21.4-71.7)			5.38 (1.04-12.3)		
No	0.65 (0.12-9.41)			0.14 (0.01-0.49)			35.6 (19.1-63.3)			4.66 (1.68-10.7)		

Continued on next page.

Table A22. Continued from previous page.

	IFN-γ	P	P adj.	sCD40L	P	P adj.	TNF-α	P	P adj.
BV		**3.6e-12**	**9.3e-12**		**2.6e-05**	**2.8e-05**		**< 2e-16**	**< 2e-16**
Positive	3.44 (1.83-8.28)			4.78 (0.07-14.8)			2.39 (0.89-8.93)		
Intermediate	2.68 (1.04-4.58)			0.90 (0.07-14.5)			1.02 (0.17-8.90)		
Negative	1.40 (0.58-2.67)			0.26 (0.07-6.41)			0.17 (0.04-0.47)		
STI(s)		0.127	0.185		0.326	0.354		**0.007**	**0.022**
Yes	2.46 (1.01-5.98)			1.44 (0.07-11.0)			1.03 (0.22-6.45)		
No	2.09 (1.04-3.84)			1.09 (0.07-7.49)			0.49 (0.11-2.92)		
C. trachomatis		0.113	0.293		0.107	0.293		0.147	0.319
Yes	2.68 (1.01-5.92)			2.36 (0.07-12.8)			1.03 (0.16-6.29)		
No	2.09 (1.02-3.87)			0.90 (0.07-7.63)			0.61 (0.13-3.04)		
N. gonorrhaea		0.596	0.646		0.596	0.646		0.137	0.408
Yes	2.06 (1.01-5.98)			1.12 (0.07-9.31)			1.03 (0.28-11.04)		
No	2.18 (1.02-4.46)			1.20 (0.07-7.95)			0.61 (0.12-3.39)		
T. vaginalis		0.060	0.116		0.365	0.365		**0.007**	**0.046**
Yes	4.28 (1.98-9.59)			9.79 (0.07-14.6)			2.01 (0.67-28.4)		
No	2.11 (1.02-4.33)			1.20 (0.07-7.95)			0.61 (0.12-3.21)		
Yeast cells		0.359	0.932		0.897	0.976		0.611	0.976
Yes	2.64 (1.03-5.26)			0.90 (0.07-11.6)			0.57 (0.09-2.90)		
No	2.11 (1.02-4.46)			1.57 (0.07-7.95)			0.69 (0.15-3.52)		

P values calculated using Kruskal-Wallis test and Wilcoxon Rank Sum test. BV, bacterial vaginosis; STI, sexually transmitted infection.

