



Inflammation and immune responses within the female reproductive tract

Cindy Adapen

► To cite this version:

Cindy Adapen. Inflammation and immune responses within the female reproductive tract. Immunology. Université Paris-Saclay, 2021. English. NNT : 2021UPASQ046 . tel-03602831

HAL Id: tel-03602831

<https://theses.hal.science/tel-03602831>

Submitted on 9 Mar 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Inflammation et réponses immunitaires au sein du tractus reproducteur féminin

Inflammation and immune responses within the female reproductive tract

Thèse de doctorat de l'université Paris-Saclay

École doctorale n° 569, Innovation thérapeutique du fondamental à l'appliqué (ITFA)

Spécialité de doctorat : Immunologie

Graduate School : Santé et médicaments. Référent : Faculté de pharmacie

Thèse préparée dans l'unité de recherche **ImVA-HB (Université Paris-Saclay, Inserm, CEA)**
sous la direction de **Elisabeth Menu**, directrice de recherche Inserm et cheffe de groupe IP

Thèse soutenue à Paris-Saclay, le 07 décembre 2021, par

Cindy ADAPEN

Composition du Jury

Constance DELAUGERRE

Professeure Université de Paris et praticienne hospitalière
Service de Virologie, Hôpital Saint-Louis, Paris

Présidente

Kristina BROLIDEN

Professeur et cheffe de groupe
Département de Médecine Solna, Division des maladies infectieuses
Karolinska Institut, Stockholm

Rapporteuse &
Examinatrice

Frank FOLLMANN

Directeur du département d'immunologie des maladies infectieuses
Statens Serum Institut, Copenhague

Rapporteur &
Examineur

Luc DE CHAISEMARTIN

Maître de conférence Université Paris Saclay et praticien hospitalier
Service d'hématologie immunologie, Hôpital Bichat, Paris

Examineur

Agathe SUBTIL

Directrice de recherche CNRS (DR1) et cheffe de groupe IP
Département de biologie cellulaire et infection
Institut Pasteur, Paris

Examinatrice

Elisabeth MENU

Directrice de recherche INSERM (DR2) et cheffe de groupe IP
Université Paris Saclay/Inserm/CEA/IDMIT/ImVA-HB
Département de Virologie, Institut Pasteur, Paris

Directrice de
thèse

Acknowledgments

J'aimerais remercier toutes les personnes impliquées de près comme de loin dans mon projet de thèse. Il y a évidemment mes collègues mais aussi mes amis, ma famille et belle-famille. Je n'aurais pas pu continuer et terminer ma thèse sans leur soutien constant et leur conseil. La fin de la thèse a été éprouvante et légèrement rallongée (pratiquement 5 ans de thèse quand même !), donc merci d'avoir été là.

Tout d'abord, je voudrais remercier le directeur de l'unité Roger Legrand pour son aide et ses encouragements tout au long de la thèse. Merci d'avoir cru en moi et de m'avoir permis de faire cette thèse.

Ensuite, je voudrais remercier les membres de mon jury, Kristina Broliden, Frank Follmann, Constance Delaugerre, Luc de Chaisemartin et Agathe Subtil d'avoir accepté d'évaluer mon manuscrit de thèse et de m'avoir conseillé.

Merci à Hervé Jacquier pour son aide dans l'établissement du score de Nugent chez le macaque.

Un grand merci à tous les membres d'IDMIT incluant les équipes de recherche mais aussi les plateformes L2i, L3i, LFC, HSS, ASW et IBI qui m'ont accompagnée et avec qui j'ai passé des moments inoubliables. Je m'excuse si j'ai oublié quelqu'un mais merci aux « vieux thésards » Jean-Louis, Candie, Sixtine, Laura, Julien, André, Thibault et aux plus jeunes Margaux, Mathieu, Yanis, Elodie P, Noémie, Florian, Justina. Merci également à tous ceux qui ont fait que IDMIT soit un super lieu de travail comme Fanny, Laetitia, Marco, Ernesto, Maxence, Yann, Sébastien J, Maxime, Julie, Sylvie, Keltouma, Mathilde, Elodie G, Isabelle, Sabine, Martine, Fabienne, Mymy, Julien D, Mario, Jérôme, Wesley, Anne-Sophie G, Brice, Jean-Marie, Quentin, Benoît, Nina, Oscar, Antoine, Raphaël, Sébastien, Delphine, Vanessa, Frédéric D, Pauline, Anne-Sophie B, Mariangela, Nabilla, Nathalie, Catherine. Merci à tous d'avoir simplement été là. Merci à mon super garant technique Frédéric M !

Merci beaucoup à Romain, le super chef de projet qui m'a suivi du début à la fin. Merci d'avoir été toujours disponible pour discuter des analyses, de la mise au point des études et de m'avoir appris à utiliser le Fortessa ☺

Je voudrais également remercier l'équipe de Life&Soft pour leur aide et leur conseil.

Merci à l'équipe d'animalliance ! et plus particulièrement à Marjo et Thierry. Merci pour votre constante bonne humeur et pour les super fous rire en animalerie !

Acknowledgments

Merci à Mö et Stéphane avec qui j'ai beaucoup ri, mangé des ramens et beaucoup de glaces ! Merci pour vos conseils, votre amitié et tous les moments passés ensemble au labo et en dehors.

J'aimerais maintenant remercier la meilleure équipe ! Je pense que je n'aurais pas pu rêver mieux comme environnement de travail que cette superbe équipe MISTIC. Merci à Claude, Marie-Thérèse, Louis, Natalia, Camille, Fahd de m'avoir aidée, supportée, conseillée tout au long de la thèse. Merci à ma géniale directrice de thèse, Elisabeth, pour avoir cru en moi, m'avoir poussée à faire des choses que je ne pensais jamais faire (ma tête sera à jamais sur internet grâce à toi...), de m'avoir encouragée, soutenue, guidée. Tu as toujours été disponible si j'en avais besoin et j'ai vraiment beaucoup appris grâce à toi, merci beaucoup.

Il n'y a pas que l'environnement de travail qui importe car la thèse te suit partout ! Même au cours d'un repas de famille ou d'une soirée entre amis. J'aimerais donc remercier tous mes amis pour les séances de fous rires, de films d'horreur, jeux de sociétés et de restaurants à gogo. Emilie, Vincent, Taima, Julie C, Thomas, Pierre, Emilie S, Julie B, Yohan et Perrine merci de m'avoir supportée tout ce temps 😊

Merci également à ma famille, ma maman, ma sœur, mon frère, mon beau-frère et ma belle-sœur. Merci également aux petits monstres Chloé, Malan, Léna, Timéo et Noa. Merci à toi, Papa, pour m'avoir encouragée et poussée à ne pas abandonner malgré les difficultés.

Je souhaiterais remercier ma belle-mère, mon beau-père ainsi que Jérémy, Dan, Amélie et Guillaume pour m'avoir soutenu et encouragé.

Et pour finir, j'aimerais te remercier, Antoine, pour m'avoir consolée, remotivée et d'avoir toujours été à mes côtés. Je n'aurais pas réussi sans toi. J'ai hâte de tourner cette page, pour enfin commencer cette nouvelle aventure ensemble.

Table of content

Acknowledgments	1
Table of content	3
List of figures	8
List of tables	10
List of abbreviations	11
Preface	13
Introduction	15
Chapter I: Sexually transmitted infections (STIs)	16
I. General data	16
II. Chlamydia trachomatis (CT)	18
a. Epidemiology in Europe and France	18
b. Physiopathology of the disease & CT feature	20
i. Physiopathology	20
ii. <i>Chlamydia trachomatis</i> description and growth cycle	22
c. Immune response induced by CT infection	24
i. Innate immune response to CT infection	24
1. Human <i>in vitro</i> and <i>in vivo</i> study:	24
2. Animal models:	26
ii. Adaptative immune response	32
1. CT infection in Human:	32
2. Experimental infections in animal:	33
3. Contribution of vaccine studies:	34
III. CT/HIV-1 co-infections	36
a. Epidemiology	36
b. Mechanisms involved in increasing the risk of HIV-1 acquisition (based on <i>in vitro</i> studies)	37
IV. STI gateway	39
Chapter II: The Female reproductive Tract (FRT)	40
I. Structure and composition	40
II. Immune cell composition in the FRT.	42
a. Innate immunity	42
i. General points	42

ii.	Neutrophils	42
1.	Hematopoiesis	43
2.	Activation status and role in immunity.....	45
3.	Genital neutrophils.....	48
b.	Adaptive immunity.....	50
i.	T cell responses.....	50
ii.	B cell responses.....	51
III.	Menstrual cycle	51
a.	Regulation of the immune cell composition by the menstrual cycle	53
b.	Regulation of antimicrobial peptides by the menstrual cycle.....	53
c.	Regulation of cytokines and chemokines by the menstrual cycle.....	54
d.	Regulation of antibody production by the menstrual cycle	55
e.	Impact of external hormonal stimulation.....	56
f.	Hormonal cycle and STI susceptibility.....	56
	Chapter III: Vaginal microbiota	58
I.	General information about the microbiota	58
II.	Vaginal microbiota composition (eubiotic vs dysbiotic)	59
a.	<i>Lactobacillus spp.</i>	61
b.	<i>G. vaginalis</i>	63
III.	Factors impacting the vaginal microbiota.....	64
a.	Hormones.....	65
b.	Contraceptive use.....	67
c.	Ethnic status	67
d.	Sexual behaviors, hygiene & STI.....	68
IV.	Modulation of the immune system by the vaginal microbiota	69
V.	Impact of the microbiota on STI.....	73
a.	Microbiota & <i>Chlamydia trachomatis</i>	73
b.	Microbiota & HIV-1	75
VI.	Relationship between the microbiota & neutrophils.....	78
	Chapter IV: Pre-clinical animal model	82
I.	Cynomolgus macaques.....	82
II.	Immune system in macaques	83
III.	Female Reproductive Tract (FRT) in macaques	84
a.	Menstrual cycle	84
b.	Immune cell distribution.....	85
c.	Vaginal microbiota.....	85
d.	STI infections in cynomolgus macaques.....	87

i. Simian Immunodeficiency Virus (SIV) infection.....	87
ii. CT infection in cynomolgus macaques	87
Hypothesis and Objectives	93
Hypothesis.....	94
Objectives	95
Materials and Methods	96
I. <i>Lactobacillus spp.</i> enrichment of the cynomolgus macaque vaginal microbiota.....	97
II. <i>In vitro</i> study: Effect of <i>L. crispatus</i> and <i>G. vaginalis</i> on CT induced inflammation. Consequences on neutrophil survival and phenotype as well as on HIV-1 co-infection.....	103
Results	105
First article: Dynamics of vaginal and rectal microbiota over several menstrual cycles in female cynomolgus macaques	106
I. Introduction and objectives.....	106
II. Data summary.....	107
III. Article	108
Second article: Local innate markers and vaginal microbiota composition are influenced by hormonal cycle phases	109
I. Introduction and objectives.....	109
II. Data summary.....	110
III. Article	112
Strategies tested <i>in vivo</i> for the <i>Lactobacillus spp.</i> enrichment of the cynomolgus macaque vaginal microbiota	165
I. Introduction and objectives.....	165
II. Results.....	167
a. Single inoculation of <i>L. crispatus</i> capsule	167
b. Metronidazole pretreatment followed by <i>L. crispatus</i> capsule inoculation.....	169
c. Sucrose intravaginal treatment.....	173
d. Repeated metronidazole treatments before <i>L. crispatus</i> inoculations.....	174
e. Repeated treatments with azithromycin and <i>L. crispatus</i> gel.....	181
III. Data summary.....	183
Third article: Influence of vaginal microbiota composition on cervicovaginal and systemic inflammation induced by <i>Chlamydia trachomatis</i> infection	185
I. Introduction and objectives.....	185
II. Data summary.....	187
III. Article	189
<i>In vitro</i> study: Effect of <i>L. crispatus</i> and <i>G. vaginalis</i> on CT induced inflammation. Consequences on neutrophil function and phenotype as well as HIV-1 co-infection	251
I. Introduction and objectives.....	251

II. Results.....	253
a. <i>Chlamydia trachomatis</i> infection of epithelial cells.....	253
b. <i>L. crispatus</i> and <i>G. vaginalis</i> exposition of A2EN epithelial cells	255
c. <i>L. crispatus</i> and <i>G. vaginalis</i> exposition on CT infected epithelial cells.....	258
d. Effect of supernatants of <i>L. crispatus</i> or <i>G. vaginalis</i> in contact of CT infected A2EN cells on neutrophil phenotype and survival	261
e. Effect of supernatants of <i>L. crispatus</i> or <i>G. vaginalis</i> in contact of CT infected A2EN cells on HIV-1 infection.....	263
III. Data summary.....	264
Discussion	266
General discussion	267
I. Establishment of a cynomolgus macaque model to study STI acquisition	268
a. Vaginal microbiota composition	269
b. Impact of menstrual phases on immune markers	271
c. Study perspectives	271
II. Vaginal microbiota dysbiosis in Human vs naturally diverse vaginal microbiota in macaques.....	274
a. Association between local inflammation and vaginal microbiota	274
b. Impact of <i>L. crispatus</i> presence on the local environment	274
c. Study perspectives	275
III. Effect of vaginal microbiota composition on CT infection and inflammation	276
a. Antibiotic effect on CT infection and immunity.....	276
b. Effect of <i>L. crispatus</i> enrichment on immune responses	277
c. Do human and macaque <i>Lactobacillus spp</i> have similar anti-inflammatory effect?....	278
d. Study perspectives	279
IV. Modification of the inflammation induced by CT and its effect on neutrophil phenotype/survival and HIV-1 co-infection.....	280
a. Inflammation: cytokine production and neutrophil activation and survival	280
b. Impact of CT on HIV-1 susceptibility	281
c. Strength and limitation of the <i>in vitro</i> model.....	281
Conclusion and Perspectives	283
Conclusion.....	284
Perspectives.....	286
Bibliography	287
Appendix	303
French detailed summary	304
I. Introduction.....	304
II. Hypothèses et Objectifs.....	308

III.	Résultats.....	309
IV.	Conclusion et Perspectives	315

List of figures

Figure 1: CT infections by transmission, category and gender.....	19
Figure 2: CT cases per 100 000 population categorized by age groups.....	19
Figure 3: CT growth cycle.....	24
Figure 4: Neutrophil role during CT infection.	28
Figure 5: Innate immune response induce by CT infection in the FRT.	32
Figure 6: Adaptive immune response induced by CT infection in the FRT.	35
Figure 7: Female reproductive tract epithelium and immune cell composition.	41
Figure 8: Bone marrow neutrophil subpopulations in Human.	45
Figure 9: Neutrophils	46
Figure 10: Cytokine expression by neutrophils.....	48
Figure 11: Female hormone cycle.....	52
Figure 12: Factors including immune cells and soluble factors influenced by menstrual cycle in Human.	55
Figure 13: Microbiota composition at the phylum level in the different mucosal human compartments.....	58
Figure 14: Vaginal microbiota composition and impact on STI acquisition.....	61
Figure 15: <i>Lactobacillus spp.</i> growth cycle.....	66
Figure 16: Diagram representing a wide array of factors influencing vaginal microbiota composition.....	69
Figure 17: Eubiotic vaginal microbiota.	72
Figure 18: Dysbiotic vaginal microbiota.....	73
Figure 19: Gut microbiota regulation of neutrophil production and activation.	80
Figure 20: Cynomolgus macaque.	83
Figure 21: CT detection in cervicovaginal swabs by PCR or culture.	90
Figure 22: MOMP IgG and IgA serum level.....	90
Figure 23: Heat map representing the fold change expression of cytokines and chemokines in cervicovaginal fluids upon infection.....	91
Figure 24: Heat map representing the fold change expression of cytokines and chemokines in peripheral blood plasma upon infection.	92
Figure 25: Futur aims.	166
Figure 26: Nugent score and vaginal pH of three female cynomolgus macaques.....	168
Figure 27: Nugent scores and vaginal pH of cynomolgus macaques.....	170
Figure 28: Isolated colonies from the vaginal microbiota of three cynomolgus macaques.	170
Figure 29: Nugent scores and vaginal pH of cynomolgus macaques after metronidazole treatment.	172
Figure 30: Nugent scores and vaginal pH in three cynomolgus macaques after Depo-provera treatment.....	172
Figure 31: Nugent scores and vaginal pH of cynomolgus macaques after sucrose treatment.	173
Figure 32: Nugent scores and vaginal pH of cynomolgus macaques.....	177
Figure 33: Vaginal microbiota composition at baseline, after metronidazole treatment and <i>L. crispatus</i> inoculations in five cynomolgus macaques.....	179
Figure 34: Cytokine and chemokine concentrations in cervicovaginal fluids before and after metronidazole and <i>L. crispatus</i> inoculations.	180
Figure 35: Neutrophil accumulation within the vagina of cynomolgus macaques.....	180

Figure 36: Nugent score and vaginal pH value after azithromycin treatment and <i>L. crispatus</i> gel inoculations.	182
Figure 37: Summary of the strategies tested during this thesis in cynomolgus macaques to induce <i>L. crispatus</i> implantation and growth in the vaginal microbiota.	184
Figure 38: Experimental design.	252
Figure 39: CT infection of A2EN cells.	255
Figure 40: <i>G. vaginalis</i> and <i>L. crispatus</i> interaction with A2EN cells.	257
Figure 41: Interaction of <i>L. crispatus</i> or <i>G. vaginalis</i> with CT infected A2EN cells.	260
Figure 42: Thesis objectives.	268
Figure 43: Thesis main results.	270
Figure 44: Thesis main perspectives.	273

List of tables

Table 1: Data on the numbers of infection cases and most infected individuals in Europe in 2018.	18
Table 2: Evolution of CT cases in France between 2013 and 2017.....	20
Table 3: Major roles and producers of main pro and anti-inflammatory cytokines.....	31
Table 4: Major roles and producers of the growth factors GM-CSF, G-CSF and VEGF.	31
Table 5: Major roles and producers of chemokines.....	31
Table 6 : Antibody panel.....	101
Table 7: <i>Lactobacillus spp.</i> species isolated in the vaginal microbiota of five cynomolgus macaques.....	171

List of abbreviations

AMP	Antimicrobial peptide
BM	Bone Marrow
BV	Bacterial Vaginosis
BVAB	Bacterial Vaginosis Associated Bacterium
CCL	C-C motif ligand
CCVR	Combined Contraceptive Vaginal Ring
COC	Combined Oral Contraceptives
CST	Community State Type
CT	<i>Chlamydia trachomatis</i>
CVL	Cervicovaginal Lavage
CVM	Cervicovaginal Mucus
CXCL	C-X-C motif Ligand
CXCR	C-X-C motif Receptor
DC	Dendritic Cell
DMPA	Depot Medroxyprogesterone Acetate
EB	Elementary Body
ECDC	European Center for Disease Center
FRT	Female Reproductive Tract
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Granulocyte Monocyte Progenitor
HBD	Human Beta Defensin
HIV-1	Human Immunodeficiency Virus type-1
HNP	Human Neutrophil Peptide
HPV	Human Papillomavirus
HSP	Heat Shock Protein
HSPC	Hematopoietic Stem and Progenitor Cell
HSV-2	Herpes Simplex Virus 2
IDO	Indoleamine Dioxygenase
IFN	Interferon
IL-	Interleukin
LC	Langerhans cell
LNG-IUS	Levonorgestrel releasing Intrauterine System
LPS	Lipopolysaccharide
LVG	Lymphogranuloma Venerum
MMP	Matrix Metalloproteinase
MOMP	Major Outer Membrane Protein
MPA	Medroxyprogesterone acetate
MPO	Myeloperoxidase

MRS	Mann Rogosa and Sharpe
MSM	Men having Sex with Men
NET	Neutrophil Extracellular Trap
Net-EN	Norethisterone Enanthate
NHP	Non-Human Primate
NK cell	Natural Killer cell
NOD	Nucleotide binding oligomerisation domain containing protein
PD-L1	Programmed Death Ligand 1
pIgR	Polymeric Ig Receptor
PrEP	Pre-Exposure Prophylaxis
RB	Reticulate Body
RM	Rhesus Macaque
ROS	Reactive Oxygen Species
SCFA	Short Chain Fatty Acid
SIV	Simian Immunodeficiency Virus
SLPI	Secretory Leukocyte Protease Inhibitor
STI	Sexually Transmitted Infection
SvD	Serovar D
T3SS	Type III Secretion System
TFV	Tenofovir
TGF	Transforming Growth Factor
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
Treg	Regulatory T cell
VEGF	Vascular Endothelial Growth Factor
VMT	Vaginal Microbiota Transplantation

Preface

Sexually transmitted infections (STI) are a major global health issue. This statement includes HIV-1 but also curable bacterial infections, such as chlamydia. Bacterial STI are not easily detected in an infected patient since there are often asymptomatic or induce mild symptoms. Unfortunately, the absence of symptoms is not associated with less pathogenicity since an untreated infection can migrate to the upper genital tract leading to severe pathologies. *Chlamydia trachomatis* infection is highly frequent in young women. The female reproductive tract (FRT) mucosa is the first line of defence against pathogens, however, the local microenvironment is weakly studied. So far, we know that the FRT is composed of a wide array of factors, but few informations are known about their interactions with each other and their impact on local inflammation. Inflammation is usually beneficial and essential for pathogen eradication, however, a sustained inflammation will favour the acquisition of STI. Indeed, it can increase the recruitment of target cells, induce epithelium breaches, disrupt the mucus layer or affect vaginal microbiota composition facilitating the passage of pathogens. Recent discoveries highlight a central role of the vaginal microbiota in the regulation of FRT inflammation and susceptibility to STI.

During my PhD, I have studied the interaction of inflammatory markers within the FRT and their influence on the local and systemic inflammation at basal state but also during a Chlamydia infection. First, I introduce essential topics of the project including Chlamydia infection, FRT microenvironment, vaginal microbiota and the animal model used throughout this study. Secondly, I present the hypothesis and objectives of the work follow by the main results. The results include three articles and additional data. The first article on the vaginal and rectal microbiota composition of cynomolgus macaques has already been published. The second article on the evolution of inflammatory markers in the FRT has recently been submitted to an international journal. The third one on the impact of the vaginal microbiota composition on Chlamydia infection and inflammation is in preparation and analysis are still

ongoing. Additional data include: (1) the development of a cynomolgus macaque model with a vaginal microbiota enriched with *L. crispatus* and (2) *in vitro* analysis of the impact of *L. crispatus* and *G. vaginalis* on the inflammation induced by CT and the consequences on neutrophil phenotype/survival but also on HIV-1 co-infection. The final part includes a global discussion of my PhD work as well as a general conclusion and perspectives.

Introduction

Chapter I: Sexually transmitted infections (STIs)

I. General data

Every day, more than a million people get infected by a sexually transmitted infection (STI) [1]. Indeed, more than thirty bacteria, viruses and parasites can be transmitted through sexual contacts. The main pathogens linked to STIs are *Treponema pallidum* (syphilis), *Neisseria gonorrhoeae*, *Chlamydia trachomatis* (CT), *Trichomonas vaginalis*, hepatitis B virus (HBV), herpes simplex virus (HSV2), type 1 human immunodeficiency virus (HIV-1) and human Papillomavirus (HPV). The World Health Organization (WHO) estimates that every year, 357 million people catch one of the following curable STI: chlamydia, gonorrhea, syphilis or trichomonas. In most of the cases, STIs are asymptomatic or with mild symptoms which are not specific of one STI. The usual symptoms for women are vaginal discharge, genital ulceration and stomach pains. In some cases, STIs can have severe consequences, including infertility or mother-to-child transmission. Moreover, by inducing inflammation, several STIs can increase the susceptibility to another infection. The best way to avoid STI acquisition is to use men or women condoms but also to educate the young population. Several studies were conducted to understand factors associated with STI acquisition: these factors include knowledge, risk perception, peer norms for condom use, gender and economic inequality. Adolescents who feel confident in using condoms, who are not afraid to negotiate condom use with their partners, have lower rates of STI acquisition [2]. The global incidence of chlamydia, gonorrhea and trichomonas infections varies according to the country income classification. Indeed, 56% of these infections are observed in upper-middle income, 23% in lower-middle income, 12% in low income and 9% in high-income countries [3]. Furthermore, STI acquisition has been associated with gender-based violence due to several facts including stigma and stereotyping.

Up to now, there are only two vaccines available and efficient against STIs: against hepatitis B and HPV. Microbicide gel (Tenofovir) and pre-exposure prophylaxis (PrEP) treatment can be used as preventive therapy to avoid HIV-1 transmission. Antiviral treatments are used for HSV2 and HIV-1 to slow down the disease without eradicating the virus

Nowadays there are four STIs which can be cured using antibiotics: chlamydia, gonorrhea, syphilis and trichomonas. However, multi-resistance strains has been seen for *N. gonorrhoeae*.

In the 80s, thanks to HIV-1 prevention strategy, the incidence of several STIs decreased in Europe. However, at the end of the 90s, there was an increase of STI acquisition with also the reappearance of some STIs that were almost eradicated, like the syphilis [4]. Moreover, a recent study performed in men who have sex with men (MSM) in Germany have determined a highest prevalence of STI in HIV negative PrEP users (40.3%) followed by HIV+ (30.8%) and HIV- who do not use PrEP (25%) [5]. STI acquisition represents a major public health issue.

Among women aged 15 to 49 years old, the estimated global prevalence of several STIs based on data from 2005 to 2012 was the following: Chlamydia 4.2%, Gonorrhea 0.8%, trichomonas 0.6% and syphilis 0.48%. L. Newman et al. made a global estimation of 131 million new cases of chlamydia, 78 million of gonorrhea, 143 million of trichomoniasis and 6 million of syphilis in women and men aged 15-49 years in 2012 [3]. In an Australian cohort, 877 women aged from 16 to 25 years old were tested for CT infection. The incidence rate was 4.4 per 100 person-years with a cumulative risk of reinfection of 22.3 per 100 person-years [6]. This paper highlights another problem of STI acquisition: a previous acquisition of an STI is not associated to protection when re-exposed to the same pathogen, on the contrary individuals that have acquired an STI are more at risk to be reinfected.

In 2017, there is an estimation of 534 063 individuals infected in Europe by CT including Lymphogranuloma venerum (LGV), *N. gonorrhea* or *T. pallidum* (Table 1). Depending of the STI, the population affected varies. Indeed, chlamydia infection is predominantly observed in young heterosexuals adult women whereas LGV infection is mainly acquired by MSM [7].

STI	Cases of infection	Most affected individuals
Chlamydia	406,406 in 26 countries	50% of young adult women & heterosexuals
Lymphogranuloma venerum (disease induced by Chlamydia)	2,389 in 22 countries	Men who have sex with men. 59% were HIV positive
Gonorrhea	100, 673 in 28 countries	48% of men who have sex with men
Syphilis	33,927 in 29 countries	69% of men who have sex with men

Table 1: Data on the numbers of infection cases and most infected individuals in Europe in 2018. Data extracted from the European Center for Disease Center (ECDC) [7].

II. Chlamydia trachomatis (CT)

a. Epidemiology in Europe and France

“Genital CT infection is the leading STI in Europe” according to the European Center for Disease Control (ECDC). In 2018, in 26 countries in Europe, the rate of CT infection was 146 cases per 100 000 persons with 176 096 cases reported in men compared to 228 306 cases among women (data on gender is on 99.5% of reported cases). Around 50% of CT infections are observed in young heterosexual women and 34% in heterosexual men [Fig 1] (data on transmission were obtained only on 47% of reported cases). Moreover, the biggest proportion of cases was observed among 20-24 year old (39% of cases), before the age group 25-34 years old (28%). Women in the age group 20-24 years old had the highest rates (1 373 cases per 100 000 population) followed by the age group 15-19 years (1052 per 100 000 population) [Fig 2]. Young women between 15-24 years that are sexually active have the highest risk of CT acquisition [7].

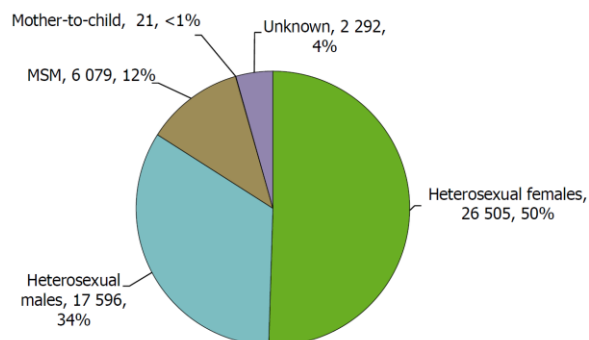


Figure 1: CT infections by transmission, category and gender.

Data of 2018 from Greece, Hungary, Lithuania, the Netherlands, Portugal, Romania, Slovakia and Sweden [7].

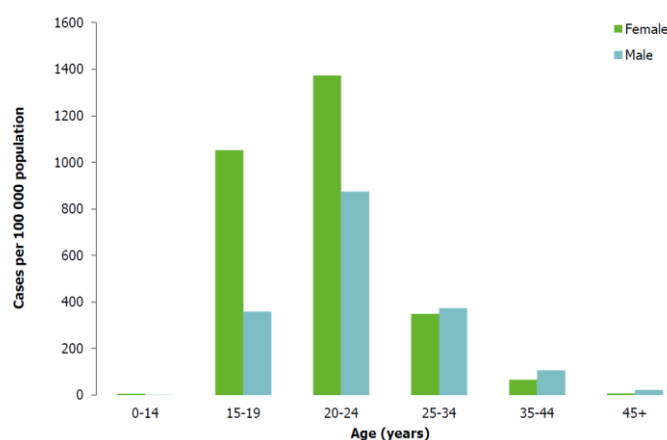


Figure 2: CT cases per 100 000 population categorized by age groups.

Country reports from Bulgaria, Croatia, Cyprus, Denmark, Estonia, Finland, Hungary, Iceland, Ireland, Latvia, Lithuania, Luxembourg, Malta, Norway, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden and the United Kingdom [7].

In the NatChla study conducted between 2005 and 2006, 4 957 people participated in a chlamydia home sampling program in France. A CT incidence of 1.6% in women and 1.4% in men was observed, however the incidence rate was higher in women and men aged 18 to 29 years old (3.2% and 2.5% respectively) [8].

In France, CT infection cases were relatively stable between 2014 and 2016 but there was an increase of CT infected individuals in 2017 with 17 672 individuals reported infected with CT [Table 2]. In the “Santé publique” website, 2.5 million people were diagnosed with CT infection in 2019. In France, women aged of more than 15 years old are at higher risk for CT acquisition with 148/100 000 in men vs 329/100 000 in women where young women from 15 to 24 years old are the most at risk (781/100 000 individuals) confirming the observation

made by the ECDC [9]. However, it is important to notice that this increased number of new CT cases might be in part due to an increase of CT screening [9]. Screening was also observed to be three times more important in women than men in 2019. However, “Santé publique France” found a global increase of 20% of CT screening between 2017 and 2019, with a highest rate of screening in men aged less than 30 years old (+46%) compared to women aged less than 25 years old (+34%) .

	2014	2015	2016	2017	2018
Confirmed cases of CT infection	14227	14971	13624	17672	No data reported

Table 2: Evolution of CT cases in France between 2013 and 2017.

Data extracted from ECDC [7].

b. Physiopathology of the disease & CT feature

i. Physiopathology

CT infection, as an STI, can be transmitted through sexual contact but also during foreplay (buccogenital or anogenital). CT is also detected in the rectum of women suffering from urogenital infection who did not report anal intercourses suggesting a dissemination of CT in the gastrointestinal tract (GI) [10] as discussed by others [11, 12]. Interestingly, gut persistence of CT could act as a reservoir for FRT reinfection [13].

Chlamydia trachomatis is an obligate intracellular Gram negative bacterium. CT is part of the family *Chlamydiaceae* and the genus *Chlamydia* along with *C. pneumoniae*, *C. psittaci*, and *C. pecorum*. *Chlamydia* is known to induce: (1) infections that can be long lasting without treatment, (2) repeated infections after natural clearance or antibiotic treatment,

(3) infections that are often asymptomatic or with mild symptoms and (4) infections that induce a local inflammation [14].

Around 70% to 90% of the infection in women are asymptomatic and without an active screening, individuals will stay infected and continue to spread the infection [15].

An untreated CT infection can either: persist without symptoms for a long period, progress to cause more severe sequelae or resolve spontaneously without treatment. About 20-54% of infected patients self-resolve within a year whereas 23-30% become repeatedly infected [16]. Repeated CT infections can lead to the development of chronic inflammation [15, 17, 18]. Even if individuals that suffer from symptomatic CT infection are at risk to develop more severe symptoms, asymptomatic CT infection can also result in sequelae like tubal scarring [14].

In women, the infection can induce a slow cervicitis with leukorrhea (thick vaginal discharge) but also cystalgias (painful bladder syndrome), urethral syndrome (infection and inflammation of the urethra), dyspareunia (painful intercourse), spotting/bleeding between periods or after vaginal intercourse. Acute forms are a combination of adnexal/uterin pain and metrorrhagia (uterine bleeding at irregular intervals). Untreated, the infection can progress to the upper genital tract, including endometrium and fallopian tubes, and induce a salpingitis (Fallopian tube infection and inflammation) with chronic pelvic pain, sterility, pelvic inflammatory disease (10%) and ectopic pregnancy [14, 19]. Pelvic inflammatory disease and scarred fallopian tube account for 20 to 40% of the symptom induced by the infection. Neonatal transmission can also occur during childbirth [15]. LGV, also induced by CT, is characterized by genital ulcerations followed by adenopathy and an inflammation of the rectum/anus.

Urethritis, cervicitis and asymptomatic form are treated with a single dose of 1g of azithromycine. In contrast, LGV is treated with another antibiotic, the doxycycline, for 3 to 4 weeks. The meta-analysis of Kong et al. has shown that the difference of efficacy between doxycycline and azitromycine treatment, is from 1,5% to 2,6% in favour of doxycycline for urogenital chlamydia infection (study composed of men and women) [20].

ii. *Chlamydia trachomatis* description and growth cycle

CT is divided into 19 serovars: trachoma serovars involved in ocular and urogenital infections (A-K) and serovars involved in LGV (L1-L3). Ocular infections are induced by the serovars A to C, accountable for the infection of the epithelial surface of the conjunctiva and chronic ocular inflammation. These serovars are still endemic in many developing countries and are the first cause of infectious blindness. Serovars D to K are responsible for genital infection. As for LGV, they primarily infect the lymphatics system and lymph nodes, and MSM are more commonly infected by LGV than women [15]. Serovars A and C appear to be strictly associated with trachoma. Indeed, these serovars differ from the genital strains by several gene. For example, the A and C strains lack an intact *trpA* gene indispensable for transforming indole into tryptophan, which is essential for bacterial survival in the cell [21]. However, genital strains also induce conjunctivitis [22].

CT preferentially infects columnar epithelial cells. CT has a unique biphasic developmental cycle that alternate between two forms: the elementary bodies (EB) that are infectious but not metabolically active, and the reticulate bodies (RB) that are metabolically active, therefore able to replicate, but not infectious. The EB interact with a cell surface protein, and upon attachment, CT is internalized into the cell through an endocytosis like mechanism, using a vesicle called an inclusion which does not fused with the endosomal or lysosomal membranes [23]. More precisely, the entry of CT in the cell can be mediated by different mechanisms involving different proteins. All the mechanisms are not fully understood and it seems that there is not a single pathway involved in CT entry. One of the mechanisms involved a type 3 secretion system (T3SS) that is delivered into the cell by CT and stimulates the Rho family GTPase Rac1. Cytoskeletal rearrangements and the formation of lamellipodia or filopodia will then be induced, facilitating the entry of CT [24]. A study revealed that filopodia could be associated with macropinocytosis entry, mediated by the sorting nexin 9 protein (SNX9) [25]. Furthermore, the T3SS allows the secretion of effectors such as TmeA. TmeA, by activating neural Wiskott-Aldrich syndrome protein (N-WASP), will promote Arp2/3-dependent actin polymerization allowing EB entry [26]. Several host cell

receptors might be involved in CT entry as well. Mannose receptor, cystic fibrosis transmembrane conductance (CFTR), Ephrin A2 and protein disulfide isomerase (PDI) have been proved to enhance CT adhesion and entry. Interestingly, silencing of Ephrin A2 and PDI reduced CT invasion by 87% [27].

After CT entry, the disulfide bonds between the outer membrane proteins of the EB, essential for EB extracellular stability, are reduced during the internalization. Within the inclusion, CT development will depend of the interaction between the inclusion and the organelles, notably for providing nutrients essential to the bacterial survival. Acquisition of lipids by the inclusion is an essential step for the inclusion maturation and reproduction. CT can synthesize its own lipids but will preferentially use host lipids, indeed they will acquire sphingolipids, cholesterol, glycerophospholipids that will be incorporate into EB/RB membrane [14, 28]. CT is also able to regulate ATP synthesis by the host mitochondria in order to use it for its intracellular growth [29]. Host iron is also necessary for a correct infectious cycle, as well as tryptophan that is necessary for its growth and survival [30, 31].

Meanwhile, the conversion of EB to RB is triggered. RB will then replicate within the inclusion. Halfway of the infection cycle, bacterial replication become asynchronous and the RB will reverse back to EB. The inclusion is now full of EBs and fill almost the entire volume of the cell. The infectious element will then be released either by cell lysis or inclusion extrusion and will be able to infect other cells [Fig 3] [28].

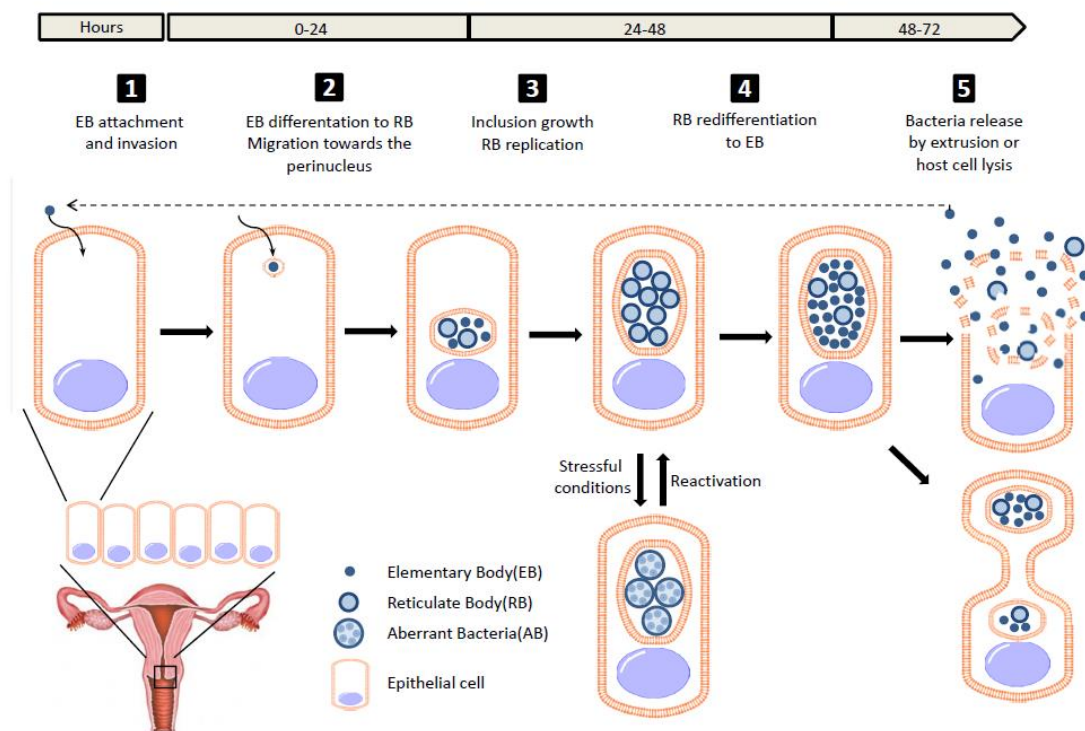


Figure 3: CT growth cycle.

Elementary bodies (EB) attach to epithelial cells to infect the cells. (2) EB differentiate into reticulate bodies (RB) within a vacuole called an inclusion. (3) RB replicate within the inclusion. (4) RB differentiate back to EB leading to a mixture of EB and RB within the inclusion. (5) Bacteria are released from the cell either by extrusion or host cell lysis and infect new epithelial cells. Image extracted from Lujan A et al., 2016 [32].

c. Immune response induced by CT infection

i. Innate immune response to CT infection

CT preferentially infects the endocervical columnar epithelial cells of the FRT and activates the immune system by inducing pro-inflammatory cytokine expression that recruit immune cells such as neutrophils, Natural Killer (NK) cells and monocytes. CT can also infect macrophages leading to lymphangitis/lymphadenitis diseases.

1. Human *in vitro* and *in vivo* study:

In vitro models were used to study the production of cytokines and chemokines by epithelial cell lines upon CT infection. Rasmussen et al. have shown that HeLa cells infected

with CT induce an increase of pro-inflammatory cytokines: IL-8, Granulocyte-macrophage colony stimulating factor (GM-CSF), IL-6 and IL-1 α [33]. Using a different epithelial cell line, the team of Quayle has observed, in polarized A2EN cells infected with CT, a mild increase of pro-inflammatory cytokines, with an increase of IL-1 α secretion and a decrease of CXCL10 and CCL5. A2EN is a human endocervical epithelial cell line (A2EN) derived from primary epithelial cells and immortalized using papilloma virus gene E6 and E7 [34]. Moreover, infection of three different epithelial cell lines (HeLa, A549 and HT-29 cells) with CT induce the expression of the mature form of IL-18 through caspase-1 activation. This production of mature IL-18 was not observed in fibroblasts infected with CT [35]. In oviduct cell lines, toll like receptor 3 (TLR) was disrupted, leading to a decrease of inflammation biomarkers (IL-6, IFN β , IL-8, IL-20, IL-26, IL-34, matrix metalloproteinase (MMP) family...) upon CT infection, suggesting a role of TLR3 in genital tract scarring and inflammation [36].

An increase of IL-1 α and IL-8 was also observed by immunohistochemistry of human fallopian tube organ culture (FTOC) infected with CT serovar D (svD). Furthermore, severe tissue damages were observed. IL-1RA adding prior to CT infection reduces the tissue damage observed previously (percentage of infection remains the same; reduce expression of IL-1 α , IL-8). The authors hypothesized that IL-1 α production by epithelial cells after CT infection induce tissue damage and the expression of IL-8, which attract neutrophils [37].

Cohort studies confirmed and supplemented *in vitro* and *ex vivo* studies on the production of immune mediators by CT infection. Immune mediators of five women suffering from CT infection were measured in vaginal fluids and compared to a non-infected group (n=13). The authors noticed in CT infected women an increase of IL-1 β , lactoferrin, TNF α , IL-8, vascular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF), IL-10, IL-3, IL-7, IL-12 and IL-6 [38]. Interestingly, cervical fluids, collected from women suffering from both cervical and upper genital tract infection, exhibit an increased expression of CXCL10, TNF α , IL-17A, CXCL9, CXCL11, CCL4 and CXCL13 compared to women suffering from cervical infection. The authors observed that an elevated ratio of IL-15/CXCL10, IL-16/TNF α , CXCL14/IL-17A were negatively associated with endometrial infection. As expected, 79% of the cytokines observed in cervical secretions were positively

associated with CT burden [39]. Women suffering from cervicitis exhibited high levels of pro-inflammatory cytokines in vaginal fluids (IL-1 β , IL-6 and IL-8). The authors also observed, in women with fertility disorders, a significant increase in levels of IL-6, IL-8, IL-10 and IFN γ [40]. Another study has shown that the increased level of IFN γ in cervical fluids of women infected with CT (n=47) was five times higher compared to uninfected women (n=52) [41]. *In vitro*, CT infection of epithelial cells in stress condition such as during IFN γ exposition or iron deprivation induces the development of morphologically aberrant non-infectious developmental forms of CT [42]. Interestingly, addition of recombinant TNF α to an epithelial cell line (Hep2 cells) immediately after CT infection (serovar L2) resulted in smaller inclusion. In a similar setting, the combination of TNF α and IFN γ pretreatment showed an inhibition of chlamydial replication [43]. Moreover, in an *in vitro* study using mouse DC and NK cell lines, the authors have shown an induction of exosome release by CT infected DC. Those exosomes do not contain CT but enhance IFN γ production by NK cells through the interaction between TNF α and NK cell associated TNF receptor [44].

2. Animal models:

The mouse model has been highly used to understand the pathogenicity and immune response upon CT infection. *Chlamydia muridarum* infects the columnar epithelial cells and lower genital tract infection mimics acute genital tract infection observed in human after CT infection. Mice clear *C. muridarum* infection after a month post-challenge. However, after 28 to 50 days after infection, mice develop sequelae in the upper genital tract and develop hydrosalpinx that correlated with infertility. *IL-1 α ^{-/-}* mice infected with *C. muridarum* were protected from oviduct damage and fibrosis despite a similar CT burden observed in CT control mice. Indeed, the oviduct epithelium were thicker and the tissue structure was maintained with less tissue scarring. Furthermore, a reduced infiltration of neutrophils is observed in the oviduct and uterine horns of *IL-1 α ^{-/-}* mice. There is also a reduced expression of pro-inflammatory cytokines (IL-1 β , G-CSF, IL-6, CCL4, CXCL1, IL-17A, IFN γ , IL-12p70) at the peak of infection in oviduct tissue of *IL-1 α ^{-/-}* mice compared to WT mice [45]. In mice infected with CT svD, authors observed a major recruitment to the site of infection of

neutrophils and monocytes and to a lesser extent macrophages, DC and NK cells. G-CSF, GM-CSF and IL-8 are involved in neutrophil recruitment. Cervical fluids collected from women suffering from CT infection show high level of cytokines involved in neutrophil activation, survival and chemotaxis, such as YKL40, CXCL5, CXCL1, G-CSF, IL-8 and CXCL6 [39]. Depletion of neutrophils in CT infected mice improves the immunopathology in the upper genital tract showing that neutrophil is the major immune cell contributing to pathology, furthermore neutrophils seem to be unnecessary for CT clearance because the bacterial burden was not impacted by neutrophil depletion [46]. CXC chemokine receptor 2 (CXCR-2) deficient mice, which is involved in neutrophil recruitment, and matrix metalloprotease-9 (MMP9) or phagocyte oxidase deficient mice, mainly produced by neutrophils, display a reduce severity of the disease. MMP9 induces degradation of extracellular matrix and inflammation and phagocyte oxidase is a membrane-bound complex that is used to engulf pathogens. GM-CSF production of epithelial cells upon CT infection has been link to increase neutrophil survival in an *in vitro* model using supernatant of HeLa cells infected with L2 strain of CT on neutrophils isolated from human blood, treated with anti-GM-CSF [47]. Neutrophils could also be involved in CT survival, indeed K. Rajeeve et al. have described that neutrophils infected by CT fail to induce neutrophil extracellular traps (NET). This mechanism is mediated by the binding between Chlamydia protease-like activating factor (CPAF) and the formyl peptide receptor 2 (FPR2) present on the neutrophil membrane. This binding will then prevent the activation of the NET mechanism and enable pathogen survival [48]. However, Naglak et al. have shown that neutrophils are highly involved in antibody-mediated anti-chlamydial immunity using serum passive transfer of mice that resolve CT infection to a reinfected mouse model. This immunity has been proved to be involved in reducing the bacterial burden observed invaginal swab but no information on pathology was given [49]. A summary figure on neutrophil involvement during CT was done [Fig4].

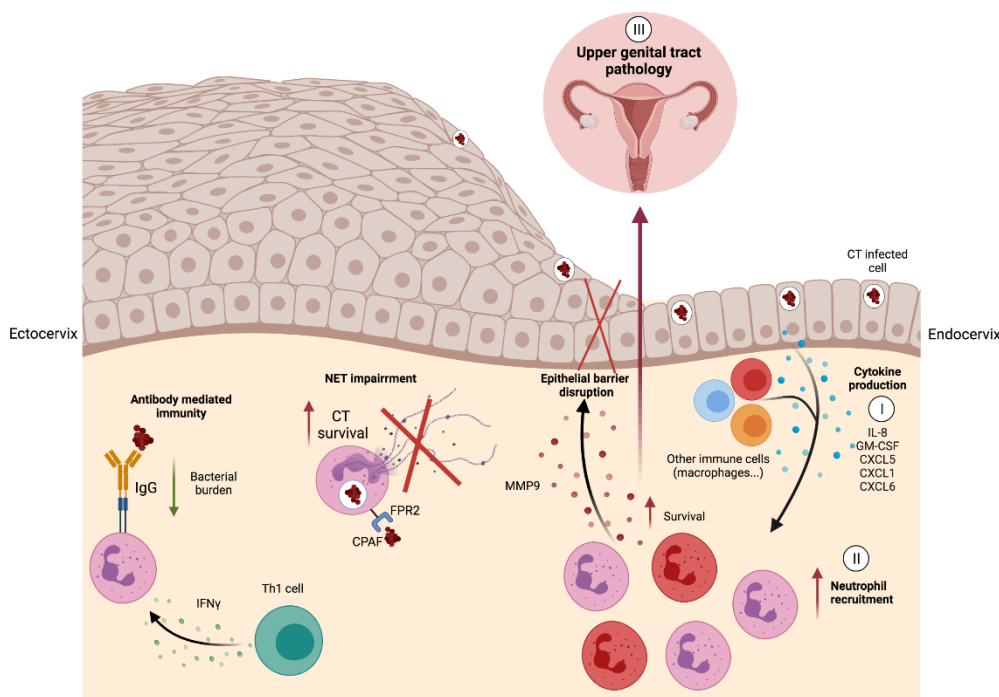


Figure 4: Neutrophil role during CT infection.

Epithelial cells infected with CT in the cervix as well as other immune cells will produce cytokines. (II) The cytokines produced by epithelial cells and other immune cells induce the recruitment of neutrophil within the mucosae. (II) Neutrophils will have an increase survival and will produce cytokine and peptides such as MMP9. (III) The overactivation of neutrophils will lead to upper genital tract pathology. CT infection of neutrophils can also favour CT survival by impairing NET production. Neutrophils was also described to be involved in antibody mediated immunity allowing bacterial burden reduction. Created with Biorender.com.

A metabolically active non replicative form of CT was also observed to persist in macrophages [50]. Macrophages are recruited at the site of infection and sense pathogen associated molecular patterns (PAMPs) like lipopolysaccharide (LPS) or Heat Shock Protein 60 (HSP60). Recruited macrophages are part of lymphoid follicles along with B cells in the center and T cells in periphery [51]. They engulf the EB supposedly by phagocytosis and induce the expression of several cytokines, chemokines and growth factors like IL-1 α , IL-6, IL-12, TNF α , CXCL10, GM-CSF [15].

Based on *in vitro/ex vivo* and *in vivo* results, CT infection induces a strong expression of several cytokines including IL-1, IL-6, IL-8, TNF α , IL-12, IL-18, IFN γ but also growth factor like VEGF, GM-CSF and G-CSF. IL-12 and IL-18 in combination are strong inducers of IFN γ

secretion by CD4⁺ T cells and NK cells, described to be essential for CT clearance. Pathogenesis is induced by several factors including IL-1, IL-18, IL-8. IL-1 β secretion, highly expressed by neutrophils and macrophages that have been shown to significantly contribute to oviduct pathology in mice [52]. IL-18 is involved in both pathogenesis and CT clearance highlighting the balance between sufficient inflammation that is necessary for bacterial clearance and excessive inflammation that induced pathogenesis. IL-1 β active form is mediated through caspase-1 cleavage of pro-IL-1 β . Furthermore, the cytosolic pattern recognition receptors (PRR), Nucleotide-binding oligomerisation domain-containing protein 1 (NOD1), can enhance caspase 1 IL-1 β production as well as IL-8 production. IL-8 is a chemokine involved in cell recruitment including neutrophils and macrophages. Active form of IL-8 and IL-18 also increased upon CT infection are dependent of the caspase 1, highlighting a central role of caspase 1 in contributing to pathogenesis [15, 35]. Tables 3, 4 and 5 recapitulate main cytokine, chemokine and growth factor functions.

	Cytokines	Main cell producers	Main relevant functions
Pro-inflammatory cytokines	IL-1 family (IL-1 α , IL-1 β , IL-18)	Epithelial cells Myeloid cells	Cytokine production (DC, macrophages) Increase survival/adhesion/oxidative burst and protease release (Neutrophils) T cells responses IL-18 \rightarrow IFN γ by NK/T cells IL-18 \rightarrow increase cytolytic NK functions IL-1 β \rightarrow IL-6 production B cell proliferation
	IL-12 family (IL-12, IL-23, IL-27, IL-35)	Myeloid cells B cells	T cells differentiation IL-12 \rightarrow IFN γ by NK/T cells
	IL-6	Endothelial cells Fibroblasts Epithelial cells	Production of: C-reactive protein, serum amyloid A, fibrinogen, haptogen Regulation of Treg/Th17/cytotoxic T cell differentiation Induction of antibody production
	IL-8	Endothelial cells Epithelial cells	Neutrophil recruitment/activation
	TNFα	Myeloid cells Th1 cells	Production of TNF α , IL-8 and GM-CSF
	Type I interferons (IFN β / ϵ / ω / κ)	Plasmacytoid DC	Cell viability and function Apoptosis, autophagy, cell differentiation, migration
	Type II interferon (IFN γ)	NK cells, Th1	Inhibition of Th17 development Generation/activation of Tregs Macrophage activation Production of TNF α , GM-CSF
Anti-inflammatory & regulatory cytokines	IL-1RA	Epithelial cells Myeloid cells	Blocked IL-1 α , IL-1 β signaling
	IL-10	T/B cells Macrophages DC NK cells	Inhibition of pro-inflammatory cytokine/growth factor release (macrophages/monocytes) Inhibition of IL-12/23 \rightarrow impact on CD4+ T cell differentiation/proliferation
	TGF-β	Treg	T cell tolerance: regulation of T cell differentiation Production of IL-10, inhibition of GM-CSF production Inhibition of NK cell proliferation/function & CD8+ T cells

Table 3: Major roles and producers of main pro and anti-inflammatory cytokines.

Data extracted from [53–63].

Growth factors	Main cell producers	Main relevant functions
GM-CSF	Macrophages Endothelial cells T cells	Survival, differentiation and proliferation (granulocyte/macrophages)
G-CSF	Bone marrow stromal cells (monocyte/macrophage lineage) Vascular endothelial cells	Basophil and neutrophil differentiation/proliferation
VEGF	Macrophages, Fibroblasts	Angiogenesis Vasculogenesis

Table 4: Major roles and producers of the growth factors GM-CSF, G-CSF and VEGF.

Data extracted from [64–66].

Chemokines	Production	Cells producers	Chemokine ligand	Main relevant functions
Chemokine ligand 2 (CCL2)	Upon inflammatory stimuli (IL-1, IL-4, IL-6, TNF α , TGF β ...)	Epithelial cells, T cells, myeloid cells...	CCR2 ligand	Recruitment of immune cells including T cells, NK cells, DC, monocytes or macrophages
Chemokine ligand 3 (CCL3)	Upon inflammatory stimuli (IL-1, TNF α , IFN γ ...)	Macrophages, T and B cells, neutrophils, DC...	CCR1, CCR4 and CCR5 ligand	
Chemokine ligand 4 (CCL4)	Upon inflammatory stimuli (IL-1, TNF α , IFN γ ...)	Macrophages, T and B cells, neutrophils, DC...	CCR5, CCR8 and CCR9 ligand	
Chemokine ligand 5 (CCL5)	Upon inflammatory stimuli (viral stimulation, TNF α , IFN γ ...)	Macrophages, T cells, epithelial cells, eosinophils...	CCR1, CCR3 and CCR5 ligand	
CXCL10	IFN γ , TNF α , LPS...	Monocytes, endothelial cells, fibroblasts...	CXCR3 ligand	

Table 5: Major roles and producers of chemokines.

Data extracted from [67–72].

CT infection of host cells are sufficient to induce chronic and intense inflammation that promote cellular proliferation, tissue remodeling and scarring of tissue [Fig5] [51].

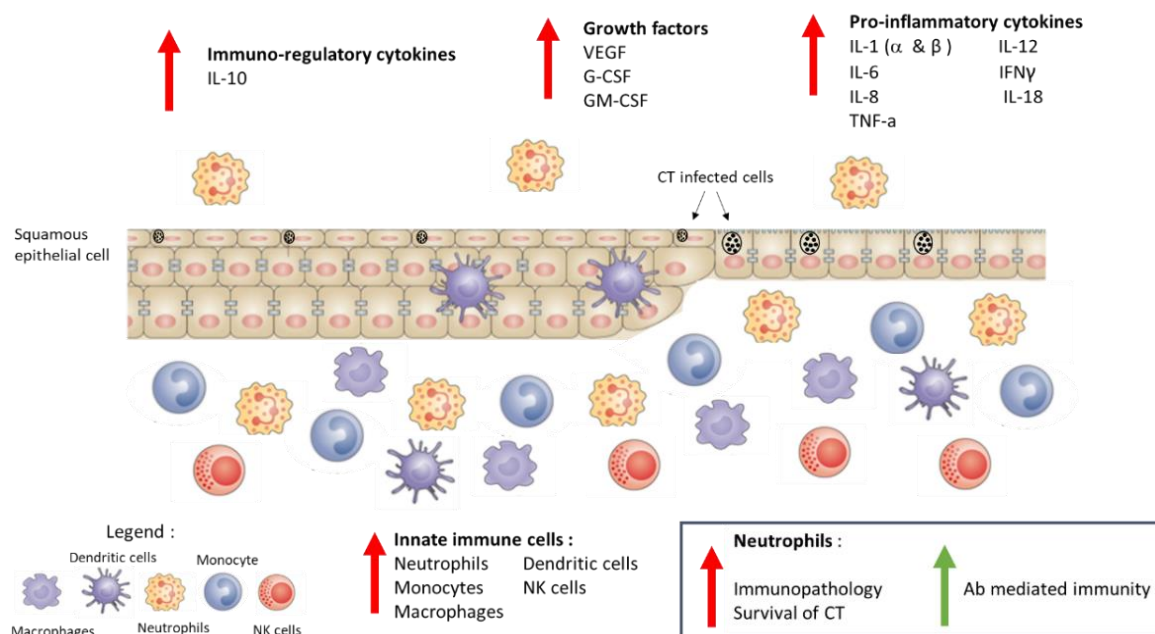


Figure 5: Innate immune response induced by CT infection in the FRT.