Appendix IX: Cytokine concentrations and hormonal contraception at crossover

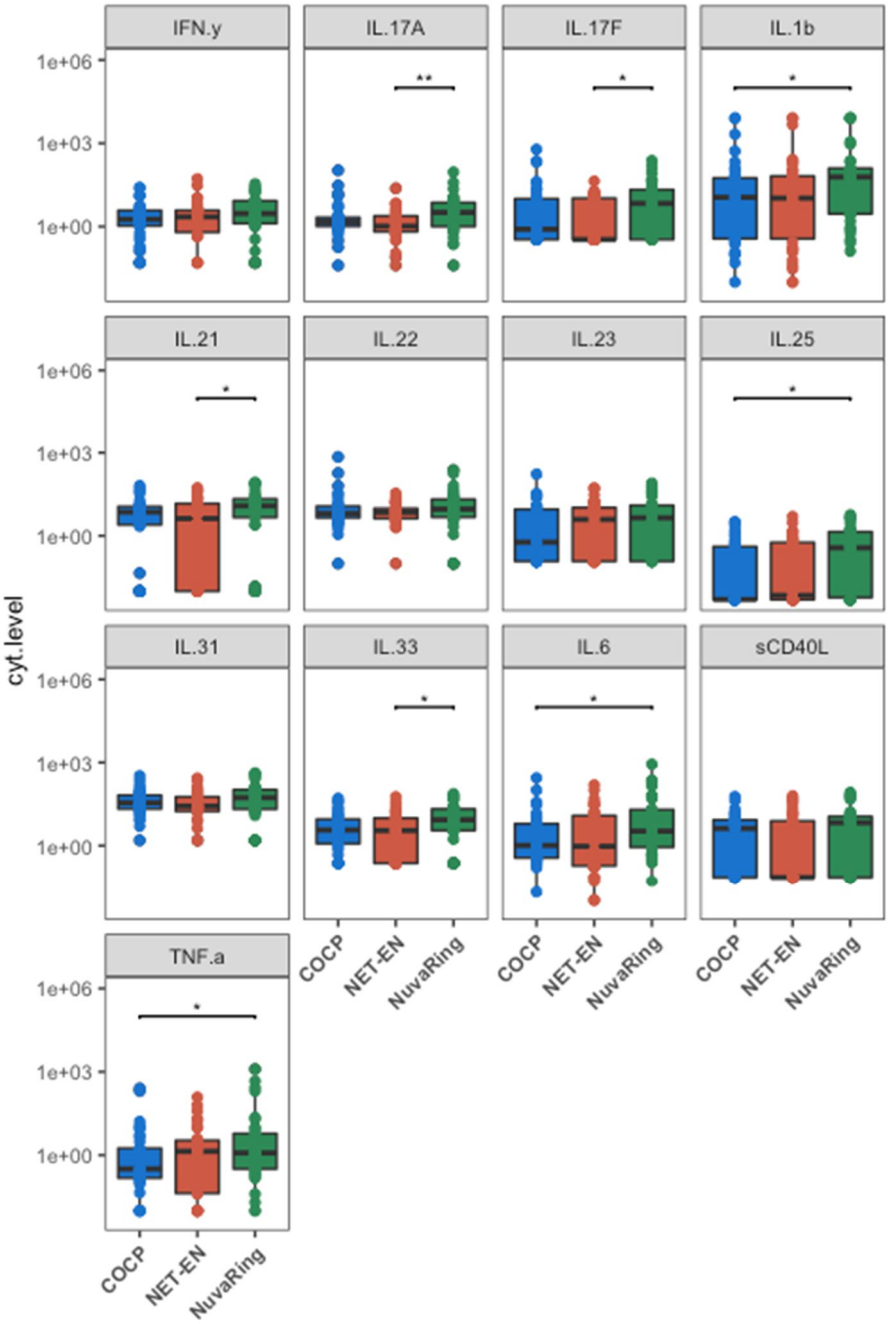


Figure A16. Cytokine levels according to study arm at crossover (intention-to-treat). Boxplots showing the levels of 13 cytokines in 105 participants stratified by study arm at crossover. P values were generated using the Wilcoxon Rank Sum test adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. Y-axis log10 transformed. ****= p<0.0001, ***= p<0.001, **= p<0.01, *= adj. p<0.05, ns=p>0.05.

Table A23. Differences in cytokine concentrations according to study arm (intention-to-treat).

	COCP	Net-En	NuvaRing	P value	Adj. P value	COCP vs. Net-En		COCP vs. NuvaRing		Net-En vs. NuvaRing	
	(median (IQR))	(median (IQR))	(median (IQR))			P	Adj. P	P	Adj. P	P	Adj. P
IL-1β	11.3 (0.37-56.2)	10.7 (0.42-66.0)	61.1 (2.88-126)	0.067	0.161	0.972	0.972	**0.033**	0.208	0.061	0.208
IL-6	1.01 (0.36-6.15)	1.01 (0.19-12.2)	3.43 (0.90-20.0)	0.074	0.161	0.945	0.972	**0.031**	0.208	0.077	0.232
IL-17A	1.48 (1.02-2.14)	1.05 (0.65-2.42)	3.24 (1.01-7.33)	**0.029**	0.161	0.225	0.401	0.111	0.298	**0.010**	0.208
IL-17F	0.80 (0.34-10.1)	0.34 (0.34-10.5)	6.83 (0.34-21.4)	0.090	0.168	0.659	0.779	0.118	0.298	**0.034**	0.208
IL-21	7.22 (2.49-11.4)	4.51 (0.01-14.7)	12.1 (4.66-21.6)	0.060	0.161	0.519	0.675	0.051	0.208	**0.040**	0.208
IL-22	6.43 (4.49-11.7)	7.00 (4.19-9.77)	9.23 (4.67-20.9)	0.363	0.393	0.854	0.925	0.284	0.482	0.173	0.375
IL-23	0.59 (0.12-9.07)	3.99 (0.12-10.5)	4.57 (0.12-12.6)	0.408	0.408	0.736	0.845	0.185	0.379	0.387	0.539
IL-25	0.01 (0.01-0.41)	0.01 (0.01-0.58)	0.37 (0.01-1.41)	0.067	0.161	0.819	0.913	**0.038**	0.208	0.060	0.208
IL-31	34.5 (21.0-65.8)	26.6 (17.3-57.8)	54.1 (20.6-104)	0.272	0.371	0.372	0.538	0.360	0.538	0.131	0.301
IL-33	3.65 (1.18-9.01)	3.49 (0.23-9.83)	8.52 (3.52-21.1)	0.066	0.161	0.606	0.739	0.064	0.208	**0.035**	0.208
IFN-γ	1.81 (1.06-3.83)	2.25 (0.51-3.84)	2.95 (1.29-8.50)	0.347	0.393	0.949	0.972	0.195	0.380	0.226	0.401
sCD40L	4.17 (0.07-8.46)	0.07 (0.07-7.79)	6.70 (0.07-11.3)	0.286	0.371	0.447	0.601	0.370	0.538	0.122	0.298
TNF-α	0.32 (0.15-1.78)	1.38 (0.04-3.42)	1.21 (0.32-6.03)	0.177	0.287	0.583	0.734	**0.046**	0.208	0.344	0.538