Cervicovaginal CT infection of epithelial cells favours the production of several cytokines and chemokines inducing the recruitment of innate immune cells. Red arrows represent negative impact on the FRT and green arrows positive impact on the FRT. Neutrophils were described to have a dual role during CT infection. Figure adapted from Wira C. et al., 2005 [61].

ii. Adaptative immune response

1. CT infection in Human:

Upon infection, there is a significant number of T cell infiltration to the infected tissue. The protective immunity against CT has been reported to be characterized by antigen-specific IFN γ secreting CD4⁺ T cells [16]. IFN γ has a central role in the immune response against CT and might be essential for infection clearance [73, 74]. IFN γ , by depleting the intracellular pool of tryptophan upon induction of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), induces the inhibition of chlamydia growth but maintains persistence of aberrant intracellular CT [31, 51, 75, 76]. Ziklo et al. have observed an increased expression of IDO1, TGF- β 1 in cervical fluid of CT repeatedly infected women,

and those that cleared the infection thanks to antibiotic treatment compared to uninfected or single CT infected women. Only women that cleared CT infection after antibiotic treatment exhibit high level of FoxP3. Interestingly, no IFN γ production was observed, suggesting that IDO1 is produced independently of IFN γ secretion. Based on *in vitro* experiment as well, the authors suggest that IDO1 and TGF- β 1 could be involved in the immune regulation in repeatedly infected individuals [77].

Association between antibodies and protection is unclear. Seropositivity in women was not associated with reduce risk of ascending CT infection or new CT infection. However, they found that seropositivity was associated to reduce cervical bacterial burden but not endometrium bacterial burden [78]. A recent study, performed in 411 women with single CT infection, and in 62 women with recurrent CT infection, have described an anti-CT MOMP specific IgG antibodies level, which is stable for 3 to 10 years after CT infection. Despite the stable level of antibodies, reinfection in patients still occurs [79]. IgA and IgG against MOMP antigen levels in cervical fluids were higher in women with primary CT infections (n=44) than women with recurrent infections (n=81). On the contrary, women suffering from recurrent infection had a higher level of IgA and IgG against HSP10 and HSP60 [80]. Elevated titers of anti-HSP60 are a recognized marker of persistent infection. The presence of immunocomplexes formed by anti-CT-HSP60 antibodies and HSP60 are supposed to worsen the course of the disease [81]. Moreover, in a cohort composed of women suffering from tubal damage, the authors have shown that chlamydia specific antibodies in sera are positively correlated to the severity of tubal damage [82]. However, a study on a sex working cohort in Kenya, have shown that levels of EB specific or HSP60 IgG and IgA in sera and vaginal fluids were not correlated with an increased risk of CT acquisition [83].

2. Experimental infections in animal model:

In a mouse model infected by CT svD, Lijek et al. observed that antigen-specific CD4⁺ and CD8⁺ T cells are sufficient for clearance of the bacterial burden. In addition, another study determined that B cells are essential for CD4⁺ T cell priming upon CT infection and in

preventing bacterial dissemination [84]. However, the influx of non-specific T cells that occurs after the CT infection might contribute to immunopathology. Indeed, CXCR3 blockade in mice results in an improvement in the immunopathology of the upper tract. CXCR3 is the common receptor of the chemokine CXCL9/10/11 [46].

During CT infection, the CD4⁺ T CD25⁺ FoxP3 Treg produced IL-10 are also involved in pathogenesis. Depletion of Treg in a mouse model infected with CT reduces the inflammation, neutrophil recruitment and oviduct pathology. Moreover, this depletion induces a decrease of Th17 but not CD4⁺ Th1 cells [85].

3. Contribution of vaccine studies:

In a mouse model, immunization with a surface protein of CT (Major outer membrane protein - MOMP) and Cholera Toxin/Cytosine-phosphate-Guanine-oligodeoxynucleotide (ChoT/CpG) followed by an intravaginal challenge elicited an increase of IFN γ and TNF α that were associated with protection through duration of the infection but also oviduct pathology. The authors observed in non-immunized animal an increase of caspase 1 activated cytokines IL-1 β and IL-18 [74]. Intranasal immunization of live EB of *C. muridarum* induces a Th1 CD4⁺ T cell response characterized by a strong induction of IFN γ but also a low level of IL-17. On the contrary, intramuscular immunization of live EB induces Th17/Th2 responses with a strong immunopathology of the upper genital tract [86]. These results highlight an impact of Th17 on immunopathology. Th17 cells that produce IL-17 recruit neutrophils to the site of infection, which are known to induce tissue damage. The role of Th17 has been studied during CT infection but their role remains unclear and need to be more explored. O'Meara et al., have observed an increase of IL-17 in immunized mice that are protected against the infection whereas mice that are protected against oviduct pathology display a lower level of IL-17. The authors suggested that IL-17 might be involved in both protection and pathogenesis and that chronic CT pathology involved an increase of IL-17 [74]. As well as the Th17, the CD8⁺ T cells does not seem to be involved in protective immunity but might be linked to pathogenesis by involving TNF α production [15]. Indeed, in a mouse model depleted of CD8⁺ T cells immunized with a MOMP vaccine, the authors

observed the same course of CT infection compared to undepleted vaccinated group upon CT challenge. This demonstrates that CD8⁺ T cells are not involved in CT protection [87]. Vaccine studies highlight a central role of B cells in protection. Indeed, in a mouse model depleted of B cells immunized with a MOMP vaccine, there is a reduced protection (slower resolution of CT infection) against CT challenge compared to the undepleted vaccinated group [87].

Immune cells seem to be involved in tissue damage such as neutrophils and Th17. The role of B cells and antibodies is not clear and need to be studied deeper, however it seems that B cells might be involved in decreasing vaginal bacterial burden but also in facilitating upper tract tissue damage. More importantly, the IFN γ producing Th1 cells have been correlated to protective immunity [Fig6].

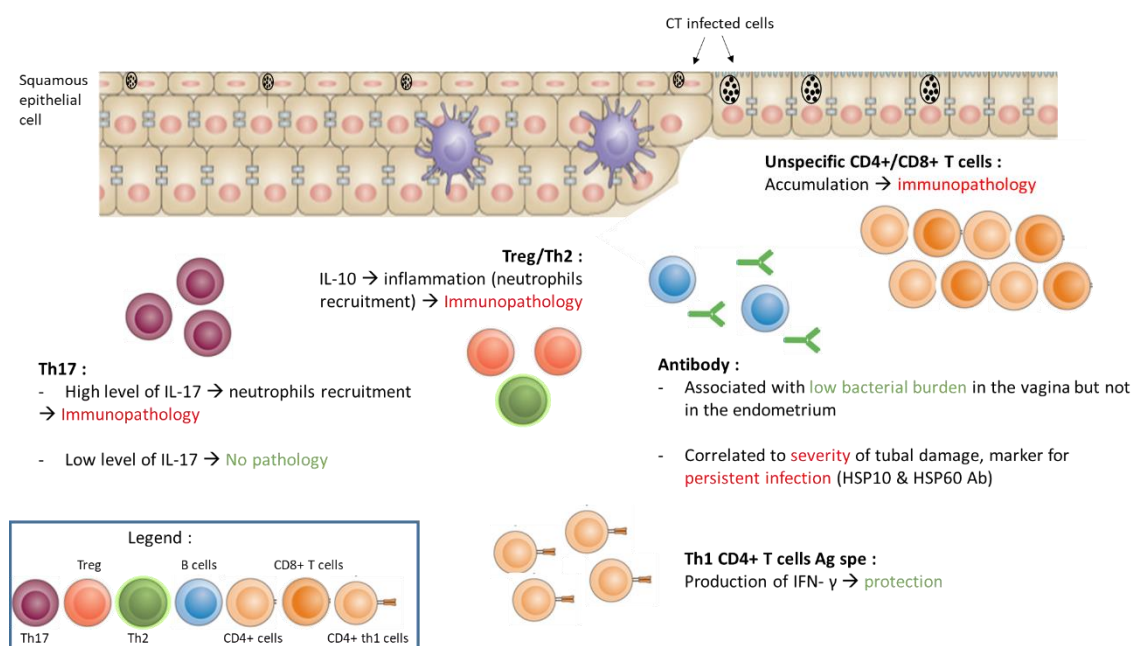


Figure 6: Adaptive immune response induced by CT infection in the FRT.

Cervicovaginal CT infected epithelial cells induce different cell recruitment/activation allowing either protection or pathology. Figure adapted from Wira C. et al., 2005 [61].

III. CT/HIV-1 co-infections

a. Epidemiology

HIV-1 is an STI that attacks the immune system by depleting CD4⁺ T cells weakening the immune system of an infected person. Two to 25 years after HIV-1 infection, individuals not under antiretroviral therapy, can develop the acquired immunodeficiency syndrome (AIDS) making them more susceptible to severe infections like tuberculosis, cryptococcal meningitis, severe bacterial infections and cancers. HIV-1 as other STIs continues to be a major global public health issue. Approximately 38 million people were living with HIV-1 at the end of 2020, 680,000 people died from HIV-1 related cause in 2020 and 1.5 million people were newly infected [88]. There are 5 000 young women aged from 15-24 years, every week that become infected with HIV-1. In sub-saharian Africa, six out of seven new infections are detected in women aged 15-19 years, moreover these young women are twice more likely to live with HIV-1 than men [89]. Adolescent and young women have an increased risk of HIV-1 acquisition.

As described earlier, CT can induce a long-term infection that will induce a local chronic inflammation that will lead to increase susceptibility to other STI acquisition including HIV-1 infection.

In a population-based incidence study in MSM, a factor associated to HIV-1 co-infection is the diagnosis of an STI in the past 12 months. Moreover, LGV infection was associated with increased risk of HIV-1 co-infection [90]. A study performed in a Brazilian cohort of women showed that CT infection was the most prevalent STI (around 20%) found in HIV+ and HIV- women [91].

Prevalence of CT infection was found to be high in HIV-1/AIDS positive women [92]. Furthermore, meta-analysis performed on several studies reported that chlamydia infection or cervicitis significantly increases the detection of HIV-1 [93]. On 609 HIV-1 seropositive sex working women in Ivory Coast, HIV-1 cervicovaginal shedding was significantly associated with CT [94]. In the MIRA trials 4,948 HIV-1 seronegative women were enrolled and followed during 12 to 24 months. The authors observed that women infected by either CT, HSV-2, *N.*

gonorrhoea or *T. vaginalis* were at increased risk of HIV-1 acquisition [95]. At last, during 2000-2009, 2,221,944 women aged from 13-59 year old were monitored for HIV-1 acquisition. In this study, HIV-1 positive diagnostic was higher in women suffering from one STI (syphilis, gonorrhea and CT) [96].

These epidemiologic studies confirmed that CT infection increases the risk for HIV-1 acquisition.

b. Mechanisms involved in increasing the risk of HIV-1 acquisition (based on *in vitro* studies)

In vitro studies have also proved that a previous CT infection increases the susceptibility of HIV-1 infection. HIV-1 can infect different parts of the female genital tract including the vagina, exocervix, endocervix but also the uterus and the fallopian tubes. The transformation zone, where the multilayer pluristratified epithelium of the vagina/ectocervix becomes a single layer of polarized epithelial cells with tight junction is highly susceptible to HIV-1 infection.

In the genital tract mucosae, HIV-1 infection can occur via three different mechanisms [97]:

- Cell free virus or cell associated virus cross the epithelial barrier through abrasions that can be induced during sexual intercourses or inflammation induce by another STI.
- In the vagina and ectocervix, CD4⁺ T cells, DCs and macrophages can penetrate the epithelium and capture cell free virions.
- Transcytosis of HIV-1 virus through the columnar epithelium can also occur.

To initiate HIV-1 infection, two surface protein the gp120 and gp41 will bind to CD4 and a co-receptor CXCR4 or CCR5. HIV-1 virus can either bind to CXCR4 co-receptor (X4 virus) or CCR5 (R5 virus) or to both co-receptors (X4R5 virus). R5 viruses are mainly transmitted. Other co-receptor can be involved to initiate HIV-1 entry such as galactosyl

ceramide (GalCer) that binds to HIV-1 gp41 and heparin sulfate proteoglycans (HSPGs) [98, 99].

In a longitudinal study, endocervical/vaginal fluids and cervicovaginal lavages (CVL) were obtained from women infected with CT. Levels of cytokines and chemokines (G-CSF, IL-1 α , CCL5, IL-10, IL-6, IL-1 β and CXCL10) in endocervical fluids were higher than in vaginal secretions or CVL. On the contrary, IFN γ and TNF α levels were higher in vaginal secretions before starting antibiotic treatment than after antibiotic treatment. This demonstrated a global inflammation with an expression pattern of cytokines and chemokines distinct, depending of the FRT compartment. Using an *in vitro* model, the authors observed an increased HIV-1 infectivity due to endocervical secretions of CT infected women before but also after antibiotic treatment [100].

To attest the mechanisms involved, Buckner et al. used a double chamber experiment to evaluate the migration of cell-associated HIV-1 and cell-free virus through an epithelial barrier. The authors observed an increased cell-associated virus migration when epithelial cells are infected with CT [101]. Moreover, CD4⁺ T cells are preferentially recruited to the upper tract of mouse infected by CT [102].

CT infection can increase HIV-1 susceptibility through several mechanisms:

- (1) Production of inflammatory cytokines inducing recruitment of HIV-1 target cells including macrophages and DCs [100].
- (2) Recruitment of a large amount of CD4⁺ T cells that express HIV-1 co-receptors CXCR4 and CCR5 [99, 102].
- (3) CT infection of endocervical epithelial cells induces the disruption of the epithelial barrier facilitating the crossing of HIV-1 cell free virus.
- (4) CT infection enhances cell associated HIV-1 migration through the epithelial barrier by transcytosis [101].

- (5) CT infection of epithelial cells induces an upregulation of CXCR4, CCR5 and Galcer [99].
- (6) CT infection enhances attachment of HIV-1 virion on CT infected epithelial cells and thus increases the transfer and replication of HIV-1 virion. In CT infected epithelial cells the levels of HIV-1 bound were higher than on non-infected cells. Moreover, co-culture of CT infected epithelial cells with CD4⁺ CCR5⁺ CXCR4⁺ T cells induce a 10 fold increase in HIV-1 viral copies compared to mock infected epithelial cell coculture with CD4⁺ T cells [99].

IV. STI gateway

A vast array of pathogens invades through mucosal surfaces: nasal/oral cavities, gut and the genitourinary tract (GU). STI pathogen invasion occurs through the male/female GU tract, the rectum (anogenital transmission) but also during foreplay (buccogenital transmission) or from the mother to the unborn child for some STIs. Blood transfusion or use of contaminate needle can also allow the transmission of viral STI like HIV-1. Concerning the male GU, pathogens will access to the urethral opening of the penis and to the inner surface of the foreskin [103]. In the FRT, pathogens will be directly in contact of the vaginal/cervix epithelium.

Heterosexual transmission from male to female is one of the major route of STIs and occurs mainly via the FRT mucosae.

Chapter II: The Female reproductive Tract (FRT)

I. Structure and composition

The FRT is divided into an upper tract, composed of the fallopian tubes, ovaries, uterus and endocervix and a lower tract, composed of the ectocervix and the vagina. The two parts differ in their architecture, with two distinct types of mucosae (type I and II) [Fig7]. Indeed, the upper tract (uterus and endocervix), is characterized by a simple columnar epithelium formed by a layer of ciliated columnar cell connected by tight junctions (type I). This epithelium is involved in mucus and egg cell movement toward the uterus and fallopian tubes thanks to the cilia [104]. Type 1 epithelium expresses polymeric Ig receptor (pIgR) facilitating the passage of dimeric IgA into the lumen [103]. The upper tract is characterized by microfold cells (M cells), the presence of mucosa associated lymphoid tissue (MALT) and secretory IgA antibodies [104]. The lower tract (vagina and ectocervix) is made of multiple layers of non-keratinized stratified squamous epithelium (type II). The type 2 epithelium is characterized by the presence of Langerhans cells (LC) and production of IgG antibodies. The critical zone for pathogen invasion is the transformation zone, between the upper and lower tract, where the multilayers squamous epithelium change to a single layer of columnar epithelial cells [105]. The transformation zone is immunologically active, the change of epithelium architecture allows the passage of small pathogens like HIV-1 to the lumen.

The lower tract epithelium, which is composed of multiple layers of epithelial cells, permits the protection of underlining tissues from abrasion during intercourse. However, the lack of tight junction allows intra-epithelial passage of molecules including small pathogens and interaction of these pathogens to potential target cells. The epithelial barrier in the upper and lower tracts acts as a physical barrier but can also recognize PAMPs through PRR like TLR and NOD, and induces the production of cytokines, chemokines and antimicrobial peptides (AMP) [106, 107]. Pathogen specific antibodies in the FRT can bind to free and cell associated pathogens mediating their elimination through phagocytosis or by the complement system. IgG amount is greater in cervicovaginal fluids compared to IgA level [108]. A layer of mucus, covering the epithelium of the FRT, is in direct contact with pathogens. The

mucus acts as a physical trap for pathogens thanks to gel-like properties. The mucus is composed of mucins, which are complex high-molecular mass O-glycoprotein of the MUC family. The cervical mucus is dominated by MUC5B and MUC5AC, which are gel-forming mucins together with MUC6. On the contrary, MUC1 and MUC16, also found in the cervical mucus, are membrane-bound mucins [109]. Mucus appearance depends on hormonal concentration. There are two types of mucus in FRT, depending of the stage of the hormonal cycle. During proliferation stage and ovulation, the estrogenic mucus is thin and watery with a low viscosity, allowing sperm movement. On the contrary, after ovulation and during the secretory phase, the progestational mucus is thick, sticky and blocks the passage of spermatozoa [104, 106]. In the lower tract, mucus is produced by goblet cells and mucus gland in the cervix and in the upper tract by mucus-secreting epithelial cells. In a normal state, the cervicovaginal mucus is acidic (pH between 4-5). The acidified environment in the vagina is mainly maintained by the production of lactic acid by *Lactobacillus spp.* present in the vaginal microbiota of healthy women. The maintenance of a low pH inhibits microbial growth. The vaginal pH is dependent on the vaginal microbiota composition. Indeed, a dominance of anaerobic bacteria, different from *Lactobacillus spp.* is correlated with a higher pH, which will increase the risk for STI acquisition.

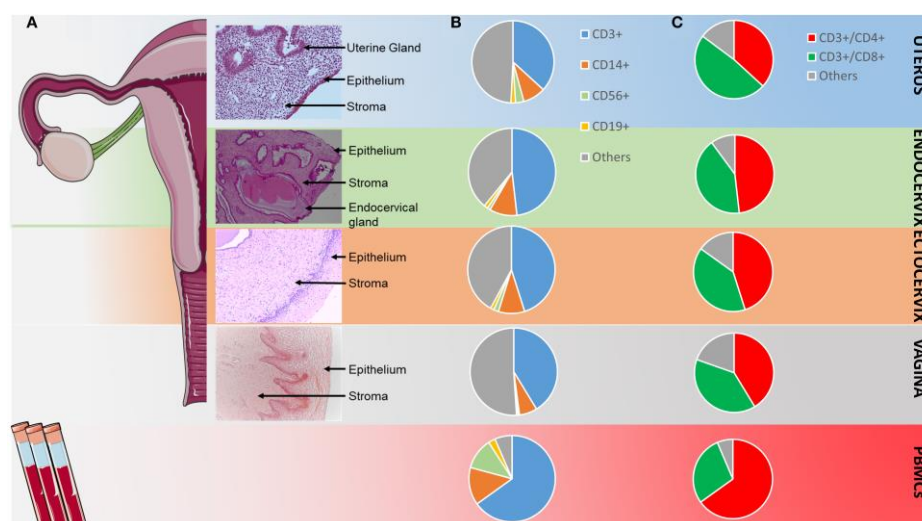


Figure 7: Female reproductive tract epithelium and immune cell composition.

(A) Representation of epithelium architecture of the upper and lower genital tract. (B) Proportion of T cells (CD3+), monocytes/macrophages (CD14+), NK cells (CD56+), B cells (CD19+) and others cells within the FRT. (C) Proportion of CD4+, CD8+ among T cells within each compartments. Extracted from Benjelloun F. et al., 2020 [110].

II. Immune cell composition in the FRT.

a. Innate immunity

i. General points

Macrophages, NK cells, DCs and neutrophils have a variable distribution according to FRT compartments and hormonal levels [106, 111]. Other innate-like lymphocyte cells such as $\gamma\delta$ and mucosal-associated invariant T (MAIT) cells are also present within the FRT. Indeed, $\gamma\delta$ T cells express predominantly V δ 1 TCR in the FRT and are decreased in the cervix of HIV-1 infected women [112, 113]. Different subsets of DC are observed in the FRT: intraepithelial Langerhans cells, lamina propria CD14⁻ DC and CD14⁺ DC [114]. At steady state, there is a high number of neutrophils, DC or LC, macrophages and NK cells in the upper genital tract, which is the opposite in the lower tract. There is around 10% of macrophages among the leucocytes present in the FRT with a highest concentration in endometrium stroma and myometrial connective tissue [106, 115]. In the lower tract, LC in the epithelium of the vagina and ectocervix are the main DC population [103]. In contrast, DC are localized in the sub-epithelial stroma of the endometrium. NK cells account for 10% to 30% of the leucocytes [106, 115, 116]. A lower number of NK cells is observed in fallopian tubes [106]. Next, neutrophils are detailed since they have been particularly studied during this thesis.

ii. Neutrophils

Neutrophils are the predominant circulating leukocytes in human and represent 50-70% of circulating leukocytes, with an estimated of 10^{11} neutrophils produced each day. Lahoz-Beneytez et al. have found a circulatory half-life of neutrophils of 19 hours and a post-mitotic transit time of around 5.7 days in healthy individuals that is consistent with the traditional dogma stipulating a rapid turnover of neutrophils in the blood [117]. Blood neutrophils follow a circadian rhythm, following the same kinetics of G-CSF level with a decrease during the night [118]. Neutrophils have an essential role in innate immunity especially in anti-microbial immunity.

1. Hematopoiesis

Hematopoietic stem and progenitor cells (HSPC) are primarily localized in the bone marrow (BM), which is also a reservoir of granulocytes that can be released in case of severe infections. Maintenance of HSPC in the bone marrow is due to the production of CXCL12, which binds to CXCR4, allowing the control of migration and retention of HSPC in the bone marrow. Furthermore, aged neutrophils expressing high level of CXCR4 are also recruited back to BM to be eliminated [119, 120]. In mice, aged neutrophils clearance by the bone marrow has been shown to follow circadian cycles. The circadian elimination of aged neutrophils leads to the decrease in size and function of the hematopoietic niche, consequently promoting the release of hematopoietic cells into the blood [120]. Neutrophils can also be eliminated by the spleen and liver [121]. HSPC, and more precisely granulocyte-monocyte progenitors (GMPs), differentiate into neutrophils thanks to extracellular (cytokines/growth factor) and intracellular stimuli (transcription factor/microRNA). GMP will give rise either to monocytes/macrophages via monoblast lineage or granulocytes (eosinophils, basophils and neutrophils) via myeloblast lineage. Following GMP, there are two major phases to obtain neutrophils: the proliferative phase and the non-proliferative phase. In the proliferative phase, GMP differentiate into myeloblast, promyelocytes (round nucleus and dark cytoplasm) and finally myelocyte (round nucleus and less dark cytoplasm). Thereafter, the non-proliferative phase starts with the differentiation of myelocytes into metamyelocytes (kidney-shaped nucleus and clear cytoplasm), banded neutrophils and finally mature circulating neutrophils. The maturation process is tightly regulated by G-CSF produced upon IL-17A stimulation, which is synthesized by neutrophil-regulatory T cells ($\gamma\delta$ T cells and natural killer T like cells) in response to IL-23, provided by activated DC and macrophages [122]. C/EBP ϵ is an essential transcription factor for the transition of GMP into pre-neutrophils ; moreover, this transcription factor is involved in the production of secondary granules [123]. Inflammatory responses involve the release of either cytoplasmic granules or secretory vesicles. Granule formation starts during the transition of myeloblast to promyelocytes and continues until the maturation of neutrophils is reached. They are composed of primary granules (or azurophil granules), secondary granules and tertiary

granules (or gelatinase granule). Primary granules contain several proteins including myeloperoxidase (MPO), serine protease and defensin (Human neutrophil peptide; HNP). Secondary granules (or peroxidase negative granules) mainly contain lactoferrin and gelatinase. Finally, tertiary granules are predominantly composed of matrix metalloproteinase 9 (MMP9 or gelatinase B). Secretory vesicles are created by endocytosis in late stage of neutrophil maturation and are composed of plasma protein [124–126]. S100A8, S100A9 and S100A12 are small calcium-binding cytosolic proteins expressed by neutrophils. S100A8 is a chemotactic agent for neutrophils, S100A9 a pro-inflammatory factor that stimulates migration, phagocytosis and degranulation. S100A8 and S100A9 can form a heterodimer called calprotectin. This heterodimer is found at high concentration in the plasma of individuals suffering from acute and chronic inflammation [127].

Release of mature neutrophils in the blood requires G-CSF alongside with CXCL1/CXCL2 chemokines. G-CSF induces a downregulation of CXCR4 expression and CXCL12 release. Furthermore, CXCL1/CXCL2 produced by BM endothelial cells will bind to CXCR2, a cell surface receptor on neutrophils leading to neutrophil released in the blood [128–130]. In the bone marrow, Evrard et al. have described three types of neutrophils in human : Pre-neutrophils (CD15⁺ CD66b⁺, CD49d⁺, CD101⁻), immature neutrophils (CD15⁺, CD66b⁺, CD49d^{mid}, CD101⁺, CD10⁻, CD16^{mid}) and mature neutrophils (CD15⁺, CD66b⁺, CD49d⁻, CD101⁺, CD10⁺, CD16⁺) [130]. CD64 is mildly expressed in pre-neutrophils, weakly expressed in immature neutrophils and mature neutrophils are CD64⁻ [Fig8]. CD64 is a high affinity receptor for IgG and is involved in antibody-dependent cell mediated cytotoxicity, phagocytosis and regulation of cytokine production. Its expression is increased on neutrophils during septicemia [131]. Blood neutrophils of HIV-1 patients express higher level of programmed death-ligand 1 (PD-L1) compared to healthy donors, and the pathway PD-1/PD-L1 contributes to T cell suppression function in HIV-1 infected individuals [132]. The elevated levels of PD-L1 on neutrophils are also observed in systemic lupus erythematosus patients, which correlate with disease severity [133]. At steady state, only mature neutrophils circulate in the blood, however, under severe infection, the BM might release immature subset of neutrophils into the blood. Indeed, in a pancreatic tumor mouse model, there is a

high amount of immature neutrophils circulating in the blood, correlated to increased tumor burden [130]. During late chronic SIV infection in cynomolgus macaque, immature neutrophils are the main subset of neutrophils in the blood, characterized by a low expression of CD11b, CD32a, CD45, CD66 and high expression of CXCR4 compared to mature neutrophils [134].

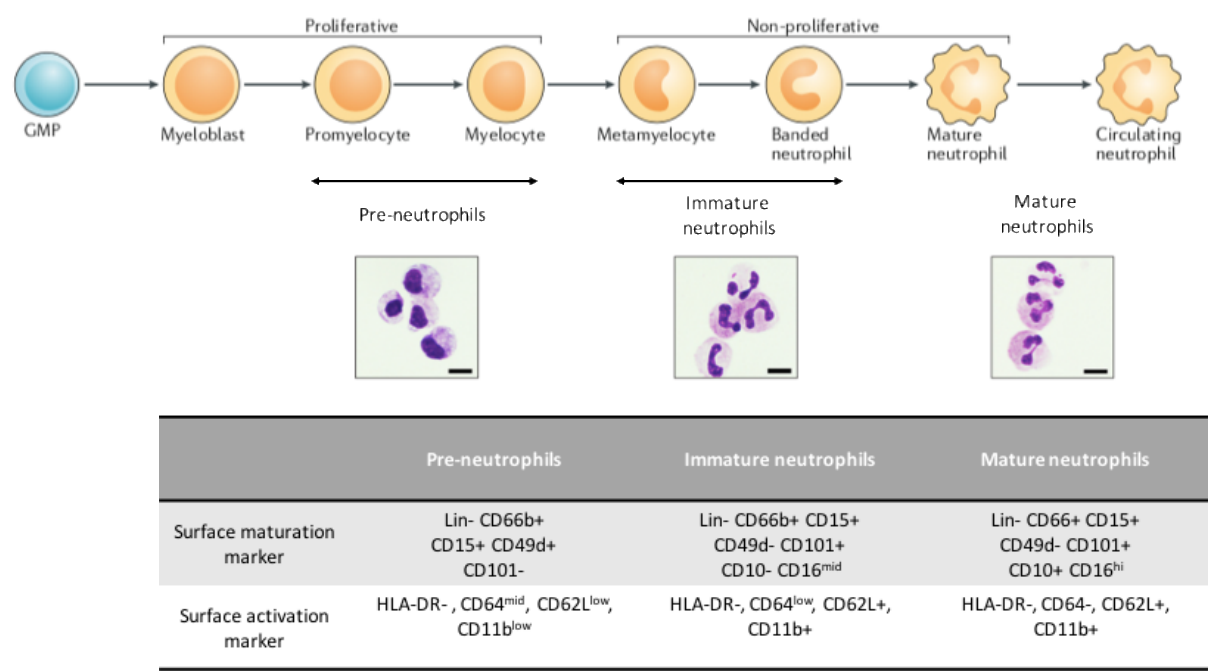


Figure 8: Bone marrow neutrophil subpopulations in Human.

Neutrophil differentiation process as well as surface activation and mature marker expression on different subsets of neutrophils (pre-neutrophils, immature and mature neutrophils). Adapted from Evrard M. et al., 2018 and Ng LG. et al., 2019 [129, 130].

2. Activation status and role in immunity

Neutrophils exhibit three main phenotypes classified as resting, primed and activated. Blood circulating neutrophils have a resting phenotype with a round morphology, are non-adherent and have a limited response to stimuli. Resting neutrophils move in the blood stream, constantly sampling the environment for signs of inflammation. Pro-inflammatory stimuli and chemokine gradient lead to adhesion, rolling, crawling and transmigration of primed neutrophils into the tissue [Fig9]. Several product including viral/bacterial products (LPS) or pro-inflammatory cytokines like TNF α , IL-18, IL-8 or GM-CSF induces neutrophil priming. Different priming agents induce different effects on the structural organization of

neutrophils. Upon priming, neutrophils become adherent and are able to transmigrate, release granules, synthesize cytokines and lipids. Priming enhance chemotaxis, respiratory burst, NET formation and results in a delayed apoptosis in response to a second stimuli [135]. CD62L shedding and CD11b high expression on neutrophils are correlated to priming in the blood [136]. CD62L, or L-selectin, is a type I transmembrane glycoprotein involved in “rolling” of neutrophils on the vascular endothelial surface [137]. Transmigration in the tissue leads to release of secretory vesicles and specific granules [138].

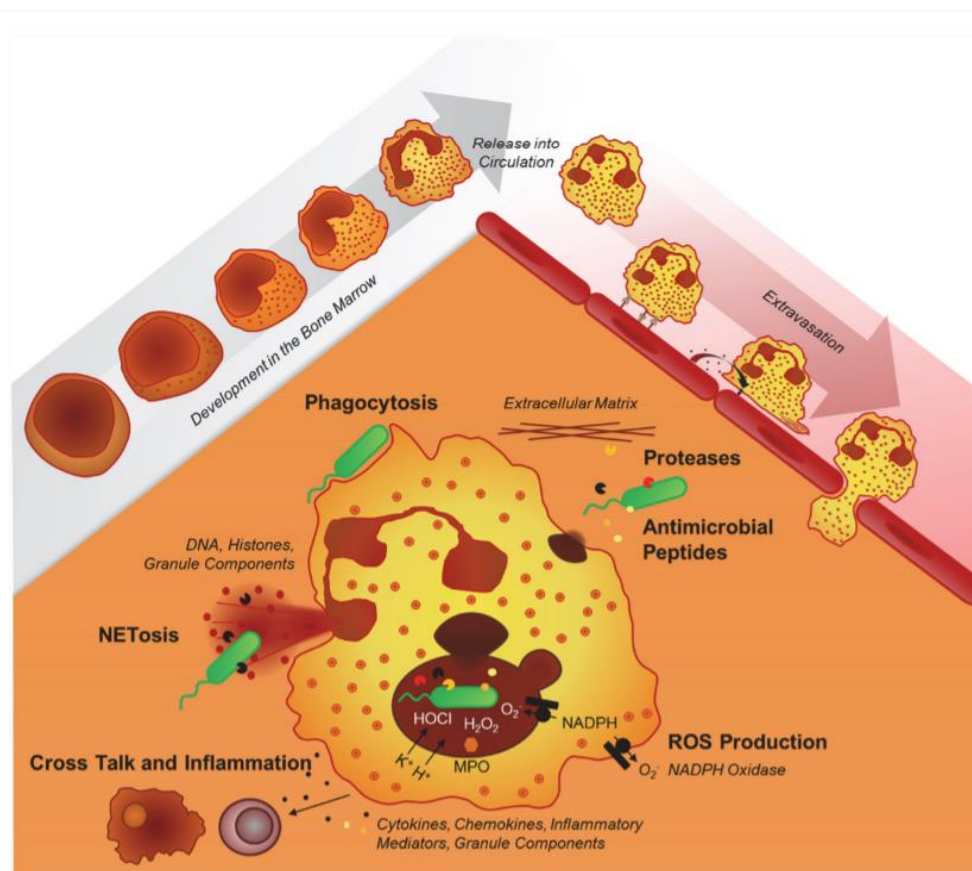


Figure 9: Neutrophils

Upon stimulation, neutrophils attach to the endothelium through selectin ligand. After attachment, chemokine lining of the endothelium luminal part induces the rolling of neutrophils. Following rolling event, crawling is initiated by integrin attachment of the neutrophils to the endothelium. Chemokine gradient leads the neutrophils to the preferential sites of transmigration. Transmigration can occur through paracellular events or transcellular events. Neutrophil immune functions include phagocytosis, NETosis, antimicrobial peptides/cytokines/ROS production. Extracted from Kruger P. et al., 2015 [138].

Within the tissue, neutrophils are able to kill pathogens by several intra and extracellular mechanisms. Phagocytosis is mediated through the binding of receptors such as Fcγ receptors, C-type lectins or complement receptors leading to engulfment of pathogens. Elimination is then induced by NADPH oxidase-dependent mechanism (reactive oxygen species (ROS) production) or antibacterial proteins released from neutrophil primary or secondary granules into the phagosomes [138]. Killing through antibacterial proteins is efficient on both intra and extracellular pathogens. Extracellular pathogens can also be eliminated using NET; it will immobilize the pathogens facilitating phagocytosis. NETs are core DNA element to which histone are attached to antimicrobial protein (for instance lactoferrin and cathepsin), protease-like (for example elastase and gelatinase) or enzymes responsible for ROS generation (MPO) [Fig9] [125, 139]. NET release can be triggered by multiple stimuli including immune complex of IgG and IgA for instance [140]. Tissue neutrophils can also interact directly or indirectly with macrophages, DC, B and T cells for instance [141]. Immature neutrophils (CD10⁻) were described to promote T cell survival, proliferation and IFN γ production by T cells [142]. On the contrary, CD10⁺ neutrophils are also involved in T cell suppression mediated by PD-L1 expression [142, 143]. Neutrophils are involved in B cell survival, maturation and differentiation by producing cytokines such as B cell activating factor of the tumour necrosis factor family (BAFF) and A Proliferation-Inducing Ligand (APRIL) [144–146].

Neutrophils express TLRs 1-9 and upon appropriate stimulation, they produce a wide range of pro/anti-inflammatory and immune-regulatory cytokines, chemokines, colony stimulating and angiogenic factors, TNF family members and growth factor [Fig10]. Those results point out several roles of neutrophils, for instance, in wound healing and repair functions, vessel growth, autoimmune diseases and inflammatory diseases. A review by N. Tamassia et al. highlights controversial results for IL-10, IL-6, IL-17A, IL-17F, IFN α , IFN γ production by neutrophils, and make several hypothesis to explain them: differential expression pattern of mouse and human neutrophils; contaminating monocytes in isolated neutrophil populations and various methodology [147, 148].

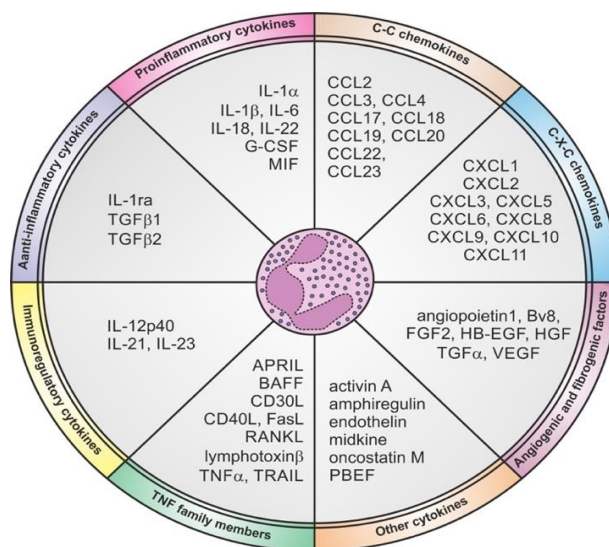


Figure 10: Cytokine expression by neutrophils.

Extracted from Tamassia N. et al., 2018 [147].

3. Genital neutrophils

Neutrophils are present in all the compartments of the FRT. The highest presence of neutrophils is in fallopian tubes and this number decreases from the upper to the lower tract [61]. Neutrophils in fallopian tubes have been described to be phenotypically and functionally different from the neutrophils in the blood. Indeed, they expressed lower level of CD66b, CD62L, IL-8 receptor (CXCR1, CXCR2) and higher level of bacterial product receptor, CD64, HLA-DR, IFN γ , TNF α , IL-12 and VEGF compared to blood neutrophils [149]. In the uterus, a strong increase of IL-8 before the menstruation leads to a significant recruitment of neutrophils. Their role during menstruation is to allow the disruption of endometrial tissue through the release of elastase, which will activate extracellular matrix-metalloproteinases and increase immune defense [106]. In animal models (rodent & pigs), insemination induces the production of a wide array of pro-inflammatory cytokines such as GM-CSF, IL-8, IL-6, CCL2 leading to an accumulation of macrophages, DC, lymphocytes and granulocytes. The great influx of neutrophils into the uterine removes unnecessary sperm, microorganisms and seminal debris [150]. In a mouse model, neutrophil depletion induces a blockade of the estrous cycle due to a dysregulation of serum steroid hormone levels, suggesting an important role of neutrophils in regulating hormone level [151]. Estradiol treatment of mice infected with *C. albicans* blocked neutrophil migration to the vagina and

accumulated them in the ectocervix and fornix. This phenomenon is mediated by an altered expression of CD44 and CD47 expression of FRT epithelial cells enabling neutrophils to migrate to the vagina. On the contrary, progesterone treatment facilitates neutrophil migration and killing. These data suggested that neutrophil migration is dependent on sex hormones. Indeed, during the ovulation, neutrophils are not present in the vaginal lumen in order to facilitate the passage of sperm cells, although it may lead to vaginal infections [152].

Chronic infections in the FRT are known to induce an increase of neutrophils. Infection with *N. gonorrhoeae* of the cervix, induces an increase of neutrophils through the induction of pro-inflammatory cytokines by epithelial cells and resident myeloid cells (IL-6, TNF α , IL1- β , IL-8) as well as IL-17 produced by Th17 cells [153]. Th17 cells, by producing IL-17 and IL-8, induce the recruitment of neutrophils, which in turn produce CCL20 and CCL2 and recruit Th17 cells [154].

The sustained neutrophils influx in the FRT is linked to tissue damage during *N. gonorrhoeae* infection. Indeed, antimicrobial components like proteases and ROS released by neutrophils enhance inflammation and neutrophil recruitment [153]. NET production is also known to induce epithelial cell damage due to NET components (histones and MPO) [155]. Symptomatic vulvovaginal candidiasis is correlated to an increased neutrophil recruitment compared to asymptomatic vulvovaginal candidiasis in women [156]. This influx has been linked, in mice, to candidalysin, a peptide toxin produced by *C. albicans*. Indeed, mice infected with *C. albicans* deficient in candidalysin exhibit a decrease in neutrophil recruitment, damage and pro-inflammatory cytokine expression [157]. However, neutrophils are also essential for pathogen clearance. *In vitro* experiments have shown that genital neutrophils are able to quickly release NETs, allowing the inactivation of HIV-1 virions [158]. Moreover, upon HSV-2 infection in mice, neutrophil recruitment mediated through IL-36 γ expression is essential for pathogen clearance [159].

b. Adaptive immunity

Adaptive immunity is a pathogen specific immunity notably mediated by presentation and activation of T cells by antigen-presenting cells (APC) such as macrophages, DC but also epithelial cells expressing major histocompatibility complex (MHC) class II.

i. T cell responses

T cells can differentiate in helper cells such as Th1, Th2 and Th17, in regulatory cells (Treg) or in cytotoxic T cells (CD8⁺ T cells). CD8⁺ T cells through binding to MHC class I of an infected cell will induce apoptosis of this cell mediated by granzyme-perforin release. CD4⁺ T cells (Th1, Th2 and Th17) will produce a wide array of cytokines involved in elimination of pathogens and B cell priming for instance [160]. Each FRT compartment has its own immune cell distribution with the T cells as predominant immune cells within the FRT [Fig7]. T lymphocytes are the most abundant leukocytes in the FRT. There is a high abundance of T cells in the cervix: 40% of T cells are CD4⁺ and 60% are CD8⁺ cells. Moreover, T and B cells are more abundant in the ectocervix compared to the endocervix [110, 161]. The majority of CD4⁺ T cells (70%) in the cervix have effector/memory (CD27⁻CD45RA⁻) or effector phenotype (CD27⁻CD45RA⁺) and more than 60% of the CD8⁺ T cells have an effector phenotype (CD27⁻CD45RA⁺) [161]. The transformation zone contains the highest concentration of macrophages, CD4⁺ and CD8⁺ T cells compared to all FRT organs [162]. Rodriguez-Garcia et al. observed on human endocervix and ectocervix high expression of RORC2, IL-17 and CCL20 on CD4⁺ T cells corresponding to a Th17 phenotype. CD4⁺ Th17 subset is more abundant in the cervix compared to endometrium in premenopausal women [163]. In the upper tract, there are also lymphoid aggregates (LA), composed of an inner core of B cells surrounded by CD8⁺ T cells and an outer halo of macrophages, which are not found in the lower tract [164].

ii. B cell responses

B cell maturation into antibody secreting plasma cells will induce the production of pathogen specific antibodies facilitating the elimination of extracellular pathogens or cell associated antigens. IgA and IgG are found in the FRT, and IgG concentration is higher than IgA. The pIgR present on the epithelial cells allows the crossing of IgA from tissue to lumen. pIgR expression is hormonally regulated as well as antibody secretion. Indeed, pIgR level is increased during the secretory phase when estrogen level is high [165]. B cells represent 0,9% of immune cells in the cervix [161]. They are mainly found in the cervix and vagina and they are more abundant in the ectocervix compared to the endocervix in premenopausal women [108, 161]. In a mouse model, primary infection of naïve mice with HSV-2 wild type strain (WT) failed to induce a B cell recruitment in the FRT. Nevertheless, vaginal immunization of mice with thymidine kinase (TK) - HSV-2 strain, followed by challenge with WT strain, induces a robust recruitment of IgG⁺ B cells to the vagina, as well as an increased number in cervix and uterus. They also showed that CD4⁺ resident memory T cells were necessary for B cell recruitment by secreting IFN γ which induces CXCL9 and CXCL10 [166]. Those results have shown that antibody secretion upon infection is not efficient, due to the lack of tissue-resident plasma cells, and that local immunization before infection is necessary to elicit a recruitment of memory B cells secreting high levels of pathogen specific IgG in the vagina. Moreover, circulating antibodies failed to enter vaginal lumen without barrier disruption, showing an essential role of tissue specific antibody secreting plasma cells.

III. Menstrual cycle

The FRT undergoes cyclic changes starting from the puberty until menopause, which are essential for preparing pregnancy. The menstrual cycle is composed of four phases: menstruation, ovulation phase, proliferative phase (or follicular phase) and secretory phase (or luteal phase), characterized by hormonal variation. There is an average of 28 days between menstruation bleeding, which last between 3 to 7 days. Following menstruation bleeding, the proliferative phase begins. This phase is characterized by an increase of estrogen produced by a mature follicle on the ovary.

This phenomenon is induced by Follicle stimulating hormone (FSH), secreted by the pituitary gland (localized on the base of the brain). The production of estrogen peaks 1 to 2 days prior ovulation. Due to estrogen level, the lining of the endometrium becomes thicker and induces luteinizing hormone (LH) by the pituitary gland through the stimulation of gonadotropin-releasing hormone (GnRH). The increase of LH and FSH promotes the release of the ovocyte from the follicle. After the release of the ovocyte, the secretory phase starts with a decrease of LH and FSH. Corpus luteum formed inside the ovocyte, produces progesterone and estrogens. If fertilization occurs, the corpus luteum will continue to produce progesterone, and to a lesser extend estrogens, which prevent the endometrium lining to shed. On the contrary, without fertilization, the corpus luteum will be degraded and progesterone, as well as estrogen level, will drop inducing menstrual bleeding [Fig11]. All these changes in the FRT affect the immune system; Wira et al. proposed that, during the secretory phase, there is a “window of vulnerability” where the FRT is more susceptible to STI acquisition [108].

Hormonal changes in the FRT induce several modifications of the immune system involving secreted molecules (antimicrobial peptides, cytokines and chemokines), epithelial cells, fibroblasts and immune cells [108].

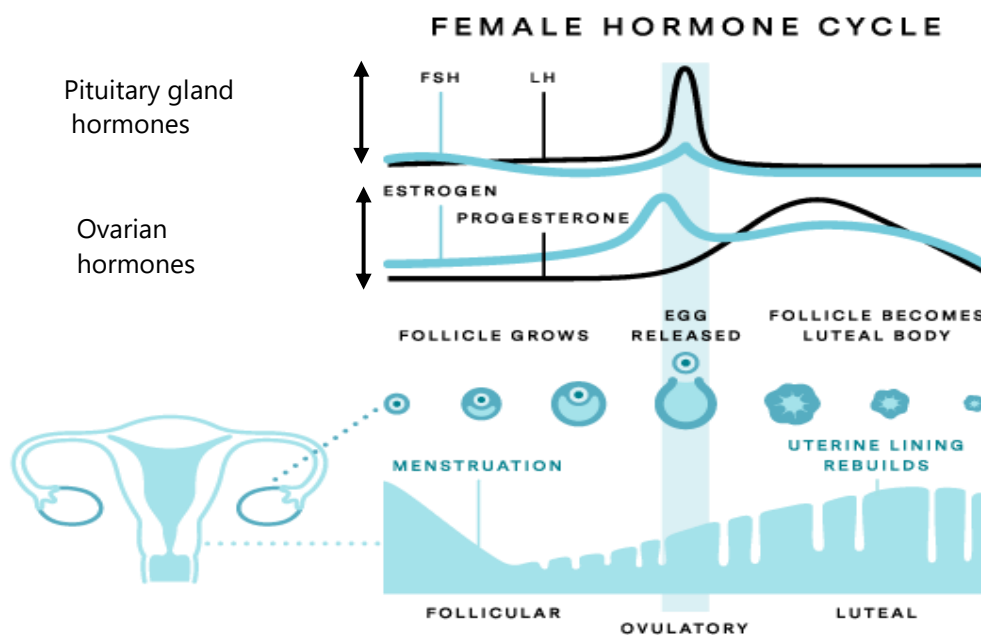


Figure 11: Female hormone cycle.

Adapted from (source: <https://ouraring.com/menstrual-cycle-impacts-on-health>).

a. Regulation of the immune cell composition by the menstrual cycle

Concerning immune cell regulation, several studies have shown a variation of immune cell numbers in the uterus but not in the vagina throughout the menstrual cycle [115, 162]. Indeed, at the ovulation and during the menstruation, there is an increased number of NK cells, neutrophils and macrophages in the uterus [106, 115]. NK cells number can reach up to 70% of total cells in the endometrium after the peak of progesterone [106, 115, 116]. In the upper tract, the size of lymphoid aggregate (LA) varies due to the hormonal cycle, with a larger size during secretory stage compared to the proliferative stage. Moreover, LA are absent in the uterus of postmenopausal women. LA might be involved in cell-mediated immunity that occurs in the uterus during the secretory phase when ovulation and implantation are most likely to occur [164]. In postmenopausal women, Th17 subset is increased in the endometrium but not in the cervix compared to premenopausal women. The lower concentration of Th17 cells in premenopausal women might be necessary for efficient fertilization and implantation [108, 163].

b. Regulation of antimicrobial peptides by the menstrual cycle

AMP are polypeptides, which are effective against bacteria, virus, fungi or protozoa when secreted at physiological level [167]. They can reduce the infection by preventing the growth of pathogens, or killing those pathogens, by direct or indirect mechanisms. Several cells are able to produce AMP, including epithelial cells and immune cells. In the FRT, they are mainly produced by epithelial cells and phagocytic cells, such as neutrophils. A wide range of AMP are present in the FRT including secretory leukocyte protease inhibitor (SLPI), defensin, human neutrophils peptide (HNP), lactoferrin, and elafin. The two main AMP families are defensins, including α and β defensin and cathelicidins. α -defensin group is composed of HNP 1 to 4, predominantly produced by neutrophils and human defensin (HD) 5 and 6. Human β -defensin (HBD) group includes HBD-1 to 4, which are mainly expressed

by epithelial cells. β -defensins induce the recruitment of CD4⁺ memory T cells, monocytes, macrophages and neutrophils, but also the maturation of immune cells like DC and mast cells. Protease inhibitor such as SLPI and elafin are also classified as AMP because of their anti-microbial effect and immunomodulatory activities. SLPI promotes for instance cell proliferation, suppresses neutrophil activation and inhibits NF- κ B pathway. Elafin maintains the barrier integrity by binding to extracellular matrix protein. Lactoferrin is an iron-binding glycoprotein, primarily stored in the secondary granules of neutrophils, whereas HNP-1 to 4 are stored in primary granules of neutrophils. One of the effect of lactoferrin is mediated by its ability to bind to iron, thus entering in competition for iron with pathogens [168].

Some studies have shown that the concentration of antimicrobial peptides varies during the menstrual cycle. Indeed, the concentration of SLPI, HBD-2, HNP-1, HNP-2, HNP-3 and lactoferrin are decreased during ovulation and 7-14 days after, during the secretory phase, and return to higher levels after menstruation during the proliferative phase [169].

c. Regulation of cytokines and chemokines by the menstrual cycle

Boomsma et al. have observed a higher expression of IL-1 β , IL-6, IL-12, IL-18, CCL2, VEGF, CCL10, MIF in the cervical fluids compared to IL-15, IL-10, IL-17, TNF α , MIF, and CCL11 [170]. Concentration of proinflammatory and immunoregulatory cytokines were measured in cervical secretions of 72 healthy women and IL-8, IFN γ , IL-2, IL-4, IL-5, IL-13 as well as IL-10, IL-12, IL-1 β and IL-6 were detected [171]. At steady state, several cytokines and chemokines are regulated by hormonal levels, whereas some cytokines were described to not be affected by the menstrual cycle [172].

Several cytokine/chemokine/growth factor expressions in the uterus are different from the cervix, with a higher expression of IL-1 β , IL-6, IL-10, IL-18, CCL2, and VEGF. IL-12 and IL-15 expression is lower in CVL compared to endometrial secretions of women prior to embryo transfer [170]. Cytokine levels, such as IL-6 and IL-8 in CVL, are increased during the follicular phase. Interestingly, the authors also observed an increase of plasma IL-8 during the follicular phase [173]. Another study, performed on CVL collected from women at different

periods during the menstrual cycle, has shown an increased expression of IL-18, IL-10, IL-17 and TGF- β and a decrease in CCL2 and stromal cell derived factor (SDF)-1 β in samples collected post-ovulation [174]. Expression of CXCR4, CCR5 and CD4 is higher in uterine epithelial cells after ovulation, and stays high during the secretory phase for CXCR4 and CD4, or decreases for CCR5. A higher expression of CCR5 and CD4 is also observed during the secretory phase in ectocervix epithelial cells [175].

d. Regulation of antibody production by the menstrual cycle

An increase in IgGA, IgG2 and total IgG levels was observed in CVL collected during ovulation [174]. The total amount of IgA, IgG and IgM reaches their maximum 3 days before ovulation and decreases by day 3-post ovulation in cervical mucus [172]. This decrease might be involved in reducing the levels of sperm-specific antibodies, permitting fertilization [108]. A summary of factors that are impacted by menstrual cycle can be seen on figure 12.

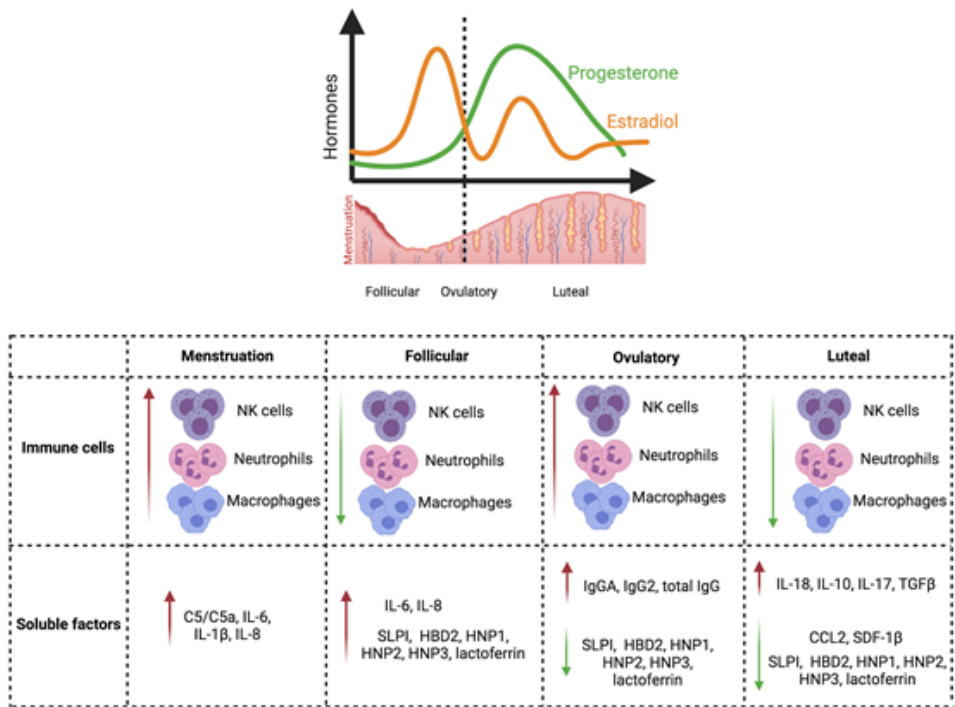


Figure 12: Factors including immune cells and soluble factors influenced by menstrual cycle in Human.

Created with Biorender.com.

e. Impact of external hormonal stimulation

Estradiol stimulation of uterine epithelial cells induces the expression of HBD1/2, SLPI and ELAFIN and decreases the expression of MIF, TNF α , IL-6 and IL-8. In contrast, estradiol decreases the expression of HBD2 and Elafin induced by vaginal epithelial cells [175].

In animal model, estrogen has been shown to stimulate epithelial cell proliferation in the vagina, as well as in the uterus, through stimulation of the insuline like growth factor receptor (IGF1R) (mouse model). In contrast, progesterone has been described to reduce the thickness of the vaginal epithelium (macaque rhesus model) [176, 177]. Moreover, women using injectable depot medroxyprogesterone acetate (DMPA) have a significantly altered expression of genes involved in maintenance of mucosal barrier function [178]. DMPA users have higher expression of several cytokines, such as IL-1 α , IL-1 β , IL-6, TNF α , IL-2, IL-4, IFN γ , CCL3, CCL4, CCL8, CXCL10, IL-8, TGF β , as well as IgG and IgA in CVL [174]. Cytokine production after *in vitro* progesterone treatment depends on the type of cells used. Indeed, PBMC treated with progesterone induce an increased expression of IL-1 β , TNF α , IL-6, IFN γ and a decrease of IL-2, TGF β , IL-4 and IL-10 mRNA. In contrast, monocytes (THP-1) treated with progesterone increase IL-10 as well as IL-1 β and TNF α mRNA level. Jurkat T cells treated with progesterone induced a decrease in IL-2 and TNF α expression, and an increase in IL-10 expression [179].

f. Hormonal cycle and STI susceptibility

Wira et al. suggested that a suppression of the immune system occurs at the end of the secretory phase to prevent rejection of the fetus, thanks to cell recruitment and decrease of AMP for instance. The authors proposed that during this phase, there is a “window of vulnerability” that increased susceptibility for STI acquisition (HIV-1) [108]. Kersh et al. ete have infected pigtailed macaque with Simian Human immunodeficiency virus (SHIV) once a week at different times of the menstrual cycle. The authors have observed that 88% of the females were infected during the follicular phase (or proliferative phase). When taking into account the viral eclipse, they estimated that the infection occurs in late secretory phase [180]. However, in a study using ecto and endocervical explant collected at different stage

of the menstrual cycle and infected with HIV-1, there were no differences in HIV-1 infection according to menstrual stage. Nevertheless, high level of progesterone was correlated with high number of CD4⁺ α4β7⁺ T cells in ectocervix and high expression of pro-inflammatory cytokines (IL-1β, IL-17, TNFα) [181]. For CT infection, cervicovaginal fluids of women suffering from either a primary or a recurrent CT infection were analyzed and the authors observed a negative correlation between IL-1β and progesterone in recurrent patient. In contrast, in primary infected patients, there was a negative correlation between IL-10, IL-1β and IL-6 with β-estradiol [80]. Moreover, intrauterine CT inoculation of minipigs during the diestrus phase (high level of progesterone) induce a longer duration of CT infection (10 days) compared to animal inoculated during estrus (3-5 days) [182]. This shows a regulation of cytokine production by hormones during infection with different effects depending of the stage of the infection.