P values calculated using the Kruskal-Wallis and Wilcoxon Rank Sum tests adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. The data is from 105 participants at crossover. The data is from 105 participants at crossover. COCP: n=37; Net-En: n=34; NuvaRing: n=34. Adj., adjusted; IQR, interquartile range.

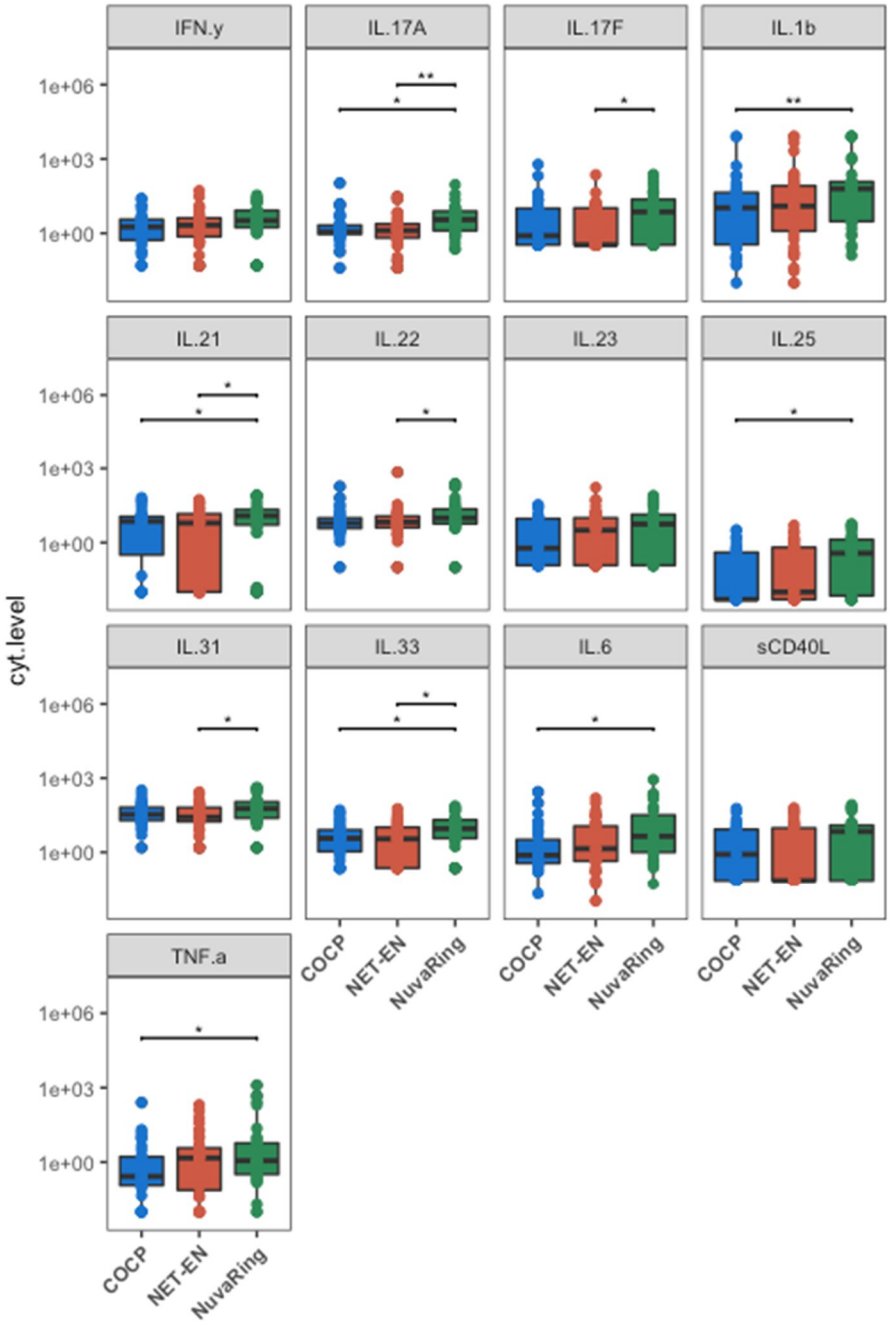


Figure A17. Cytokine levels according to hormonal contraception at crossover (according to protocol). Boxplots showing the levels of 13 cytokines in 105 participants stratified by hormonal contraception at crossover. P values were generated using the Wilcoxon Rank Sum test adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. Y-axis log10 transformed. ****= p<0.0001, ***= p<0.001, **= p<0.01, *= adj. p<0.05, ns=p>0.05.

Table A24. Differences in cytokine concentrations according to hormonal contraception (per protocol).

	COCP (median (IQR))	Net-En (median (IQR))	NuvaRing (median (IQR))	P value	Adj. P value	COCP vs. Net-En		COCP vs. NuvaRing		Net-En vs. NuvaRing	
						P	Adj. P	P	Adj. P	P	Adj. P
IL-1β	10.63 (0.35-44.6)	12.4 (1.30-84.3)	62.3 (3.03-121)	**0.034**	0.108	0.471	0.613	**0.001**	0.103	0.078	0.180
IL-6	0.76 (0.36-3.50)	1.40 (0.44-11.5)	4.44 (1.03-35.3)	**0.041**	0.108	0.613	0.445	**0.013**	0.103	0.083	0.180
IL-17A	1.14 (0.93-2.12)	1.29 (0.66-2.40)	3.53 (1.28-7.84)	**0.011**	0.108	0.516	0.637	**0.024**	0.103	**0.005**	0.103