The FRT mucosae is rich in immune and non-immune factors including the vaginal microbiota that are influence by hormonal cycle.

Chapter III: Vaginal microbiota

I. General information about the microbiota

The term microbiota was defined by Joshua Lederberg as “the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease”. The microbiota represents 90% of the total cell number of the human body, and only 10% are human cells [183]. The microbes present in and on the human body are commensal and are essential for our health. In the Human Microbiome Project, 300 subjects between 18 and 40 years old were sampled at 15 in man or 18 body parts in women [Fig13]. As expected, each body habitat has its own microbe signature, community dynamics and interaction with host tissue, and the abundance and diversity of each microbial habitat varies among subjects [184].

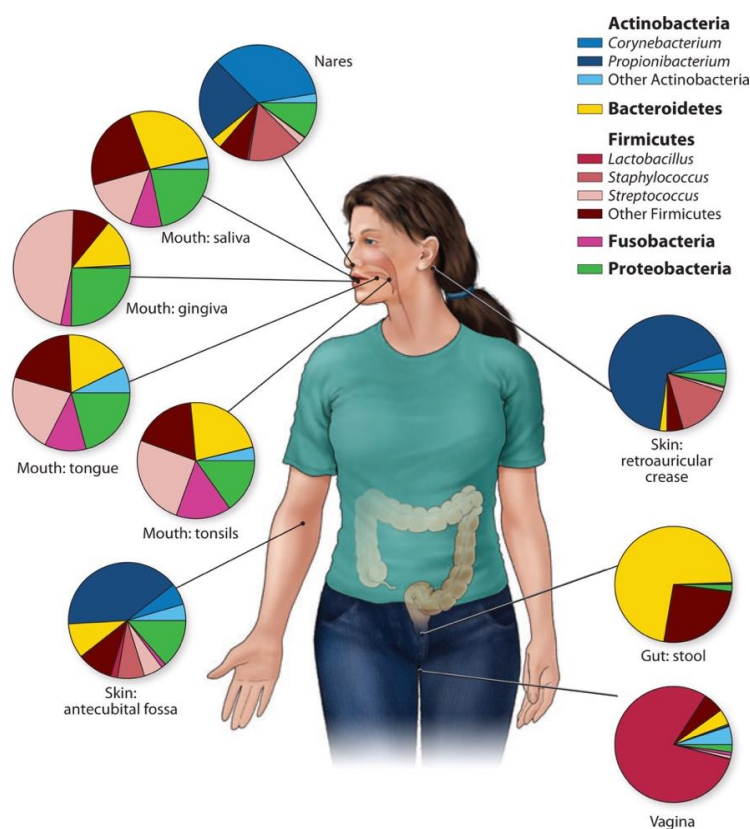


Figure 13: Microbiota composition at the phylum level in the different mucosal human compartments.

Data extracted from the human microbiome project [185].

The microbiota composition has been described to modulate host physiology such as host metabolism [186], immune regulation [187], but also anti-PD1 immunotherapy [188], autoimmunity and allergic inflammation. Hagan et al. have shown that the gut microbiota has a central role in vaccine-induced immunity [189].

Recent studies have highlighted the impact of the vaginal microbiota on STI acquisition: for HIV-1 infection [190, 191] and *Chlamydia trachomatis* infection [192] for instance.

II. Vaginal microbiota composition (eubiotic vs dysbiotic)

In humans, eubiotic vaginal microbiota is characterized by the dominance of one genus, *Lactobacillus spp.*, which maintains an acidic pH in the vagina (pH = 4 to 5) thanks to lactic acid production. It also maintains a low inflammatory environment through the production of bactericide and bacteriostatic components like H₂O₂.

Analysis of the vaginal microbiota by 16S rRNA gene sequencing and vaginal pH measurement of 396 North-American women allowed a community clustering into five groups [193]. Group I, II, III and V are dominated by one species of *Lactobacillus spp.*: *L. crispatus* (group I), *L. gasseri* (group II), *L. iners* (group III) and *L. jensenii* (group V). In this cohort, 73% of women had a vaginal microbiota dominated by *Lactobacillus spp.* Women from the group I have a pH around 4, and the other groups dominated by *Lactobacillus spp.* have a slightly higher pH between 4,4 to 5. The remaining 23% of women had a diverse vaginal microbiota (group IV) characterized by higher proportions of anaerobic bacteria (facultative or strict other than *lactobacillus spp.*) including *Prevotella*, *Dialister*, *Atopium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Mobiluncus*, with a vaginal pH starting at 5 and rising up to more than 5,5. The group IV can be divided into two groups : microbiota with a modest proportion of *Lactobacillus spp.* and low number of various species of anaerobic bacteria like *Anaerococcus*, *Corynebacterium*, *Finnegoldia* or *Streptococcus* (group IV-A), or dominated by diverse anaerobic bacteria belonging to genus *Atopium*, *Prevotella*, *Parvimonas*, *Sneathia*, *Gardnerella* or *Mobiluncus* (group IV-B) [194]. The increased diversity

and abundance of anaerobic bacteria and the decrease of *Lactobacillus spp.* leads to a clinical condition called bacterial vaginosis (BV). This diverse vaginal microbiota composition has been associated with local inflammation, characterized by an increase in pro-inflammatory cytokines and presence of activated cells, which in turn increase the susceptibility to STI but also poor obstetrics events [Fig14] [195]. In subjects suffering from BV, *L. crispatus* was not detected whereas *L. iners* was present in 5 patients out of 9 [196]. Common bacteria detected in BV + women are : *Mycoplasma hominis*, *Gardnerella vaginalis*, *Prevotella spp.*, *Mobiluncus spp.*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus spp*, *E. coli* [104]. BV is mostly asymptomatic or induces mild symptoms.

In clinics, two methods are used to determine bacterial vaginosis: Nugent score and Amsel criteria in combination with vaginal pH measurement. Amsel criteria is based on 5 variables : milky thin homogeneous vaginal discharge, a vaginal pH greater than 4,5, a fishy amine odor, abnormal amines in vaginal fluids (putrescines, cadaverines and trimethylamine) and significant presence of clue cells [197]. Clue cells are epithelial cells coated with bacteria. Nugent score is a Gram staining of vaginal fluid and quantification of the number of bacteria including *Lactobacillus spp.* Three groups are determined: normal (0-3), intermediate (3-6), BV (7-10) [198].

Srinivasan et al. have demonstrated a strong metabolic signature in women suffering from BV, correlating with the presence and abundance of specific bacteria. In BV+ women, several metabolites are associated with BV clinical criteria: elevated pH (increase of tyramine, cadaverine, N-acetylputrescine, sphingosine and decrease in tyrosine and glutamate), vaginal discharge (increase cadaverine, agmatine), presence of clue cells (increase of deoxycarnitine, pipecolate and decrease of glutathione, glycylproline) and amine odor (increase of N-acetylputrescine and decrease in lactate, phenylalanine). A group of BV associated bacteria including *Megashaera*, *G. vaginalis*, *A. vaginae*, *Sneathia sanguinegens*, *Parvimonas micra*, *Dialister*, *Prevotella timonensis* and *BVAB2* were highly correlated with metabolites associated with BV (succinate, cadaverine, putrescine, tyramine and deoxycarnitine). On the contrary, *L. crispatus*, *L. jensenii* and *L. iners* exhibit opposite metabolites correlation patterns [199].

Metronidazole is the antibiotic often used by women suffering from BV. BV+ women, that cured BV after treatment with metronidazole gel for 5 days, have a reduction of several BV associated bacteria (Bacterial vaginosis associated bacterium (BVAB1/2/3), *Megasphaera*, *Atopium*, *Sneathia* and *G. vaginalis*) [200]. Moreover, monthly oral treatment with metronidazole and fluconazole (antifungal treatment) was associated with an increase in *Lactobacillus* dominant microbiota [201]. However, in a cohort suffering from recurrent BV, after an application of metronidazole gel twice weekly for 16 weeks followed by an interruption of the treatment for 12 weeks, there were 51% of the women exhibiting a BV recurrence [202]. Recently, vaginal microbiota transplantation (VMT) have shown interesting results to cure recurrent bacterial vaginosis. Indeed, VMT of four patients out of five induced a full remission until 5-21 months after VMT [203].

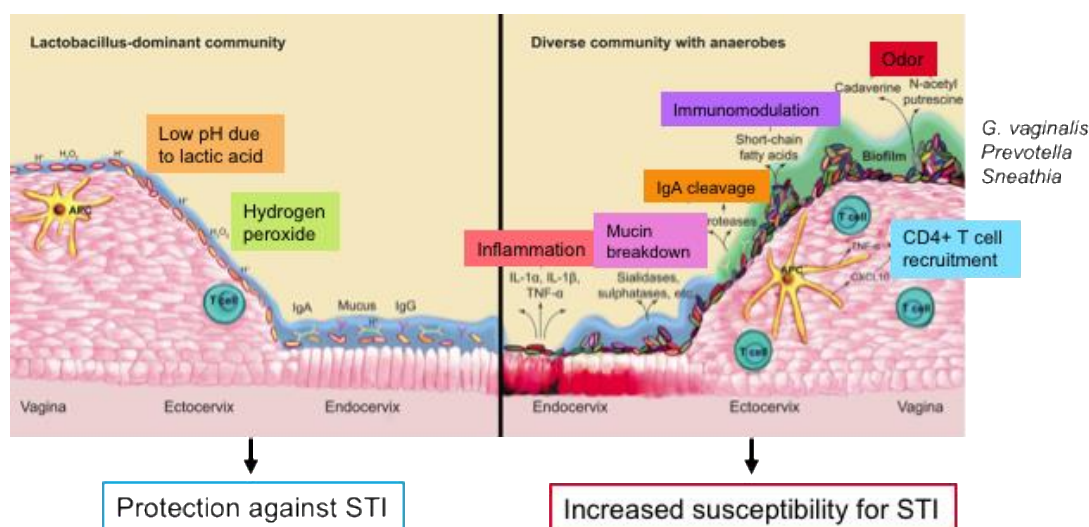


Figure 14: Vaginal microbiota composition and impact on STI acquisition.

Left panel represent a *Lactobacillus spp* dominant microbiota and right panel a diverse vaginal microbiota composed of anaerobic bacteria (ie *G.vaginalis*, *Prevotella spp.*, *Sneathia spp.*...). Adapted from Petrova MI et al., 2013 and Anahtar MN et al., 2018 [104, 204].

a. *Lactobacillus spp.*

Lactobacillus spp. are rod shape (bacillus) Gram-positive facultative anaerobic bacteria. Different species of *Lactobacillus spp.* are found in the vaginal microbiota, such as *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. plantarum*, *L. casei*, *L. vaginalis*, *L. iners*.

L. crispatus and *L. jensenii* concentration have been described to correlate positively with metabolites, such as sugars, lactate, amino acids and dipeptides, but negatively correlated with tyramine, pipecolate, cadaverine putrescine and agmatine, which are increased in BV+ women. *L. iners* exhibited correlation patterns that are intermediate between *L. crispatus*/*L. jensenii* and BV associated bacteria [199].

Lactobacillus have been proposed as probiotic agents to be used against the overgrowth of pathogenic microorganisms in the vagina. They could enter in competition with pathogens for attachment site on epithelial cells, co-aggregate with pathogens and produce antimicrobial compounds. *Lactobacillus* can produce : (1) biofilm allowing to mask epithelial cell receptors, adhesion to mucin and epithelial cells [205, 206] (2) lactic acid that can inhibit pathogen growth (3) bacteriocins and bacteriostatic agents like H₂O₂. Vaginal lactic acid exists as D and L-isomers. Epithelial cells can only produce L-lactic acid but *Lactobacillus*, depending of the species, produce both isomers. In axenic culture, *L. crispatus* and *L. gasseri* are able to produce both isomers. On the contrary, *L. jensenii* only produces D-lactic acid and *L. iners* can only produce L-lactic acid [207]. *L. iners* colonization has been linked to a lower vaginal pH compared to other species of *Lactobacillus* [208]. *Lactobacillus* will produce lactic acid in order to acidify the vagina, but when they reach a pH range between 3,2 and 4,8, they stop their growth and acid lactic production (*in vitro* study) [209]. MMP-8, which is able to degrade the cervical plug, is down regulated by D-lactic acid through decrease of extracellular metalloproteinase inducer (EMMPRIN), thus inhibiting pathogen migration to the uterus [207]. H₂O₂ was described as a potent antimicrobial factor produced by *Lactobacillus*, however, under hypoxic condition found in the vagina, *Lactobacillus* production of H₂O₂ is undetectable [210, 211]. Moreover, physiological concentration of H₂O₂ are not sufficient to have a bactericidal effect on pathogens, suggesting that the main antimicrobial agent produced by *Lactobacillus* is lactic acid [211]. A recent paper have demonstrated different inflammatory properties of *Lactobacillus* depending on if they were isolated from optimal or non-optimal vaginal microbiota. Indeed, stimulation of vaginal epithelial cell line with *Lactobacillus*, obtained from women with intermediate flora, or BV,

induced an increased inflammation with higher level of IL-6, IL-8, IL-1 α , CCL3, CCL4 and IL-1RA compared to the ones isolated in optimal microbiota. Interestingly, *Lactobacillus* isolated from women with non-optimal microbiota produced less D-lactate and lactic acid, compared to *Lactobacillus* isolated from women with optimal microbiota. Moreover, *Lactobacillus* cell adhesion was negatively correlated with cytokine expression. All those results suggest that anti-inflammatory properties of *Lactobacillus* depend greatly on the strain studied [212].

b. *G. vaginalis*

G. vaginalis is a coccobacillus Gram variable facultative anaerobe bacteria often associated with BV. *G. vaginalis* is the predominant agent inducing biofilm, indeed, a study has shown that most of BV⁺ women had a *G. vaginalis* biofilm covering the endometrium [213]. Moreover, *G. vaginalis* production of vaginolysin, a cholesterol-dependent cytolysin, induces adherence of the bacteria with host epithelial cells, its subsequent multiplication and biofilm formation, critical for its survival [214]. *G. vaginalis* predominates the vaginal microbiota during menstruation, possibly due to iron increase [215]. This bacteria was present in all the patients (n=27) suffering from BV. However, it was also observed in 59% of BV⁻ patients (n=46), suggesting that *G. vaginalis* is also present in healthy women [196]. Genome sequencing of 17 clinical isolates of *G. vaginalis* demonstrated a high genotype diversity, with two sets of strains (A and B) divided in four clades (group 1 and 2, and group 3 and 4 respectively) [216]. Hilbert et al. have observed that the four clades of *G. vaginalis* are present in most of the vaginal fluids sampled (n=394), independently of their BV status (normal, intermediate or BV) but the abundance of the four clades are higher in BV⁺ patients compared to normal flora. Clade 1 and 2 decrease upon antibiotic (Clindamycin, metronidazole and tinidazole) treatment but increase post-treatment in women suffering from recurrent BV suggesting an association of clade 1 and 2 with BV status [217].

Metronidazole is partially effective against *G. vaginalis*. Indeed, in a cohort study, only 58% of treated patient showed an eradication or a significant decrease of *G. vaginalis*, suggesting either a resistance of some strains to metronidazole, or a presence of biofilms

protecting *G. vaginalis* from eradication [218]. *G. vaginalis* alone does not systematically induce BV [219], but it has a central role in BV acquisition by being a part of a group of organisms including *Atopium*, *Megashaera*, *Sneathia* and BVAB1/2/3 involved in the induction of a dysbiotic environment. A recent review discusses a BV model in four steps involving several bacteria: (1) *G. vaginalis* colonization will remove *Lactobacillus* and initiates biofilm production, (2) *G. vaginalis* will facilitate the growth of *Prevotella bivia*, (3) production of enzymes along with vaginal sialidase by *G. vaginalis* and *P. bivia* will induce the breakdown of the mucus layer (4), the loss of the mucus barrier will favour the colonization by other anaerobic bacteria, such as *A. vaginae*, inducing a pro-inflammatory environment [220].

III. Factors impacting the vaginal microbiota

Various factors are known to influence the vaginal microbiota, such as hormones, contraceptive use, sexual behaviours, ethnic status etc. Recently, others factors including nutrition, stress or tobacco use have also been shown to influence the vaginal microbiota composition. Indeed, a relationship between oral and vaginal microbiota have been observed in 94 adolescent women. Authors have observed an increased diversity in the salivary microbiota of women suffering from BV [221]. In addition, vegetarian diet is associated to a higher vaginal microbiota diversity compared to non-vegetarian. In the same study, the authors also described an increased alpha-diversity with exercise time per day, suggesting an impact of diet and metabolism on the vaginal microbiota composition [222]. In a cohort composed of 20 smoker's women and 20 non-smokers women, the authors found an association between low proportions of *Lactobacillus spp.* in the vaginal microbiota of smoking women [223]. Finally, a review discussed the importance of stress hormone such as cortisol in regulating vaginal glycogen deposition, thus in the maintenance of a healthy environment (eubiosis) [224].

a. Hormones

The vaginal microbiota composition changes drastically throughout a woman life. After birth, infant vaginal microbiota is mainly composed of bacteria from the gut microbiota through natural ascension [104]. Low estrogen level and thin vaginal epithelium in prepubertal girls allow the colonization of the vagina by Gram-negative anaerobe bacteria such as *Veillonella*, *Bacteroides*, *Fusobacteria* and Gram-positive bacteria including *Actinomyces*, *Bifidobacteria*, *Peptococcus*, *Peptostreptococcus*, *Propionibacterium*. Gram-positive aerobes are also found in prepubertal girls including *S. aureus*, *Streptococcus viridans*, *Enterococcus faecalis*, *Corynebacteria* and *Diphtheroids* [225].

At the puberty, the vaginal microbiota composition drastically changes with an increase of *Lactobacillus spp* and a decrease of vaginal pH. Those changes coincide with an increased level of estrogen, inducing thickness of the vaginal epithelium and rise in glycogen deposits in vaginal epithelial cells. In a cohort study of African-American women, free glycogen in vaginal fluids has been associated with a vaginal microbiota predominantly composed of *Lactobacillus* and a low vaginal pH [208]. Moreover, *L. crispatus* and *L. jensenii* levels were higher in vaginal fluids exhibiting high concentrations of free glycogen. However, *L. iners* was not found at higher level in sample containing high amount of free glycogen. No positive correlation was observed between free glycogen and estrogen level measured in vaginal fluids. However, a negative correlation was observed between progesterone and cell free glycogen [226]. The authors hypothesis that the absence of positive correlation between estrogen and cell free glycogen is due to a lag time between estrogen increased, epithelial cells shedding and glycogen release. Nevertheless, *Lactobacillus* do not use glycogen but rather glycogen break-down products. Indeed, α -amylase present in vaginal fluids will degrade glycogen into maltose, maltotriose and maltotetraose which are necessary for *Lactobacillus spp.* growth [227] [Fig15]. In a recent paper, community change rate and diversity measured by alpha diversity were negatively correlated with estradiol levels, whereas the relative abundance of *Lactobacillus* was positively correlated to estradiol levels. This hormone was not measured but obtained from previously established values by Minassian and Wu, Int J Gynecol Obstets 1993 [222, 228]. During menstruation, quantity of

Lactobacillus is inversely related to *G. vaginalis* concentration [229]. In addition, *Streptococcus spp.*, *Peptostreptococcus spp.* and *Anaerococcus spp.* tend to increase during menstruation [222].

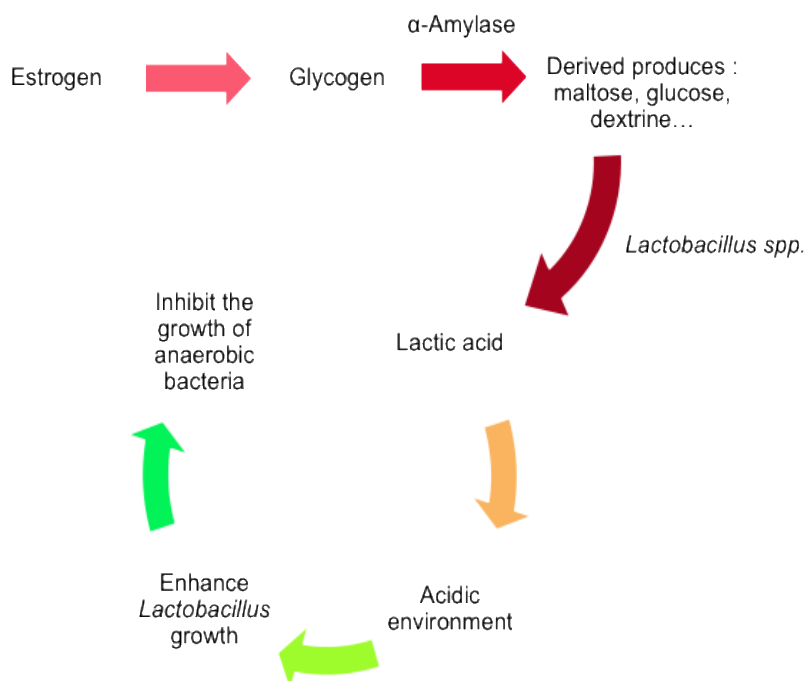


Figure 15: *Lactobacillus spp.* growth cycle.

Increased level of estrogen enhances epithelial shedding and release of glycogen in the vagina. The glycogen is then degraded by α -amylase into glycogen break down like maltose, which is taken up by *Lactobacillus spp.* to produce lactic acid. Then lactic acid induces an acidic environment that enhances lactobacilli growth and inhibits anaerobic bacteria growth.

In postmenopausal women, there is a decrease of estrogen level, inducing change in vaginal microbiota composition. Indeed, the vaginal microbiota in postmenopausal women is dominated by *L. iners* and *G. vaginalis* and a lower abundance of several bacteria including *Sneathia*, *Mobiluncus*, *Bifidobacterium*, *Gemella* [230]. Glycogen levels in vaginal fluids of postmenopausal women is significantly lower compared to premenopausal women, that correlate with a lower median vaginal pH (4 vs 4,6) [231].

b. Contraceptive use

Contraceptive use is also involved in the modulation of the vaginal microbiota. Brooks et al. have shown that women using combined oral contraceptives (COC) were significantly associated with vaginal *Lactobacillus spp.* colonization and were less likely to be colonized by BV associated bacteria [232]. A study, performed in 130 HIV negative women, described that COC users have a lower vaginal microbiota diversity compared to norethisterone enanthate (Net-En) and etonorgesterol/ethinyl estradiol combined contraceptive vaginal ring (CCVR) users [233]. Nevertheless, use of condom and levonorgestrel-releasing intrauterine system (LNG-IUS) are associated with the presence of BV associated bacteria in the vaginal microbiota. DMPA users have an intermediate vaginal microbiota composition: between COC users and LNG-IUS users. In a second study performed on 266 asymptomatic healthy women that initiated different contraceptives, BV prevalence was increased in women using copper intrauterine devices with an increased *G. vaginalis* and *Atopium vaginae* colonization [234]. Moreover, in terms of STI acquisition, medroxyprogesterone acetate has been correlated to an increased HIV-1 infection using cervical explant [235]. Net-En or CCVR users have also an increased abundance of species associated with an increased risk of HIV-1 such as *Prevotella*, *Sneathia*, *Parvimonas* and *Mycoplasma*. In addition, a higher local inflammation was observed in CCVR users, exhibited by a higher level of cytokines (IFN γ , IL-1 β , IL-21, IL-33, IL-6, TNF α) in cervicovaginal fluids compared to Net-En and COC users [233]. Another study showed that women using progestin-only contraceptives have a modified periodic variation of the vaginal microbiota and a lower *Lactobacillus* abundance compared to women using combined contraceptives [222].

c. Ethnic status

Ravel et al. have studied the vaginal microbiota of North American women from four ethnic groups (white, Hispanic, black and Asian). Vaginal microbiota dominated by *Lactobacillus spp.* were observed in 80.2% and 89.7% of asian and white women respectively. However, only 59.6% and 61.9% of hispanic and black women display a vaginal microbiota

dominated by *Lactobacillus*. A higher prevalence of non-*Lactobacillus* dominated vaginal microbiota was observed in hispanic and black women as well as a higher vaginal pH [193]. A recent study has also shown that a minority of South African women have a *Lactobacillus* dominant microbiota (37%), among them 77% have a vaginal microbiota dominated by *L. iners* [195]. The authors also observed that 45% had a *Gardnerella* dominant microbiota and the remaining 55% had a diverse vaginal microbiota with at least 10% of *Prevotella*. Despite the lack of *Lactobacillus* dominancy, those women are considered as healthy.

d. Sexual behaviors, hygiene & STI

BV acquisition has been correlated with several factors: history of prior STI, high number of sexual partners, new sexual partner when the symptoms occur and douching [236]. Douching has been reported to alter vaginal flora, and is associated with BV. This practice is more common among black women, who exhibit a more diverse microbiota, compared to hispanic or white women [237]. Vaginal douching is also associated to a higher local inflammation in BV+ women, which is known to increase the risk of STI acquisition [238]. The male partner of BV+ women have penile skin communities similar to the vaginal communities of their sexual partner, supporting the hypothesis of BV transmission through sexual contact [239]. A study performed in heterosexual couples showed that the enrichment of penile *Dialister* and *Megasphaera* species was positively associated with BV. Moreover, those species were also increased in BV positive women, suggesting that BV associated bacteria could come from the penile microbiota of the sexual partner [240].

STI acquisition is also an important factor that induced vaginal microbiota modification. In a cohort-based study, 46% of the women had bacterial vaginosis and with a higher risk for *N. gonorrhoeae* or CT infection. The authors were not able to determine if bacterial vaginosis were present before STI acquisition, thus facilitating STI acquisition, or if the presence of the STI modified the vaginal microbiota, inducing a bacterial vaginosis [241]. Tamarelle et al have observed that, out of 21 CT infected young African American women, 21.6% were belonging to group III (*L. iners* dominated microbiota) and 17.2% had a group IV (highly diverse microbiota) microbiota [242]. Furthermore, in another study, CT+ women

had mainly a CST IV microbiota confirmed by an increase concentration of putrescine and cadaverine, compared to uninfected women [243]. The authors also observed that metabolomes of CT+ and uninfected women clustered separately. Several metabolites were increased in CT+ women compared to uninfected women after microbiota adjustment, suggesting a role of CT in modifying the metabolome. Indeed, higher concentration of multiple long chain fatty acids and fatty alcohols were observed in CT+ individuals [243]. Finally, HSV-2 infection in a South African cohort of 2750 women has been positively associated with BV acquisition [244].

To conclude, many different factors can influence the composition of the vaginal microbiota, they are summarized in the figure below [Fig16].

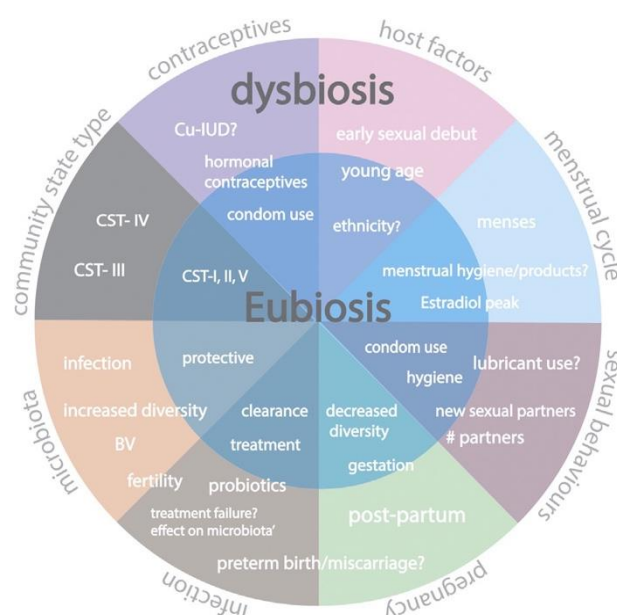


Figure 16: Diagram representing a wide array of factors influencing vaginal microbiota composition.

Extracted from Kroon et al., 2018 [245].

IV. Modulation of the immune system by the vaginal microbiota

The vaginal microbiota of 236 HIV uninfected women from the FRESH (Female Rising through Education, Support and Health) cohort was studied and divided into 4 community state type (CST). Only 37% of the women had a *Lactobacillus* dominant microbiota divided

in non *L. iners* *Lactobacillus* (CST1) and *L. iners* predominant microbiota (CST2), 45% had a *G. vaginalis* dominant microbiota (CST3) and 18% had no consistent predominant bacteria but had at least 10% of *Prevotella* (CST4). Women with CST4 were four times more likely to have elevated genital pro-inflammatory cytokines, compared to those with CST1-3. More precisely, IL-1 α , IL-1 β , TNF α , IFN γ , IL-8, IL-12-p70, IL-4, IL-10, and FMS-like tyrosine 3 ligand (FLT-3L) were significantly increased in women with CST4 compared to women with CST1. Women with CST3 exhibited a less pronounced increase in cytokines levels, with only significant higher IFN γ and a trend towards higher TNF α , IL-8 and IL-10. IL-1 α , IL-1 β and TNF α were strongly associated with CT in women that had a change in microbial communities during follow-up visits. Six bacteria in women with CST4 were highly correlated with inflammation: *Fusobacterium*, *Aerococcus*, *Sneathia*, *Gemella*, *Mobiluncus* and *Prevotella* [195]. K. Lennard et al. identified the same bacteria as well as other species associated with inflammation, such as *BVAB1/2/3*, *Prevotella amni* and *pallens*, *Parvimonas micra*, *Megasphaera*, *G. vaginalis* and *A. vaginae* in South African women aged 16 to 22 years old [246]. In addition, L. Masson et al. have determined that elevated expression of IL-1 α , IL-1 β and decrease of CXCL10 were associated with asymptomatic STI or BV in HIV negative young women (>500). Those markers were proposed by the authors to be used as biomarker for asymptomatic STI or BV [247]. In another study conducted on CVL, obtained from an African cohort, the authors observed an increase of IL-1 α , IL-1 β , G-CSF, GM-CSF and a decrease of CXCL10 in women suffering from dysbiosis [248]. In *in vitro* experiments, epithelial endocervical (End1)/ectocervical (Ect1) and vaginal cell line (VK2) were exposed to high concentration of bacteria from the vaginal microbiota. Cells showed an increased inflammation when they were exposed to *G. vaginalis* or *A. vaginae* compared to *L. johnsonii*. Indeed, 24h post interaction, the authors observed significant higher level of IL-1 β , IL-6, IL-8, G-CSF, PDGF, CXCL10, MIP-1 β and Gro- α in all epithelial cell lines exposed to *G. vaginalis* or *A. vaginae*, with *A. vaginae* exhibiting a more robust cytokine response than *G. vaginalis*. Other cytokines were increased such as IL-1RA, IL-10, IL12 (p70), CCL5 and IL-1 α . Moreover, *A. vaginae* induced an increased expression of defensins (HBD2, HBD3) only in

End1 cells. One difference has to be noted between *in vitro* and *in vivo* analysis: the difference in CXCL10 expression [249].

In BV+ mouse model colonized by *G. vaginalis*, oral administration of *L. acidophilus* efficiently inhibits *G. vaginalis* induced epithelial cell disruption, MPO activity, iNOS and COX-2 expression, NF- κ B pathways as well as IL-1 β , IL-17, TNF α and increases IL-10 and Foxp3 expression. Moreover, the *Lactobacillus* species inhibited, *in vitro*, the differentiation of mice splenocytes into Th17 cells but increased their differentiation into Tregs [250]. In human cervical cytobrushes, there were no differences in CD11⁺ DC or CD14⁺ monocytes and macrophages frequency when comparing BV⁺ and BV⁻ women. However, in women with highly diverse microbiota, there were gene upregulations for NF- κ B, TLR, NOD like receptor and TNF α signaling pathways in cervical APCs, most likely with LPS acting as the upstream regulator. CD4⁺ T cells frequency, obtained from cervical cytobrushes for those women, were higher and cells were more activated (HLA-DR⁺, CD38⁺, CCR5⁺) [195]. Interestingly, Lennard et al. did not found any differences in the frequency of CD4⁺ CCR5⁺ cells in cervical cytobrushes of BV⁺ women suggesting the implication of other factors in the increased susceptibility of BV⁺ women to HIV-1 [246]. BV⁺ women, treated with 500mg metronidazole for 7 days (twice daily), that did not resolve the infection, exhibited high level of IL-1 β , TNF α and ICAM-1 and low level of SLPI and GRO- α compared to BV⁻ women. *Gardnerella* species were higher in samples of women having an unresolved BV. Ectocervical tissues of BV⁻ women harbored higher number of CD45⁺ cells, including CD3⁺, CD8⁺ and HLA-DR⁺ cells. Nevertheless, BV⁺ women had higher number of CD4⁺ and CCR5⁺ cells [251].

Cervicovaginal lavage (CVL) of 50 Rwandan female sex workers were collected for the microbiota and the proteome was analyzed. The microbiota has been clustered in 4 groups: *L. crispatus* dominated microbiota (group 1), *L. iners* dominated microbiota (group 2), moderate dysbiosis (group 3) and severe dysbiosis (group 4). MUC5B was increased from group 1 to 4 and MUC5AC was increased in group 4 compared to other groups, which could be explained by an increased production of mucus, or by the degradation of the mucus by BV associated bacteria. Cytoskeleton alterations (increasing actin-organizing proteins, decreasing keratins and cornified proteins) are observed in groups 3 and 4. Furthermore,

there is an increasing trend of cell death from group 1 to 4 reflecting cell damage. Histones (HIST3H2A, HIST2H3A/C/D, HIST4H4) and AMPs such as psoriasin or calprotectin were increased from group 1 to 4. Besides, proteasome core complex proteins, proteases and proteins involved in catabolism were also induced in dysbiosis women. Moreover, a pro-inflammatory cytokine, IL-36, was increased in women suffering from dysbiosis [252]. Lactic acid treatment of cervicovaginal epithelial cell lines, with physiologically concentrations of short chain fatty acid (SCFA), induced an anti-inflammatory environment (increase of IL-1RA). Moreover, lactic acid and SCFA mixture significantly decreased the expression of IL-6, IL-8, CXCL10 and CCL20 upon stimulation with TLR1/2 and TLR3 agonists. On the contrary, higher concentration of SCFA, compared to lactic acid, induced an increased expression of TNF α and a decreased expression of CCL5 and IP-10 upon TLR stimulation, suggesting that elevated SCFA might be responsible for the increased inflammation observed in the vagina of BV⁺ women [253].

The modulation of the vaginal immune response is summarized in the figure 17 and 18.

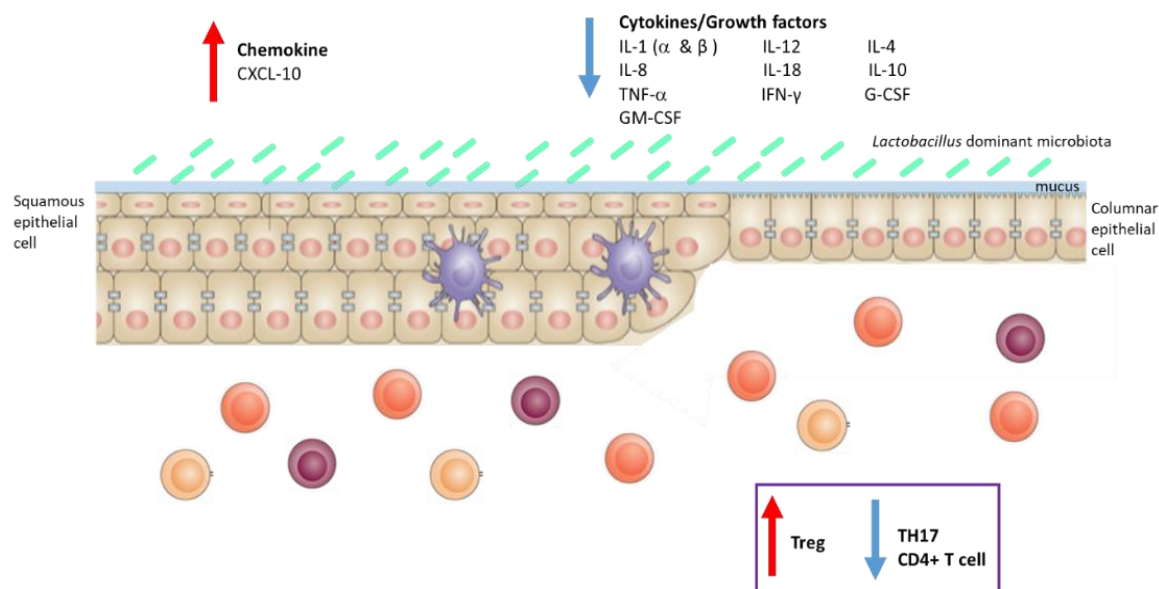


Figure 17: Eubiotic vaginal microbiota.

Representation of a *Lactobacillus spp.* dominant cervicovaginal microbiota and the immune response induced. Red and blue arrows represent respectively high and low presence of cell populations or soluble factors. Adapted from Wira et al, 2005 [61]

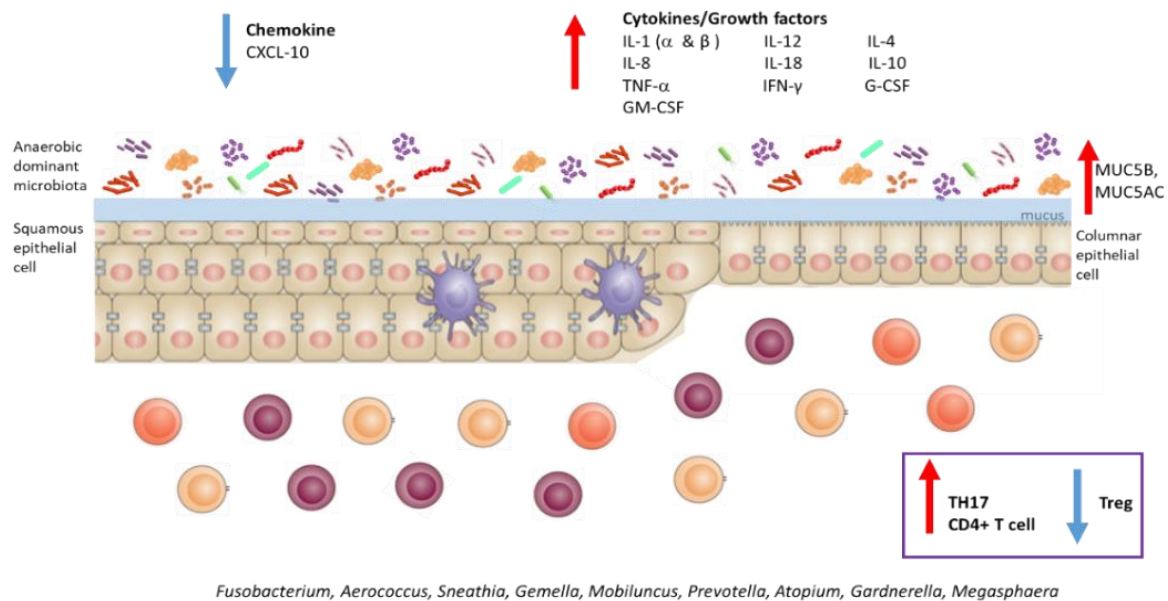


Figure 18: Dysbiotic vaginal microbiota.

Representation of a diverse cervicovaginal microbiota composed mainly by anaerobic bacteria. Immune responses induce in this context is also represented. Red and blue arrows represent respectively high and low presence of cell populations/soluble factors or mucus secretion. Adapted from Wira et al, 2005 [61]

V. Impact of the microbiota on STI

a. Microbiota & *Chlamydia trachomatis*

The vaginal microbiota of adolescent girls or women (n=72) dominated by diverse anaerobic bacteria or *L. iners* were more likely to be infected by CT. Besides, those women had higher relative abundance of several anaerobic bacteria such as *G. vaginalis*, *Porphyromonas somerae*, *Corynebacterium urealyticum*, *Dialister spp.*, *Megasphaera*, *A. vaginae*, *Prevotella disiens* compared to women with a vaginal microbiota dominated by *L. crispatus* [192]. A cohort study of 255 women demonstrated that BV+ women, were at increased risk (3.4 times) to acquire *N. gonorrhoeae* and CT, contrary to women colonized with hydrogen peroxide producing *Lactobacillus* [241].

In vitro, *Lactobacillus spp.* (*L. crispatus*, *L. gasseri*, *L. jensenii*) are able to inactivate CT through lactic acid production [254]. *In vitro*, this effect is different depending of the strains used. Indeed, *L. crispatus* strains seem to have the best anti-chlamydia effect [255]. Furthermore, *Lactobacillus* are able to decrease CT infection by either aggregating to

extracellular EB or by competition for epithelial cells attachment [256]. *In vitro*, *L. crispatus* treatment of a CT infected epithelial cell line (J774) decreased the production of IL-6, IL-8 and TNF α and induced the production of an anti-inflammatory cytokines (IL-10) [257]. A decrease availability of nutrient like glucose in the environment, thank to it consumption by *Lactobacillus spp.*, has also been shown to be another anti-CT mechanism [255].

Lactobacillus strains have different inflammatory properties. Indeed, increased risk for CT acquisition has been shown to be associated to a *L. iners* dominated microbiota in a Dutch cohort [258]. After azythromycin treatment, *L. iners* was significantly over-represented in patients that clear CT infection. Participants that were in the group IV (dominated by anaerobics bacteria) before treatment and shift to group III (dominated by *L. iners*) post treatment transitioned back to group IV or stayed in group III. Moreover, 5 out of 10 strains of *L. iners* and 1 out of 8 strains of *G. vaginalis* were resistant to high concentration of azythromycin (256ug/mL), nevertheless, doxycycline efficiently killed all the strains. The strain resistant to azythromycin seemed to be selected post treatment, suggesting that the risk for CT reinfection or another STI acquisition is not reduced [259]. In another similar study, the authors observed that the proportion of women with a vaginal microbiota dominated by anaerobics bacteria (group IV) decreased from 74.4% to 43.9% while group III (dominated by *L. iners*) increased from 23.2% to 46.3% following treatment with azithromycin to clear CT infection. Identically to the paper of Tamarelle et al., they observed that, *in vitro*, the growth of *L. iners*, as well as *G. vaginalis*, were not inhibited by a high concentration of azithromycin (640mg/L), contrary to other species of *Lactobacillus* (*L. crispatus*, *L. jensenii*, *L. gasseri*). The authors concluded that, even if azithromycin is efficient for clearing the infection, it also drives the composition of the microbiota toward a microbiota more susceptible to reinfection. *In vitro*, *L. iners* can inhibit CT infection only when the culture is supplemented by D-lactic acid, which inhibit epithelial cell proliferation. Indeed, epithelial cell proliferation is necessary to induce an efficient CT infection [260].

As described in the first part of the introduction, upon CT infection, IFN γ production induces the activation of the enzyme IDO1 which depletes the tryptophan pool within the epithelial cell into kynurenine. *In vitro*, pretreatment with IFN γ of CT (svD) infected epithelial

cell induces the depletion of the tryptophan pool, leading to no infection of the cells (36h post infection). In this experimental setting, adding indole (36h post infection) in the medium promotes epithelial cells infection with CT (72h post infection). Indeed, supernatant of indole, producing bacteria such as *Prevotella intermedia* and *Prevotella nigrescens* also promotes CT infection in presence of IFN γ [31]. Interestingly, *in vitro*, IFN γ induced persistent CT phenotype was described to have a distinct proteome profile compared to EB and RB with a high expression of TrpA and TrpB [75]. These proteins are essential for tryptophan synthesis using indole produced by anaerobic bacteria present in the FRT [21].

Ratios of kynurenine/tryptophan are higher in vaginal fluids of CT infected women, and correlated with high abundance of *Streptococcus spp.* and *Peptoniphilus spp.* Moreover, 94% of the samples of CT infected women exhibited a high abundance of indole producing bacteria such as *Porphyromonas asacharolytica*, *Propionibacterium acnes*, *Fusobacterium nucleatum*, *Enterococcus faecalis*, *Peptoniphilus harei* and *E. coli* [261]. CT can retain a subset of genes involved in tryptophan synthesis (*trpA*, *trpB* and *trpR* genes) and use them to synthesize tryptophan from indole, leading to CT growth and infection [16].

b. Microbiota & HIV-1

In the FRESH cohort, 31 women out of 236 were infected with HIV-1 despite HIV prevention counseling. Women with a high vaginal microbiota diversity and a low abundance of *Lactobacillus* (CT4) had a 4-fold increased risk for HIV acquisition compared to women with a microbiota dominated by *L. crispatus* (CT1). Moreover, the authors observed that none of the women with *L. crispatus* dominated microbiota acquired HIV-1. Women with CT4 had a 17-fold increase in HIV target cells in the FRT with elevated MIP-1 α and MIP-1 β , which attracts CCR5+ cells. CVL of women with CT4 had higher level of IL-1 α , TNF α , IL-8, IL-12p70, IFN γ , IL10, IL-17 and IL-17 inducing cytokines IL-23 and IL-1 β . Vaginal inoculation of *Prevotella bivia* in germ free mice promotes the recruitment in the FRT of CCR5+ CD4+ T cells compared to mice inoculated with *L. crispatus*. This inflammation suggested an increased susceptibility to HIV-1 for women with CT4 due to an increase of HIV-1 target cells (CD4+ T cells and Th17 cells) and local pro-inflammatory cytokines [191].

In a *in vitro* model, vaginal fluids from women were exposed to a multilayer of vaginal epithelial cells, with either CD4 T cells (TZM-bl) or monocyte derived macrophages. The vaginal fluids from women with CT1 inhibited HIV-1 infection compared to women with CT4. HIV-1 infection was even enhanced when the *in vitro* model was cultured with vaginal fluids of women with CT4 containing high abundance of *Ruminococcaceae spp.*, *Sneathia sanguinegens*, *Atopium vaginae* and *Aerococcus spp.* [262]. HIV-1 BaL (R5 tropism), exposed to CVL obtained from women, was significantly less infectious in a pH dependent manner, suggesting a role of lactic acid. Indeed, they observed a significant association between HIV-1 infectivity with percent of D and L protonated lactic acid. Low HIV-1 infectivity is associated with high percent of D and L protonated lactic acid, which correlated to a high relative abundance of *Lactobacillus spp* (particularly *L. crispatus*) [263]. Moreover, *in vitro* experiments using cervicovaginal mucus (CVM) collected from women demonstrated a better HIV-1 trapping when CVM concentration in D-lactic acid is high. These mechanisms seem to be mediated through hydrogen bonds between surface carboxyl groups and host-derived envelope glycolipids or glycoproteins. Furthermore, CVM that trapped HIV-1 generally exhibited an *L. crispatus* dominant microbiota whereas those that failed to trap HIV-1 had either a *L. iners* dominant microbiota or significant abundance of *G. vaginalis*. As already mentioned *L. crispatus* is able to produce L and D Lactic acid whereas *L. iners* can only produce L-lactic acid [264, 265]. On the contrary, Nahui Palomino et al. have shown that L-lactic acid and not D-lactic acid were the main contributors for HIV-1 inhibition on cervicovaginal explants. The authors also demonstrated that *Lactobacillus* were able to bind HIV-1 virions, thus decreasing HIV-1 infection [266].

A recent study has linked extracellular vesicles (EV) produced by *Lactobacillus* and protection against HIV-1. EV are produced by Gram negative and positive bacteria. It has been described, in Gram negative, to be produced by the pinching off of the outer membrane of the bacteria. However, the mechanism implied in EV production in Gram + bacteria is not yet determined [267]. EV produced by *Lactobacillus spp* isolated from women (*L. crispatus* BC3 and *L. gasseri* BC12) inhibit HIV-1 replication in CD4+ T cells (MT-4 cells),

human tonsillar and cervicovaginal tissues in a dose dependent manner. No HIV-1 inhibition was observed using EV from *L. crispatus* BC5 and *L. gasseri* BC13. The inhibition seemed to be mediated by a decrease of viral entry or attachment to the target cells. Metabolomic and proteomic analysis showed differences in EV composition depending of the ability to inhibit HIV-1. Indeed, HIV inhibiting EV are associated with more amino acids, and expression of several proteins such as enolase and elongation factor Tu. Those proteins were reported to play a role in adhesion to epithelial cells [268].

Finally, vaginal microbiota composition has been linked to antiretroviral microbicide gel protection against HIV-1. Treatment with an antiretroviral (tenofovir) prior to HIV-1 infection leads to a reduced efficacy in cultures colonized by bacteria from vaginal fluids of women with CT4 [262]. In the CAPRISA study, tenofovir (TFV) gel reduces HIV-1 incidence by 61% in women with a vaginal microbiota dominated by *Lactobacillus*, but only by 18% in women with a non-*Lactobacillus* dominant microbiota. Furthermore, TFV concentration in CVL negatively correlated with BV associated bacteria such as *G. vaginalis* and *Prevotella*. *G. vaginalis*, and to a lesser extend *Prevotella* species, are able to rapidly metabolized TFV before drug uptake by target cells. The mechanism involved the production of adenine by cleavage of the side chain component of TFV (oxy-methylphosphonic acid) [190]. A recent study has also described that CVL, collected from women suffering from BV, were able to degrade TFV and dapirivine (DPV) but not tenofovir alafenamide (TAF). The authors also demonstrated the importance of *Lactobacillus* in preventing drug degradation by other species of bacteria, highlighting that microbial communities are critical for TFV and DPV efficacy [269]. Interestingly, Taneva et al. observed different results, suggesting a more complex role of the microbiota in the modulation of topical antiretroviral efficacy. They observed that TFV transport into human cells is decreased at high pH. Compared to the study of Klatt et al., the authors did not observe any metabolization of TFV by *G. vaginalis*. Nevertheless, they observed adenine production in the culture medium, which is able to block endocytosis of TFV into human cells, leading to decrease protection against HIV-1. They also observed that TFV is transported and metabolized by *L. crispatus* but not *L. jensenii* and *L. iners*. The authors hypothesize that *L. crispatus* could be a reservoir that gradually

release TFV into the vagina. Finally, they observed that the inhibitory effect of the vaginal microbiota was overcome at higher drug levels, suggesting that sustained drug delivery could be sufficient to induce protection. However, decrease of drug level might lead to a decreased efficacy of antiretroviral due to adenine and high pH. The pharmacokinetics of tenofovir deoproxil fumarate and tenofovir alafenamide were not impacted by the vaginal microbiota [270]. The efficacy of daily oral PrEP for HIV prevention among women with highly diverse microbiota were not impacted, compared to women with a *Lactobacillus* dominant microbiota [271].

VI. Relationship between the microbiota & neutrophils

In the gut, neutrophils are highly impacted by the microbiota. The gut microbiota is essential for neutrophil production and priming. Furthermore, neutrophils also contribute to the containment of the microbiota [Fig19] [272]. A study, performed in patients receiving hematopoietic cell transplantation after chemotherapy, described a relationship between the gut microbiota and immune cell recovery. Indeed, *Faecalibacterium*, *Ruminococcus* and *Akkermansia* were associated with an increased number of neutrophils and, on the contrary, *Rothia* and *Clostridium* were negatively associated with neutrophil number [273].

Firstly, antibiotic treatment of pregnant mice induces an altered gut microbiota with a reduced number of intestinal bacteria in neonates. Following birth, neonates continued to receive antibiotics and exhibited an altered granulopoiesis (decrease of bone marrow neutrophils, level of plasma G-CSF and IL-17A in the intestine) compared to naïve mice. Indeed, absence of the gut microbiota decreased the number of IL-17 producing cells in the intestine and consequent production of G-CSF. LPS stimulation through TLR4/MyD88 signaling pathway promotes IL-17 producing cells in the intestine leading to granulocytosis. Antibiotics-exposed neonates treated with G-CSF had an improved resistance to bacterial infection compared to untreated antibiotics exposed neonates, and exhibited an increased number of circulating neutrophils. Those results show that the microbiota is essential in the production of neutrophils [274].

Secondly, germ free mice infected with *K. pneumoniae* produced a low amount of pro-inflammatory cytokines, decreased neutrophil influx and produced high level of IL-10. Colonization of germ free mice with a normal gut microbiota allows a better response to the infection. Moreover, treatment with LPS prior infection restored transiently the response to the infection (low level of IL-10, increased pro-inflammatory cytokines, increased number of neutrophils, low number of bacteria in the lung). Those results suggested a role of the microbiota in priming the neutrophils [275]. Furthermore, human circulating neutrophils progressively lose CD62L expression and up-regulated CXCR4 as they aged. Aged neutrophils have high levels of TLR4 and molecules involved in cell migration and intercellular interactions (CD11b, CD49d, ICAM-1), suggesting a high activation of aged neutrophils. Microbiota depletion in mice induces a reduced number of aged neutrophils, which can be restored by LPS (TLR4 ligand) or peptidoglycan (TLR2 ligand) treatment. In antibiotic treated mice, neutrophils remain in the circulation and exhibit significant reductions in neutrophil adhesion/Mac-1 activation compared to untreated mice, suggesting that the microbiota is essential for the generation of aged/activated neutrophils. Neutrophils in antibiotics treated mice exhibit a marked reduction in NET formation. Myd88, TLR2 and TLR4 knockout mice exhibit the same number of total neutrophils but with less aged neutrophils, suggesting a role of the microbiota in priming neutrophils [276].

Thirdly, metabolites such as short chain fatty acid, secondary bile acid lithocholic acid or histamine produced by the microbiota, have been shown to modulate neutrophil function, which can be mediated by histone deacetylase (HDAC) or G protein coupled receptor [272]. SCFA produced in the colon by bacterial fermentation of polysaccharides are mainly composed of butyrate, propionate and acetate. Butyrate and propionate inhibit pro-inflammatory cytokines (TNF α and cytokines induced neutrophil chemoattractant-2) and nitric oxide production by LPS stimulated neutrophils (rat model). Moreover, those SCFA are also able to inhibit HDAC and NF- κ B activation, which might explained the decrease in pro-inflammatory cytokines [277]. Finally, AMPs produced by neutrophils have been shown to modulate the microbiota. Mice deficient in cathelin-related antimicrobial peptide (CRAMP), the ortholog of cathelicidin, exhibit a significant dysbiosis of the gut (increased level of

bacteria from the oral cavity) which result in a higher susceptibility to colitis and azoxymethane-mediated carcinogenesis [278].

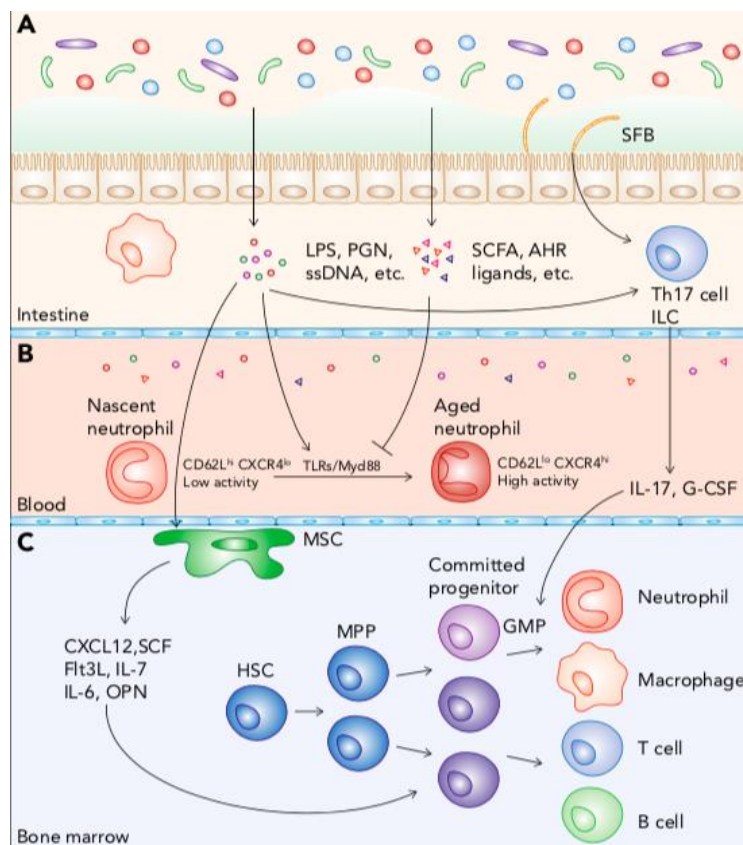


Figure 19: Gut microbiota regulation of neutrophil production and activation.

(A) Microbiota production of small molecules allows crosstalk between microbiota and cell populations locally. (B) The small molecules produced by the microbiota can also diffuse into the circulation in order to regulate neutrophil functions. (C) Microbial products can diffuse into the Bone marrow to support lineage differentiation from hematopoietic stem cell by triggering mesenchymal stem cell cytokine production. Extracted from Zhang D. and Frenette PS, 2019 [272].

Lactobacillus have been used in the gut to reduce infection severity linked to inflammation. Neutrophil accumulation is reduced upon *Lactobacillus* treatment. Oral administration of *L. plantarum* CIRM653 before *K. pneumoniae* infection in mice, induced an immunosuppressive Treg response and decreased the number of immune cells (macrophages and neutrophils) and pro-inflammatory cytokines (IL-6 and TNF α) compared to naïve mice [279]. Another paper has shown that administration of *L. acidophilus* induced a decrease in pro-inflammatory cytokines (IL-8, TNF α , IL-6), immunoregulatory cytokines (IL-

4, IL-10), neutrophil and macrophage infiltrates as well as a reduced injury severity in rats suffering from colitis [280]. Hensley-McBain et al. have shown that neutrophil accumulation in colorectal biopsies of HIV-1 infected ART treated patients is due to an increase of functional neutrophil survival. When comparing with the gut microbiota, the authors observed an altered ratio of *Lactobacillus/Prevotella* in HIV-1 positive individuals, which correlated with neutrophils survival in colorectal tissue from the same patient. *In vitro* experiments using blood neutrophils isolated from the patients and exposed to several bacteria (*Prevotella*, *Bacteroides fragilis*, *Ruminococcus bromii*), increased neutrophil survival compared to *L. plantarum* and *L. rhamnosus* independently of their HIV-1 status [281].