IL-17F	0.80 (0.34-10.1)	0.34 (0.34-10.4)	7.29 (0.34-23.8)	0.091	0.131	0.646	0.699	0.121	0.236	**0.033**	0.118
IL-21	7.22 (1.13-11.3)	6.15 (0.01-14.6)	12.1 (5.25-21.5)	**0.030**	0.108	0.702	0.740	**0.020**	0.103	**0.022**	0.103
IL-22	6.06 (3.77-10.0)	6.70 (4.02-11.8)	9.80 (5.78-22.6)	0.083	0.131	0.897	0.897	0.058	0.151	**0.046**	0.137
IL-23	0.59 (0.12-9.24)	3.15 (0.12-10.1)	5.57 (0.12-13.7)	0.330	0.330	0.752	0.772	0.134	0.238	0.309	0.464
IL-25	0.01 (0.01-0.40)	0.01 (0.01-0.64)	0.37 (0.01-1.33)	0.056	0.121	0.448	0.613	**0.021**	0.103	0.094	0.193
IL-31	33.9 (19.5-65.8)	26.1 (17.4-65.0)	58.9 (24.2-110)	0.102	0.133	0.461	0.613	0.134	0.238	**0.043**	0.137
IL-33	3.65 (1.08-8.15)	3.48 (0.23-10.3)	9.00 (3.65-20.6)	**0.030**	0.108	0.623	0.694	**0.030**	0.115	**0.017**	0.103
IFN-γ	1.81 (0.54-3.65)	2.11 (0.77-4.15)	3.26 (1.71-8.39)	0.175	0.190	0.603	0.692	0.079	0.180	0.161	0.261
sCD40L	0.83 (0.07-8.46)	0.07 (0.07-9.79)	6.96 (0.07-12.4)	0.123	0.146	0.555	0.656	0.143	0.243	0.052	0.144
TNF-α	0.27 (0.12-1.66)	1.46 (0.08-3.71)	1.14 (0.32-5.84)	0.076	0.131	0.182	0.283	**0.016**	0.103	0.523	0.637

P values calculated using the Kruskal-Wallis and Wilcoxon Rank Sum tests adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. The data is from 105 participants at crossover. COCP: n=35; Net-En: n=39; NuvaRing: n=31. Adj., adjusted; IQR, interquartile range.