Very few is known about the relationship between the vaginal microbiota and neutrophils at steady state, but the mechanisms observed in the gut might also be important in the vagina in terms of neutrophil priming and function for instance. *Lactobacillus* impact on neutrophils might also be observed in the vagina and be essential to decrease the risk of STI acquisition. Interestingly, R. Cheu et al. have observed an activation of cervicovaginal neutrophils, as well as a delay in neutrophils apoptosis leading to neutrophil accumulation within the vagina of women suffering from bacterial vaginosis [Keystone 2018].

The vaginal microbiota is a key regulator of the immune system in the FRT. Studying its composition in animal study model, used to evaluate STI induced immune response, is essential.

Chapter IV: Pre-clinical animal model

Animal models including mice, rat or Non-Human primates (NHP) have been widely used in biomedical research including STI studies. They are essential to determine vaccine or drug safety/efficacy, immune responses against infectious pathogens that induce high mortality and tissue specific immune responses for instance. Rodents are particularly used due to their numerous advantages including a low cost in terms of housing, easy breeding and numerous offsprings at each litter. Moreover, genetic engineering or microbiota modification can be easily performed in mouse model for instance [45, 282]. However, substantial differences can be highlighted between the mouse models and humans. Divergence between humans and mice occurs between 65 and 75 million years ago [283, 284]. Differences in terms of innate and adaptive immune responses can also be highlighted: the balance of leukocyte subsets (T and B cells), neutrophils and soluble factor production (cytokines and chemokines) are different for instance [284, 285]. Moreover, FRT anatomy as well as hormonal secretion differs between humans and mice. For instance, the cervical epithelium differs between the two species [286]. Furthermore, several infectious pathogens do not infect or recapitulate human pathology in mice [287]. On the contrary, NHP have been described to be susceptible to wide range of human pathogens including viral (Simian Immunodeficiency virus, the simian analogue of HIV-1, ZIKA, Ebola, Chikungunya or Influenza) or bacterial infections (Periodontal disease, Tuberculosis, *Chlamydia trachomatis*) [287–290].

I. *Cynomolgus* macaques

Three species of macaques are commonly used in biomedical research: rhesus macaque (*Macaca mulatta*), cynomolgus macaque (*Macaca fascicularis*) and pigtail macaque (*Macaca nemestrina*) [291].

Cynomolgus macaques, from the *Cercopithecidae* family, belong to the Old World monkeys, and are mainly distributed in southeast Asia. These macaques arbors different names, including crab-eating, cynomolgus or longtail macaques. The introduction of cynomolgus in Mauritius occurred in the sixteenth century, probably by European

(Portuguese or Dutch) sailors [292]. Mauritian island is assumed to be free of measles, Herpes B, Simian Betaretrovirus Serotype 1 and 2 [293]. Those macaques descend from a small founder population, which consequently led to low genetical diversity of the offspring. Indeed, Trask et al. have reported no significant difference in the genetic diversity when comparing macaques from different regions in Mauritius [294]. S.H. Lawler et al. observed a 10-fold lower variation in the mitochondrial genome of the Mauritian samples, compared to Indonesian and Filipino samples supporting very low genetic diversity in Mauritian macaques [295].



Figure 20: Cynomolgus macaque.

II. Immune system in macaques

Humans and NHP are closely similar since their divergence occurs only between 6 to 7 million years ago [283]. Therefore, it is not surprising to observe significant similitudes in terms of immune system between the two species. Indeed, immune cells including neutrophils, basophils, CD8⁺ T cells, CD4⁺ T cells, B cells, NK, pDC, classical/intermediate or non-classical monocytes in the blood are observed in macaques (rhesus and cynomolgus) and humans [285, 296]. For example, DC subsets represent less than 1% of cells in PBMC of humans and rhesus macaques (RM). Moreover, RM DC express similar TLR as observed in humans. NK cells are CD3⁻ and CD16^{+/-} in humans and RM. Both species have similar T cells

subsets (naïve, central memory or effector memory) [297]. However, some differences can be noticed between NHP and humans. Indeed, Z.B Bjornson-Hooper et al., observed more CD4⁺ CD8⁺ double positive T cells, a lower ratio of classical/non classical DC, low expression of CD11c and CD16 on neutrophils and the CD8 expression on NK cells in the blood of cynomolgus or rhesus macaques compared to humans [285]. The lower presence of B cells is also noticed in peripheral blood, mesenteric lymph node and spleen in cynomolgus macaques compared to humans [296].

Overall, cynomolgus as well as rhesus macaques are suitable animal models to study human physiological situations, pathogenesis, immune responses against infectious diseases or safety/efficacy of vaccines and therapies.

III. Female Reproductive Tract (FRT) in macaques

a. Menstrual cycle

The female reproductive tract of cynomolgus macaques is closely similar to the one in women in terms of morphology, endocrine system and menstrual cycle [298]. The menstrual cycle in rhesus and cynomolgus macaques (28-32 days) is similar to the one observed in humans (28-30 days). The follicular phase lasts 12 to 14 days, and the luteal phase around 14 to 16 days [299]. However, RM exhibit seasonal variations in their ovarian cycle: sexual activity as well as gonadal activity was reported only half of the year (October to March in this cohort), contrary to marmoset and cynomolgus macaques [299]. Shaikh et al. have observed a menstrual cycle duration of 22 to 37 days in 93 cynomolgus macaques, with 60% exhibiting a cycle length of 27-31 days, 28.8% a cycle length of 32-37 days and 7.4% a cycle length of 24 to 26 days. Menstruation lasts between 1 and 8 days, but 85% of the females exhibit a menstruation cycle of only 3 to 5 days [300].

b. Immune cell distribution

The localization and phenotype of immune cells are similar in the human and cynomolgus macaque FRT. Indeed, Marlin et al. have characterized the immune cell proportions in the vagina, cervix, uterus and fallopian tubes of cynomolgus macaques. T lymphocytes are the main leukocyte population, with a higher number of CD8⁺ T cells (40% of total leukocytes) compared to CD4⁺ T cells (20% of total leukocytes in the lower FRT, 10% in the upper tract). Moreover, T cells exhibit a memory phenotype: CD4⁺ T cell expressed makers of central memory phenotype (CD28⁺/CD95⁺) and CD8⁺ T cells exhibit a central memory and effector memory phenotype. B lymphocytes represent a minor population in the FRT. NK cells represent less than 4% of total leukocytes in the FRT, however, this percentage reaches 15% of total leukocytes in the uterus. Myeloid DC and CD14⁺ cells represent 2.2% and 5.6% of total leukocytes in the FRT. Those cells were observed in all compartments, but are increased in the uterus. Finally, neutrophils were mainly observed in the cervix (6.8% of total leukocytes) and in the upper tract (1.8% and 2.3% of total leukocytes in the uterus and fallopian tubes respectively) [301].

c. Vaginal microbiota

The vaginal microbiota of macaques exhibit very low abundance of *Lactobacillus*, in contrast to the one observed in the majority of women. RM harbor a diverse vaginal microbiota, composed of a wide range of bacteria including *Sneathia*, *Peptoniphilus*, *Porphyromonas*, *Mobiluncus*, *Atopobacter*, *Dialister*, *Thioreductor*, *Prevotella* and *Streptococcus*. Spear et al. have observed that four out of eleven RM had *Lactobacillus*, but only in two of them, the *Lactobacillus* sequences were found at a percentage higher than 1%. The vaginal microbiota of two RM were constituted by 39% and 11% of *Lactobacillus* sequences respectively at one time point. Most of the *Lactobacillus* detected in the vaginal microbiota of the rhesus were *L. johnsonii*, and few sequences correspond to *L. gasseri* and *L. salivarius*. The vaginal pH was high, with a median value of 7 [302]. Analysis of pig-tailed monkey vaginal microbiota also highlighted a polymicrobial microbiota, where the most

observed sequences belonged to *Sneathia* and *Fusobacterium*, accounting for 18.9% and 13.3% of sequences in all the samples. Other bacteria were frequently observed in all the samples, such as *Prevotella*, *Porphyromonas*, *Atopium* and *Parvimonas*. Five animals had *Lactobacillus* sequences at a percentage higher than 2% of total sequences. Furthermore, only two animals exhibited a vaginal microbiota composed of more than 10% of *Lactobacillus* sequences with the highest amount reaching 27% at one time point [303]. A comparative study between humans and rhesus macaques oral, perianal and vaginal microbiota was performed by Chen et al. They observed that oral, perianal and vaginal microbiota of rhesus macaque were distinct from the human ones. Indeed, the vaginal microbiota was, as expected, more diverse than the human vaginal microbiota. The vaginal microbiota of rhesus macaques were mainly composed of *Porphyromonas*, *Campylobacter*, *Saccharofermentans*, *Prevotella*, *Dialister*, *Fusobacterium* and *Sneathia* [304]. Interestingly, several BV associated bacteria were found in the vaginal microbiota of rhesus and pig tailed macaques, such as *Gardnerella*, *Prevotella*, *Fusobacterium*, *Sneathia*, *Atopium*, *Peptoniphilus*, *Dialister*, *Mobiluncus* and *Porphyromonas* [302–304]. Juvenile rhesus macaques exhibited a lower vaginal microbiota diversity and an enrichment in *Halomonas* compared to adult females macaques [304].

In a recent study, the vaginal microbiota was observed to be more diverse during lactation and menstruation. In addition, during lactation and menstruation, the bacterial composition of the vagina was closely related to one of the urethra of the male suggesting, a shift of the vaginal microbiota allowing minor bacterial species to dominate [305]. One hypothesis, explaining the difference in bacterial composition of the vaginal microbiota of macaques compared to humans concerns the levels of glycogen and lactic acid in the vagina of macaques. Indeed, Mirmonsef et al. have observed low levels of glycogen and lactic acid in the vaginal fluids of rhesus/pig-tailed compared to humans [306]. Diet habits might be involved in the *Lactobacillus* dominance observed in women vaginal microbiota. Indeed, Miller et al. hypothesized that high level of starch in human diets have induced an increased amount of glycogen within the vagina, which in turn promotes the proliferation of *Lactobacillus* [307].

d. STI infections in cynomolgus macaques

i. Simian Immunodeficiency Virus (SIV) infection

SIV is a lentivirus closely related to HIV, which has ~55-60% of homology with HIV-1, and ~84% of homology with HIV-2 [308]. Asian macaques are not naturally infected with SIV, but are susceptible to the infection. Rhesus and cynomolgus macaques are widely used to understand HIV-1 infection and in HIV-1 vaccine development. Cynomolgus macaques infected with SIV are known to reproduce closely HIV-1 disease progression observed in humans. Indeed, cynomolgus macaques infected with SIV have less severe levels of lymphopenia, lower peak and set-point viral loads, and slower disease progression, than rhesus macaques [309]. Intravaginal infections of cynomolgus macaques with SIV_{mac251} are well established in our laboratory (IDMIT). Vaginal inoculation of SIV_{mac251} in female cynomolgus macaques (n=4) leads to the early detection of the virus in vaginal fluids, between day 14 to 28, with a variable time of seroconversion [310]. 50% of the female cynomolgus macaques (n=12) inoculated with 0,5 animal infective dose of 50% (AID₅₀) of Simian Human Immunodeficiency Virus (SHIV_{163P3}) in the vagina got infected after two challenges [311].

ii. CT infection in cynomolgus macaques

The mouse model has been widely used to study CT infection. However, the mouse reproductive tract exhibits structural and functional differences compared to the woman FRT. For instance, rodents have a single cervix lined by stratified squamous epithelium, in contrast to women, which have an endocervix (simple columnar epithelium) and an ectocervix (stratified squamous epithelium). Moreover, throughout the estrous cycle, the vaginal mucosa of mice undergoes morphological changes (thickness of the epithelium) in contrast to the vaginal mucosa of women [312]. Besides, in the CT mouse model, or *Chlamydia muridarum* mouse, pre-treatment with progestins, such as medroxyprogesterone acetate (MPA), is necessary to induce infection. Without MPA pretreatment, *C. muridarum* infection

is inconsistent and *Chlamydia* infection does not occur [15]. Macaques such as pig-tailed and marmoset are also used to study pelvic inflammatory disease induced by CT infection [288]. However, only repeated inoculation of CT directly in the fallopian tubes of macaques induce salpingitis [288, 313]. Rhesus macaques that received repeated cervical inoculations at 10^6 IFU of CT svD exhibit nonpersistent infection and mild symptoms (some oviduct edematous, fimbriae endematous). In addition, the authors described differences in term of infection length, dissemination of CT to genital upper tract and inflammation according to animal [314]. Cervicovaginal inoculation of pig-tailed macaques with 10^6 IFU of CT (svD) leads to the infection of three animals out of six, with between 10^1 and 10^2 IFU in cervical fluids, and minor pathological change of the cervix (Erythema). The bacterial burden was detected until week 11, and at week 17 the three animals had cleared the infection. Positive anti-CT IgG was observed in infected animals at week 3 [315].

In our laboratory (IDMIT), CT infection through vaginal route has been set up in cynomolgus macaques. A single dose of CT svD at 7.10^7 IFU inoculated intravaginally induce the infection of two females macaques ($n=2$) without persistent infection (PCR+ until day 30 and day 21). No erythematous aspect of the cervix was observed as well as no mucopurulent discharge. Another strategy was then established to induce a persistent CT infection. Repeated vaginal inoculation of CT serovar D strain UW-3/Cx (obtained by F. Follmann laboratory, SSI, Danemark) in female cynomolgus macaques ($n=4$) was performed at different inclusion forming unit (10^6 and 10^7 IFU) with two animals in each group. Vaginal inoculation was performed once a week during 6 weeks. Bacterial detection in vaginal fluids was observed from day 2 after the first inoculation until day 57 (22 days after the last inoculation) in all animals. At day 91 (56 days after the last inoculation), CT was detected only in three animals: two in the low dose group (10^6 IFU) and one in the high dose group (10^7 IFU) [Fig21]. Specific anti-CT IgG in the serum was observed at day 14 after the first inoculation in both animals infected with 10^6 IFU and one animal infected with 10^7 IFU. Specific anti-CT IgG in the serum was detected at day 35 for the second animal infected with 10^7 IFU [Fig22]. Pathology report showed sub epithelial and epithelial mononuclear infiltrations of the vaginal mucosa with mild to moderate overall severity as well as mild to moderate

neutrophilic cervicitis in the vagina. All of those phenomenons were not group-specific. Cytokine and chemokine expression was determined in cervicovaginal fluids (n=2 for each group) and peripheral blood plasma (n=1 for each group) of infected animals. There was an increased expression of pro-inflammatory cytokines (IL-6, IL-8, IL-18, IFN γ), chemokines(CCL4), growth factors (TGF α , VEGF, G-CSF) and the soluble form of CD40L in both groups in cervicovaginal fluids and plasma. Interestingly, in the vaginal fluids IFN γ and TNF α expression were increased only in the animals that are still infected at day 91 after the first inoculation. In the vaginal fluids, CCL2, IL-1RA, IL-6 and IL-8 were more strongly expressed in the lower dose group. On the contrary, TNF α expression was higher in the high dose group. In the plasma, IL-8, TNF α , IL-1RA, CCL2, sCD40L and IL-13 expression was higher in the low dose group [Fig 23 and 24]. Based on all the results, the inflammation observed was stronger in the low dose group. Moreover, the low dose group show a better persistence of the infection. [Unpublished results from Martin Siguier under the supervision of Delphine Desjardins].

In summary, this overview highlight the effect of factors such as STI infection, menstrual cycle and vaginal microbiota composition on the FRT micro-environment. Moreover, it described the impact of the FRT environment on local inflammation. The main question we have here is: how these factors will interact with each others and influence local inflammation ?

	Day after first inoculation	Group 1: 10 ⁷ IFU/inoculation				Group 2: 10 ⁶ IFU/inoculation			
		#BT421		#BT250		#BT528		#CB143	
		Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR
Challenge 1	D0	-	-	-	-	-	-	-	-
Challenge 2	D2	+	+	+	+	+	+	+	+
Challenge 3	D7	+	+	+	+	+	+	+	+
Challenge 4	D9	+	+	+	+	+	+	+	+
Challenge 5	D14	+	+	+	+	+	+	+	+
Challenge 6	D17	+	+	+	+	+	+	+	+
	D22	+	+	+	+	+	+	+	+
	D25	+	+	+	+	+	+	+	+
	D28	+	+	+	+	+	+	+	+
	D31	+	+	+	+	+	+	+	+
	D35	+	+	+	+	+	+	+	+
	D38	+	+	+	+	+	+	+	+
	D43	+	+	+	+	+	+	+	+
	D50	+	+	+	+	+	+	+	+
	D57	-	+	+	+	+	+	+	+
	D64	-	-	-	+	-	-	+	+
	D91	-	-	-	+	+	-	+	+

Figure 21: CT detection in cervicovaginal swabs by PCR or culture.

Qualitative PCR for the detection of CT DNA in cervicovaginal swabs was performed in Saint-Louis hospital (Paris) by the team of Dr C. Delaugerre. Culture assays to detect infectious bacteria were performed by Dr F. Follmann's team in SSI, Copenhagen (Danemark). Figure made by M. Siguier.

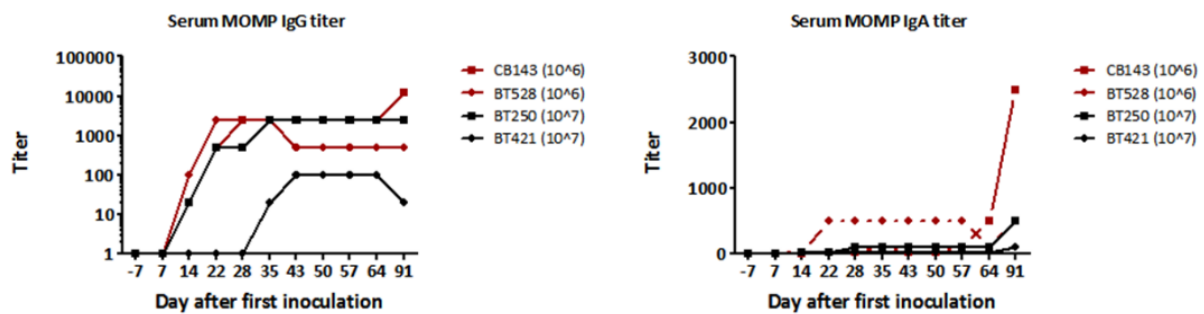


Figure 22: MOMP IgG and IgA serum level.

Specific MOMP IgG and IgGA quantification was performed in the sera of all four animals throughout the study by ELISA. Experiments were performed by Dr F. Follmann's team in SSI, Copenhagen (Danemark). Figure made by M. Siguier.

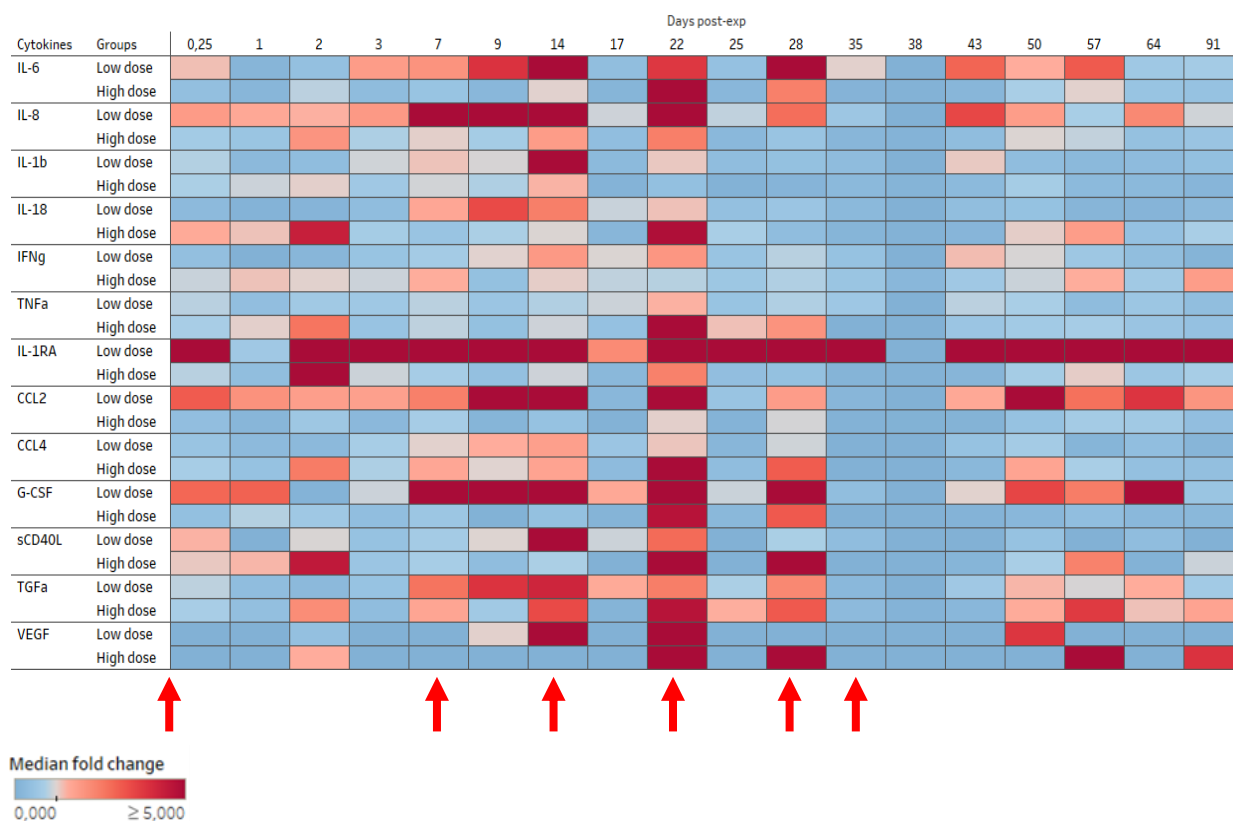


Figure 23: Heat map representing the fold change expression of cytokines and chemokines in cervicovaginal fluids upon infection.

Median fold change was calculated based on the value at D0 (before CT infection). Blue represents decrease of cytokine concentration and red an increase compare to D0 values. Low dose group corresponds to animals inoculated with 10^6 IFU of CT (n=2) and high dose to animals inoculated with 10^7 IFU of CT (n=2). Red arrows represent intravaginal CT inoculations.

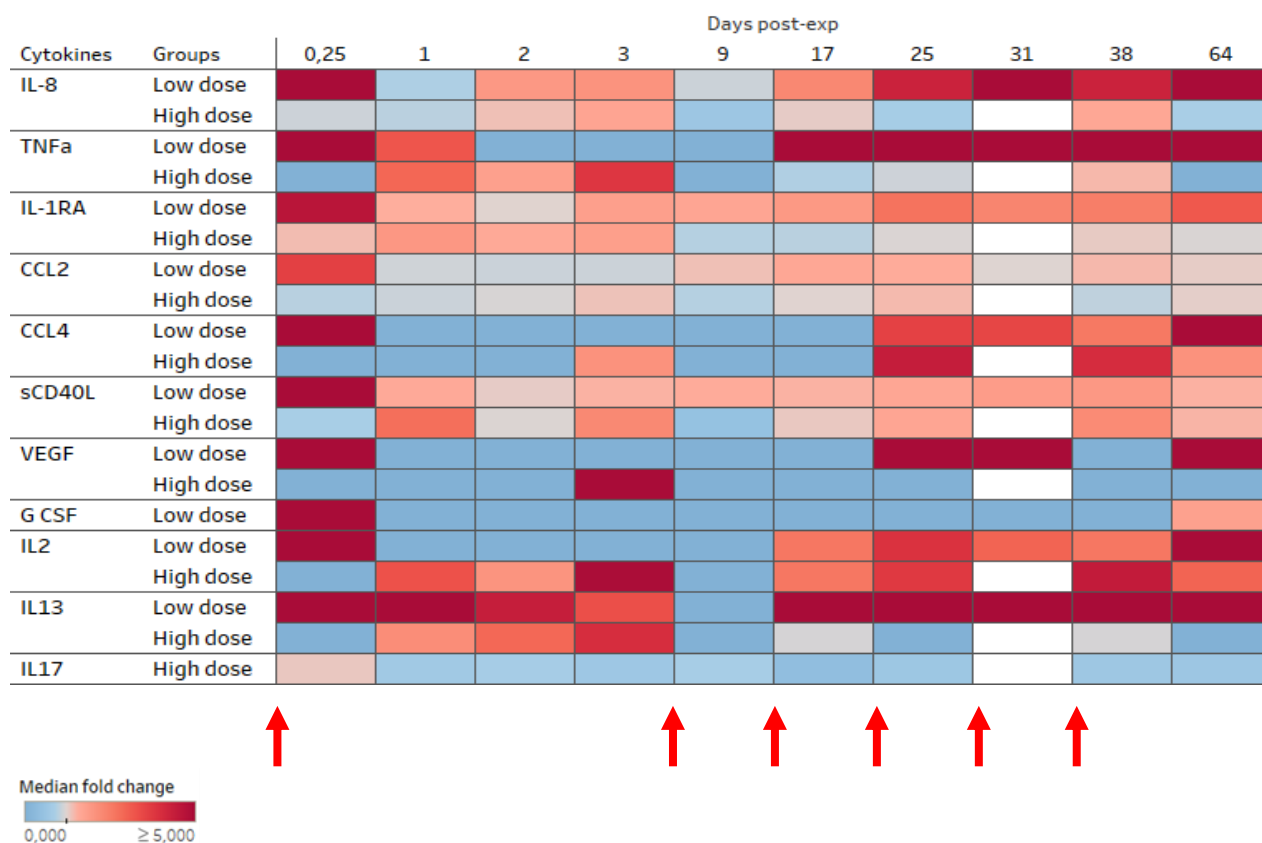


Figure 24: Heat map representing the fold change expression of cytokines and chemokines in peripheral blood plasma upon infection.

Median fold change was calculated based on the value at D0 (before CT infection). Blue represent decrease of cytokine concentration and red an increase compare to D0 values. White are null values. Low dose group corresponds to animal inoculated with 10^6 IFU of CT (n=1 ; CB143) and high dose to animal inoculated with 10^7 IFU of CT (n=1 ; BT250). Red arrows represent intravaginal CT inoculations.

Hypothesis and Objectives

Hypothesis

Heterosexual transmission from male to female is the major route of STI and occurs via the FRT mucosae. The vaginal mucosa is the first line of defense against pathogens invasion and is composed of a wide array of environment factors that are subject to hormonal cycle influence. These factors present within the FRT, including the vaginal microbiota composition, influence the susceptibility to STI by modulating the local inflammation. A high inflammatory environment has been shown to facilitate STI acquisition while a low inflammatory environment is more protective. Furthermore, a pre-existing infection increase the risk for co-infection. It is essential to understand how the hormonal cycle influences the local factors for a better understanding of the impact on STI acquisition and immune responses. *Chlamydia trachomatis* (CT) is the bacterial agent responsible of an STI that is often detected in young women. This STI induces a local inflammation that, in the worse cases, induces infertility and that enhances the risk to be infected by a second STI such as HIV-1. Therefore, a better understanding of the interaction between CT and the mucosal physiology and immunity is very important.

The aim of this project was to, on one hand, understand the interplay between inflammatory markers and vaginal microbiota according to hormonal phases *in vivo*, and on the other hand, ascertain the influence of the mucosal environment (*i.e.* vaginal microbiota composition) on CT infection and on the inflammation induced by CT *in vivo* and *in vitro*.

Our hypothesis were the following: (1) the local environment (including immune cells, soluble factors and the microbiota) and the level of inflammation is modified according to the menstrual cycle phases, (2) the manipulation of the vaginal microbiota (enrichment with *Lactobacillus spp.*) modulates the immune responses/inflammation and consequently affects CT susceptibility and/or CT-induced inflammation.

To test these hypotheses, we develop *in vivo* approaches in a non-human primate pre-clinical model and an Human *in vitro* model to dissect some of the mechanisms involved.

Objectives

The objectives were to:

- I. Characterize longitudinally the vaginal microbiota in female cynomolgus macaques
- II. Study the interplay between the vaginal microbiota, the local inflammation (neutrophil accumulation/activation, production of pro-inflammatory cytokines) and the menstrual cycle in female cynomolgus macaques.
- III. Develop an *in vivo* model to study the impact of *L. crispatus* enrichment on CT infection:
 - a. Modification of the vaginal microbiota using *L. crispatus* in cynomolgus macaque
 - b. Infection with CT of female cynomolgus macaques exhibiting an *L. crispatus* enriched or not vaginal microbiota
- IV. Set up an *in vitro* model to :
 - a. Study the modulation of the inflammation induced by the infection with CT of human endocervical epithelial cells by two species of bacteria present in the vaginal microbiota: *L. crispatus* and *G. vaginalis*,
 - b. Test subsequently the different induced environments on HIV-1 susceptibility of target cells and on neutrophil survival and phenotype.

Materials and Methods

Only the Materials and Methods not described in the articles are detailed below.

I. *Lactobacillus spp.* enrichment of the cynomolgus macaque vaginal microbiota

Experimental design

*Single inoculation of *L. crispatus* capsule*

Three female cynomolgus macaques were followed one month prior *L. crispatus* capsule inoculation (Physioflor® LP; IPRAD, Paris). Then a capsule composed of 10^9 *L. crispatus* was inoculated intravaginally once a week during four weeks. If a significant decrease of the Nugent score was observed (between 0 to 3), then the next inoculation was postponed until the loss of *Lactobacillus*. At the time of menstrual bleeding, no inoculation was performed.

*Metronidazole pretreatment followed by *L. crispatus* capsule inoculation*

Five female cynomolgus macaques were treated with a metronidazole vaginal ovule (concentration ranging from 150mg to 200mg) (Flagyl®; SANOFI Aventis, Paris) two days before each inoculation of a *L. crispatus* capsule (Physioflor® LP; IPRAD, Paris). This experimental schedule was performed at least twice with a minimum of two weeks interval between two metronidazole treatments. At the time of menstrual bleeding, no inoculation was performed.

In a second set of experiment, three animals were treated with Depo-provera (30mg) by intramuscular route (IM) one month before vaginal microbiota modification. Protocol of microbiota modification was identical to the one previously described including metronidazole and *L. crispatus* capsule inoculation.

Sucrose intravaginal treatment

Five female cynomolgus macaques were studied but only three received a Depo-provera injection (30mg) by IM route one month before the sucrose gel inoculations. All animals received intravaginally a 9% gel of sucrose twice a week during 3 weeks.

Repeated metronidazole treatment before L. crispatus inoculations

Five female cynomolgus macaques were inoculated intravaginally with an ovule of metronidazole (Flagyl®; SANOFI Aventis, Paris) three times a week during one week. Animals were then treated intravaginally with a capsule of *L. crispatus* (Physioflor® LP; IPRAD, Paris) twice a week during three weeks, then once a week during three weeks. Before each inoculation of *L. crispatus* capsule, the vaginal cavity was washed with 2mL of a 0.02% of lactic acid.

Repeated azithromycin treatment before L. crispatus gel inoculation

Five female cynomolgus macaques were treated orally with azithromycin (30mg/kg) three times a week during one week. Animals were then treated intravaginally with a gel of 10^{10} *L. crispatus* twice a week during three weeks, then once a week during three weeks. Before each inoculation of *L. crispatus* gel, the vaginal cavity was washed with 2mL of a 0.02% of lactic acid.

Sample collection*Single inoculation of L. crispatus capsule; Metronidazole pretreatment followed by L. crispatus capsule inoculation; Sucrose intravaginal treatment; Repeated azithromycin treatment before L. crispatus gel inoculation*

Cervicovaginal swabs were collected and pH measurement was done twice a week. Blood was collected once a week. Cervicovaginal samples were collected with a nylon flocked swabs (ESWABR1, Copan Diagnostics Inc., Murrieta, CA, USA) inserted in the vaginal vault and turned four to five times before storing in amies liquid. Swabs were then aliquoted and either used for Nugent score or stored frozen at -80°C with 30% glycerol (VWR international

Avantor; USA) for bacterial culture. To measure vaginal pH, a neonate nylon flocked swab (Copan Diagnostics Inc., Murrieta, CA, USA) was inserted in the vaginal vault for a few seconds to collect vaginal fluids. The fluids were then spread on a pH paper ranging from 4 to 7, and pH value was read immediately. Plasma obtained from whole blood was aliquoted and stored at -80°C before progesterone quantification.

Repeated metronidazole treatment before L. crispatus inoculations

Cervicovaginal swabs were collected and pH measurement was done twice a week. Blood and cervicovaginal fluids were collected once a week. Cervicovaginal cytobrushes was collected at the end of the protocol (D+38). Collection protocol for cervicovaginal swab was the same as previously described and several aliquots were done for Nugent score, V3/V4 16S rRNA sequencing and bacterial culture (30% glycerol). pH measurement and plasma aliquoting were done the same way as previously described. Cervicovaginal fluids were collected with a Weck-Cel Spear (Medtronic) placed in the vaginal vault for 2min. Secretions were recovered from the spears by adding 600uL of elution buffer (PBS, NaCl 0.25M and protease inhibitor mixture; Merck Millipore, Fontenay-sous-Bois, France) and centrifuged at 13000xg for 20min. Secretions were then aliquoted and stored at -80°C before cytokine/chemokine quantification. Cervicovaginal cells were collected using two successive cytobrushes (VWR avantor) inserted in the vaginal cavity and turned 4 to 5 times. After collection, the cytobrushes were put in a 15mL tube containing 5mL of RPMI with 10% Fetal Calf Serum (FCS) and 5% Penicilin streptomycin neomycin (PSN). The samples were conserved on ice block before processing.

Nugent score

Nugent score was determined as previously described in C. Adapen et al. "Local immune innate markers and vaginal microbiota composition are influenced by hormonal cycle phases".

Progesterone quantification

Progesterone level was determined in plasma sample by ELISA (IBL international; Germany) according to manufacturer's instructions.

Metronidazole ovule

Each metronidazole ovule (Flagyl[®], Sanofi Aventis; Paris) of 500mg was put in a 5mL tube and dropped in a water bath set at 37°C for few hours until complete melting. Melted ovule was then used to fill a pediatric ovule (Herboristerie du Valmont; Belgium). Ovule was then stored at 4°C until inoculation.

***Lactobacillus spp.* culture**

Cervicovaginal fluids obtained from cervicovaginal swabs were spread on a Mann Rogosa Sharp (MRS) agar plate (BD Biosciences) and cultured for 48h to 72h at 37°C with 10% CO₂. Isolated colony was then cultured in MRS broth supplemented with L-cysteine (Sigma Aldrich) for 12h at 37°C with 10% CO₂. Morphology of each colony was monitored by gram staining. Then, three rounds of washing with 1X PBS were performed (3min at 10 000xg) and bacteria were freezed with 30% glycerol (VWR avantor; USA).

Sucrose gel

Citrate buffer was obtained by mixing citric acid monohydrate, trisodium citrate dehydrate (Sigma Aldrich; USA) and H₂O MilliQ. This buffer was then mixed with 1.5% of Hydroxyethylcellulose (HEC), 0.02% of lactic acid, and 9% sucrose (Sigma Aldrich). Homogenization of the preparation was done using a magnetic shaker, and agitation lasted between 1 to 4h. Syringe were filled with 1mL of sucrose gel and freezed at -80°C until inoculation.

Cytokine and chemokine quantification

Pro-inflammatory and anti-inflammatory cytokines, as well as chemokines, were measured in cervicovaginal fluids and plasma in female cynomolgus macaques. The method

use is the same as previously described in C. Adapen et al. "Local immune innate markers and vaginal microbiota composition are influenced by hormonal cycle phases".

Neutrophil phenotyping

Neutrophil populations were analyzed in whole blood and cervicovaginal cells collected by cervicovaginal cytobrushes. Cervicovaginal cells were filtered with 35µm (Corning Falcon; USA), incubated with the antibodies listed in table 2 and washed. Red blood cells were removed using FACS lysing buffer (BD, Biosciences) and cells were fixed with BD cell Fix solution (BD, Biosciences). A Fortessa flux cytometer was used (BD, Biosciences) with DIVA (BD) and FlowJo (Tristar, USA) software.

<i>Antibody</i>	<i>Label</i>	<i>Manufacturer</i>
<i>Bluevid</i>	BUV736	Life technologies
<i>CD64</i>	BUV737	BD
<i>CD11b</i>	V450	BD
<i>CD45</i>	V500	BD
<i>CD3</i>	BV650	BD
<i>CD8a</i>	BV650	BD
<i>CD20</i>	BV650	BD
<i>CD62L</i>	BV711	BD
<i>CD14</i>	FITC	BD
<i>CD16</i>	PerCP-Cy5,5	BD
<i>CDw125</i>	PE	BD
<i>PD-L1</i>	PE-Dazzle 594	Biolegend
<i>CD89</i>	PE-Cy7	Biolegend
<i>CD32a</i>	AF647	Stemcell
<i>HLA-DR</i>	AF700	Biolegend
<i>CD66</i>	APC-Vio770	Milteny

Table 6 : Antibody panel.

DNA extraction, 16S rRNA gene sequencing, sequencing data processing and taxonomic assignation

DNA extraction and 16S rRNA gene sequencing as well as processing and taxonomic assignation of the sequences was performed as previously described in C. Adapen et al. "Local immune innate markers and vaginal microbiota composition are influenced by hormonal cycle phases".

Gel of 10^{10} *L. crispatus*

A capsule of 10^9 *L. crispatus* (Physioflor LP[®]; IPRAD PHARMA, Paris) was cultured in Mann Rogosa and Sharpe (MRS) broth (BD Bioscience) supplemented with L-cysteine (Sigma Aldrich) for 12h at 37°C with 10% CO₂. Then, 50µL were spread on a MRS agar (BD Bioscience) for 72h at 37°C with 10% CO₂. An isolated colony (*Lactobacillus crispatus* strain IP174178) was amplified in liquid MRS culture medium (BD Bioscience) supplemented with L-cysteine (Sigma Aldrich) for 12h at 37°C with 10% CO₂. The culture was then diluted 1/100 and cultured until the exponential phase was reached (DO: 0.6). Three consecutive washing steps were performed with 1X PBS and centrifugation at 4000xg for 20min to obtain the bacteria. Resuspension was performed with a gel composed of 1.5% Hydroxyethylcellulose gel (HEC; Sigma Aldrich), 9% sucrose (Sigma Aldrich), 0.05% lactic acid (Sigma Aldrich) and citrate buffer (Citric acid monohydrate; trisodium citrate dihydrate; H₂O milliQ). Syringes were filled with 1mL of *L.crispatus* gel and freezed until inoculations. Serial dilution of the gel and spreading on MRS agar were performed to confirm the concentration of 10^{10} *L. crispatus* in the gel.

II. *In vitro* study: Effect of *L. crispatus* and *G. vaginalis* on CT induced inflammation. Consequences on neutrophil survival and phenotype as well as on HIV-1 co-infection

Cell line culture

The A2EN are human endocervical epithelial cell line generated in the laboratory of Dr A. Quayle from primary epithelial cells isolated from endocervical explant and immortalized with human papilloma virus E6 and E7 [316]. A2EN cells were grown in phenol red-free serum-free medium (EpiLife; Cascade Biologics) with a growth supplement (EDGS) and 0.004M of CaCl₂ (Sigma Aldrich). The cells were grown at 37°C with 5% CO₂.

U87 CD4⁺ CCR5⁺ are a cell line isolated from a glioblastoma astrocytoma (human brain) firstly transduced with MV7neo-T4 retroviral vector and selected for G418 resistance then subsequently transduced with pBABE-puro-CCR5 and selected for puromycin resistance. Cells were cultured in DMEM with 15% SVF, 1µg/mL puromycin, 300µg/mL G418, 1% glutamine and 1% Penicillin/Streptomycin.

C. *trachomatis* infection

Chlamydia trachomatis serovar D (D/UW3/Cx) were obtained from Dr Follmann laboratory. Chlamydia inoculum were placed directly in the culture medium of A2EN cells to obtain a multiplicity of infection of 1, 4 or 12. Cultures were then centrifugated at 700xg for one hour at room temperature. After centrifugation, medium containing extracellular CT were removed and fresh medium were added. Cultures were grown for 24h or 48h at 37°C with 5% CO₂.

Bacteria from the vaginal microbiota

Lactobacillus crispatus strain IP174178 isolated from a capsule of Physioflor LP® (IPRAD PHARMA, Paris) was cultured for 48h to 72h at 37°C with 10% CO₂ in a Mann Rogosa Sharp

(MRS) agar plate (BD Bioscience). Isolated colony was then culture in MRS broth (BD Bioscience) for 12h at 37°C with 10% CO₂. Dilution of 1/100 of the overnight culture were done in MRS broth the day of the experiment. The bacteria were used as soon as they reached exponential growth phase (5-6h after dilution, DO = 0.6).

Gardnerella vaginalis 14018 (ATCC) was cultured for 72h at 37°C in anaerobic condition in *Gardnerella* agar supplemented with 5% Human blood (Becton Dickinson, Le Pont de Claix). Isolated colony was then culture in Brain Heart Infusion (BHI) broth (Sigma Aldrich) for 72h in anaerobic condition. Dilution of 1/100 of the liquid culture in BHI broth was done the day before experiment. The bacteria were used as soon as they reached exponential growth phase (24h after dilution, DO = 0.6).

Interaction assay

A2EN cells were seeded in 24 well plate at $0.5 \cdot 10^5$ cells/mL. After 96h, confluent A2EN cells were infected or not with CT svD MOI 12 for one hour. Then culture medium was removed and fresh medium was added. *L. crispatus* or *G. vaginalis*, in exponential growth phase, at a ratio of 50 bacteria for a cell were added. The cultures were maintained for 24h at 37°C with 5% CO₂. After 24h, pH measurement as well as viability test using CellTiter® were performed to confirm the good viability of the cells. Supernatants were also collected and freezed at -80°C after 0.2µM filtration.

Cytokine and Chemokine quantification

Pro-inflammatory and anti-inflammatory cytokines were measured in the supernatants collected during the interaction assays by 25plex assay for the detection of: IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNFα, IFNα, IFNγ, GM-CSF, CCL3, CCL4, CXCL10, CXCL9, CCL11, CCL5 and CCL2 (Human cytokine magnetic 25-plex panel; Life technologies).

Results

First article: Dynamics of vaginal and rectal microbiota over several menstrual cycles in female cynomolgus macaques

Marie-Thérèse Nugeyre, Nicolas Tchitchek, Cindy Adapen, Claude Cannou, Vanessa Contreras, Fahd Benjelloun, Jacques Ravel, Roger Le Grand, Romain Marlin, Elisabeth Menu

Frontiers in Cellular and Infection Microbiology 2019 ; 9
doi: 10.33.89/ fcimb.2019.00188 [317].

I. Introduction and objectives

Susceptibility to vaginal STI in women are impacted by several environmental factors including vaginal microbiota composition. For example, Gossman et al., have described a 4 fold increased risk in HIV-1 acquisition in women with a high vaginal bacterial diversity with low level of *Lactobacillus spp.* compared to women with a vaginal microbiota dominated by *L. crispatus* [191].

Cynomolgus macaques are commonly studied in biomedical research including in the field of sexually transmitted infections (STI). However, the vaginal microbiota of cynomolgus macaques was not yet characterized. Rhesus and Pig-tailed vaginal microbiota were observed to be poly-microbial with a wide array of bacteria including *Sneathia*, *Fusobacterium*, *Peptoniphilus*, *Porphyromonas* etc [302, 303]. A characterization of the vaginal microbiota composition of cynomolgus macaques was necessary to conduct subsequent studies aiming to understand the impact of the microbiota on the susceptibility to STI in this animal model.

Therefore, the team has performed a longitudinal study on five female cynomolgus macaques during 15 weeks. Rectal and vaginal swabs were collected to determine and compare the composition of the rectal and vaginal microbiota in each animals. Moreover, progesterone concentration was quantified in the plasma to monitor menstrual cycle and determine if the vaginal and rectal microbiota composition are subject to hormonal fluctuation. I participated in the analyses and writing of this paper.

II. Data summary

This study demonstrated that cynomolgus macaque rectal microbiota was dominated by two phyla: *Firmicutes*, *Bacteroidetes* whereas the vaginal microbiota was predominantly composed of three phyla: *Bacteroidetes*, *Firmicutes* and *Fusobacteria*. Very few variations were observed in the rectal microbiota according to animal with a high abundance of *Ruminococcaceae*, *Prevotella* and *Clostridiales*. In contrast, the vaginal microbiota was more variable according to animal, four genus were highly abundant and observed in all animals: *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Peptoniphilus* whereas *Sneathia* was highly frequent only in four females out of five. *Lactobacillus spp.* were weakly present in the vaginal microbiota of those females. When comparing the bacterial abundances in the rectal and vaginal microbiota in all animals we observed that both microbiota displayed its own bacterial profile with the vaginal microbiota being more heterogeneous. We have shown that the vaginal microbiota of female cynomolgus macaques was similar in term of taxonomic composition and relative abundance to the one of women harboring a high vaginal microbiota diversity (CST IV). Finally, variations according to time were observed in the vaginal microbiota compared to the rectal microbiota. Those fluctuations were determined to be hormone based. Indeed, changes of the vaginal microbiota composition were animal specific where taxa abundances (*Mobiluncus*, *Bacteroides*, *Prevotella*, *Lactobacillus*, 1-68, *Helcococcus*, *Peptoniphilus*, ph2, *Peptococcus*, *f_Peptostreptococcaceae*, *Peptostreptococcus* and *Fusobacterium*) varied according to progesterone level.

Overall, this study described in deep the rectal and vaginal microbiota of cynomolgus macaques, a model often used to study STI infection. Since the vaginal microbiota in women have been described to influence STI infection such as *Chlamydia trachomatis* or HIV-1, this project was a necessary prerequisite to further study STI susceptibility and control in this pre-clinical model.

III.Article



Dynamics of Vaginal and Rectal Microbiota Over Several Menstrual Cycles in Female Cynomolgus Macaques

OPEN ACCESS

Edited by:

Omry Koren,
Bar-Ilan University, Israel

Reviewed by:

Laurel Lagenaur,
Oselt, United States
Ronald Veazey,
Tulane National Primate
Research Center, United States
Ajay Sundaram Vishwanathan,
Centers for Disease Control and
Prevention (CDC), United States

*Correspondence:

Elisabeth Menu
elisabeth.menu@cea.fr

†These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Microbiome in Health and Disease,
a section of the journal
Frontiers in Cellular and Infection
Microbiology

Received: 20 March 2019

Accepted: 16 May 2019

Published: 12 June 2019

Citation:

Nugeyre M-T, Tchitchek N, Adapen C,
Cannou C, Contreras V, Benjelloun F,
Ravel J, Le Grand R, Marlin R and
Menu E (2019) Dynamics of Vaginal
and Rectal Microbiota Over Several
Menstrual Cycles in Female
Cynomolgus Macaques.
Front. Cell. Infect. Microbiol. 9:188.
doi: 10.3389/fcimb.2019.00188

Marie-Thérèse Nugeyre^{1,2†}, Nicolas Tchitchek^{1†}, Cindy Adapen¹, Claude Cannou^{1,2},
Vanessa Contreras¹, Fahd Benjelloun^{1,2}, Jacques Ravel³, Roger Le Grand¹,
Romain Marlin¹ and Elisabeth Menu^{1,2*}

¹CEA, Université Paris-Sud, Inserm, U1184 "Immunology of Viral Infections and Autoimmune Diseases" (IMVA), IDMIT
Department, IBFJ, Fontenay-aux-Roses, France, ²MISTIC Group, Department of Virology, Institut Pasteur, Paris, France,
³Institute for Genome Sciences and Department of Microbiology and Immunology, University of Maryland School of
Medicine, Baltimore, MD, United States

The composition of the microbiota in cynomolgus macaques is only partially characterized, although this animal model is often used to study pathogenesis and preventive strategies against infections. We thus performed, for the first time, a longitudinal characterization of the vaginal and rectal microbiota of five cycling female cynomolgus macaques. Samples were collected weekly for 15 weeks and the V3/V4 regions of the 16S rRNA gene sequenced. Sequences were analyzed with QIIME for OTU detection and taxonomic assignment. Progesterone levels were also determined to evaluate hormonal influence on bacteria relative abundance. The rectal and vaginal bacterial composition in cynomolgus macaques is polymicrobial and clearly distinct, with larger individual variability in the vagina. Rectal microbiota profiles were consistent between animals, whereas they were highly variable and animal-specific in the vagina. In the rectum, the most abundant taxa were *Ruminococcaceae*, *Prevotella*, and *Clostridiales*. In the vagina, the most abundant genera were *Sneathia*, *Porphyromonas*, *Prevotella*, and *Fusobacterium*. *Lactobacillus* were found at relative abundances higher than 1% in only one animal and were not predominant. Comparison of the vaginal cynomolgus macaque microbiota with that of humans showed similarity to community state type IV-A usually associated with dysbiosis. In the vagina, the relative abundance of 12 bacterial genera was found to be associated with progesterone levels. Our study provides a detailed characterization of the rectal and vaginal microbiota in female cynomolgus macaques and opens new perspectives of this animal model.

Keywords: cynomolgus macaques, female hormones, microbiota, mucosa, rectum, vagina

INTRODUCTION

Mucosae are the main portal of entry of sexually transmitted pathogens. Several factors of the mucosal environment are known to maintain the integrity of the epithelial barrier and protect against infections (Ferreira et al., 2014). Studies of mucosae-associated microbiota are increasing exponentially and have demonstrated their impact on the protection against sexually transmitted infections (STI). It has been shown that the microbiota can influence mucosal and systemic immune functions (Manuzak et al., 2016), as well as host physiology and in pathological states (Clemente et al., 2012; Anahtar et al., 2015).

The human gut microbiota is composed primarily of four major bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. It is involved in the maturation of the host immune system and many basic metabolic pathways, including sugar and protein fermentation (Landman and Quevrain, 2016). Dysbiosis of the gastrointestinal microbiota is associated with HIV-1 disease progression (Vujkovic-Cvijin et al., 2013).

The human vaginal microbiota plays an important role in the maintenance of an environment that protects against viral or bacterial infections, including HIV-1 (Spear et al., 2011). *Lactobacillus* spp. constitute the most common bacterial genus in women (Giorgi et al., 1987; Eschenbach et al., 1989; Antonio et al., 1999; Ravel et al., 2010). Five community state types (CST) have been defined in women: CST I (*Lactobacillus crispatus* predominantly), CST II (*Lactobacillus gasseri* predominantly), CST III (*Lactobacillus iners* predominantly), CST IV (comprises of a wide array of strict and facultative anaerobes), and CST V (*Lactobacillus jensenii* predominantly). CST IV has been further divided in CST IV-A and IV-B according to the modest proportions of *Lactobacillus* spp. (CST IV-A) and the different proportions of various species of strictly anaerobic bacteria in the sub-classes (Gajer et al., 2012). *Lactobacillus* spp. can maintain a protective environment against STI by producing several factors, including lactic acid, which decreases the pH (~4), and bacteriocins (Petrova et al., 2013; Tachedjian et al., 2018). The composition of the vaginal microbiota varies over time depending on estrogen levels over a woman's lifespan (Cribby et al., 2008). Vaginal dysbiosis is characterized by a diverse microbiota with a greater abundance of anaerobic bacteria, impaired epithelial integrity, and enhanced microbial translocation (Ziklo et al., 2016). It also induces the production of pro-inflammatory cytokines (such as TNF- α or IL-8) (Anahtar et al., 2015; Borgdorff et al., 2016a), decreases mucin expression (Borgdorff et al., 2016b), and stimulates the activation of CCR5⁺ CD4⁺ T cells (Anahtar et al., 2015). Dysbiosis can lead to bacterial vaginosis (BV), the most common vaginal condition of women in reproductive age. BV can be symptomatic or asymptomatic. In women with BV, there are reduced proportions of *Lactobacillus* spp. and increases in the number and diversity of facultative and strictly anaerobic bacteria, including species of *Gardnerella*, *Prevotella*, and other taxa of the order *Clostridiales* (Fredricks et al., 2005). Vaginal and gastrointestinal live biotherapeutic products have been proposed to prevent and cure dysbiosis, and restore a functional microbiota (Ganesh and Versalovic,

2015). These include *Lactobacillus* spp. formulated as live biotherapeutic products to prevent and treat bacterial vaginosis (BV) (Homayouni et al., 2014; Braundmeier et al., 2015).

Macaques are relevant models to study pathogenesis and validate preventive strategies against the transmission of infections (Alfson et al., 2017; Sharpe et al., 2017). The menstrual cycle length of cynomolgus macaques (28–32 days) is similar to that of humans (28–30 days) and do not exhibit seasoning menstrual cycle in contrast to rhesus macaques (Weinbauer et al., 2008). Currently, the vaginal microbiota of rhesus (Spear et al., 2010) and pigtailed macaques (Spear et al., 2012) have been described, but not that of cynomolgus macaques. Additionally, the fecal microbiota has been described in cynomolgus macaques (Seekatz et al., 2013) of different origins (Mauritius, Indonesia, and the Philippines) and the analysis was performed on samples collected over a short period of time (14 days). More recently, a gut microbiome gene catalog from cynomolgus macaques was reported and compared with pig, mouse, and human gut microbiomes (Li et al., 2018). A metagenomic comparison of the rectal microbiota between rhesus and cynomolgus macaques was also performed (Cui et al., 2018).

The aim of this study was to perform a longitudinal characterization of the vaginal and rectal microbiota of cynomolgus macaques over several menstrual cycles. Sequencing of the 16S rRNA gene was performed on samples collected once a week for 15 weeks, which covers at least three menstrual cycles per animal. This study, characterizing the mucosal bacterial composition of female cynomolgus macaques, further improve our understanding of the relationship between the microbiota and hormonal cycle.

MATERIALS AND METHODS

Animal Housing

Five sexually mature macaques (*Macaca fascicularis*) were imported from Mauritius and housed in the Infectious Disease Models and Innovative Therapies (IDMIT) facilities at the Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA, Fontenay-aux-Roses, France). The animals were housed in groups under controlled conditions of humidity, temperature, and light (12-h light/dark cycles). Water was available *ad libitum*. The animals were monitored and fed with commercial monkey chow (6020 formula, Altromin, Germany) and fruit once or twice a day by trained personnel and were provided with environmental enrichment, including toys, novel foodstuffs, and music, under the supervision of the CEA Animal Welfare Officer. The 6020 formula is a cereal-based (soy, wheat, and corn) fixed formula which is free of alfalfa and fish/animal meal and deficient in nitrosamines. This maintenance diet was designed as complete feeding stuff for adult NHP. The five animals in this study were housed in two different rooms into level-3 facilities. They were between 3 and 5 years old, weighed between 3.09 and 3.79 Kg, had different MHC genotypes (Table 1), and were never pregnant.

Experiment Design and Sample Collection

Sample collection was performed once a week for 15 weeks (Figure 1A), corresponding to approximately three menstrual

TABLE 1 | Animal characteristics.

Animal ID	Age (years)	MHC genotype	Weight (Kg)	Room #
BA890I	4	H5/H3	3.79	1
CA086	5	H2/H1	3.53	2
CB804C	4	H4/rec.H1-H5-H3	3.38	1
CBL015	4	H3/H1	3.43	1
CCA096	3	rec. H6-H1/rec.H2-H6	3.09	1

cycles. Vaginal and rectal samples were collected with nylon flocked swabs which were stored frozen at -20°C in 1 ml Amies transport medium (ESWABR1, Copan Diagnostics Inc., Murrieta, CA, USA) until DNA extraction. Room control samples (air swab) were collected in parallel with vaginal and rectal samples at each timepoint. Sequencing of the V3/V4 regions of the bacterial 16S rRNA gene was followed by bioinformatics analysis, consisting of data processing, read quality-control filtering (QC), OTU identification, and statistical analyses. The animals were anesthetized using ketamine (10 mg/kg) administered intramuscularly once a week before sampling. Blood samples were also collected weekly and plasma stored at -80°C . At least three progesterone peaks were detected per animal during the study, on average one peak every 3–4 weeks, confirming that the five female macaques had regular hormonal cycles.

Determination of Progesterone and Estradiol Concentrations

Plasma levels of progesterone (**Figure 1B**) were determined weekly for the five animals (15 samples per animal) using an ELISA kit from IBL International (Hamburg, Germany). Plasma levels of estradiol were determined at the same timepoints using the InvitrogenTM NovexTM Estradiol Human Elisa kit (Göteborg, Sweden).

DNA Extraction and 16S rRNA Gene Sequencing

The PowerFecal DNA isolation kit from MOBIO (Qiagen, Courtaboeuf, France) was used following the instructions of the manufacturer. PCR was performed using the 16S rRNA gene Amplicon PCR Forward Primer, 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTACGGGNGGCWGCAG and the 16S rRNA gene Amplicon PCR Reverse Primer, 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC, which target the 16S rRNA gene V3 and V4 regions selected from Klindworth et al. publication (Klindworth et al., 2013). PCR was performed using KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Roche, Boulogne Billancourt, France). The following conditions were used: initial denaturation at 95°C for 3 min, followed by 25 cycles consisting of denaturation (95°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 30 s) and a final extension step at 72°C for 5 min. PCR products were purified with AMPure XP beads (Beckman Coulter, Villepinte, France). To prepare DNA libraries

for Illumina MiSeq using Nextera XT Index Kit (Illumina), PCR reactions were performed with the primers provided in the kit. The following conditions were used: initial denaturation at 95°C for 3 min, followed by eight cycles consisting of denaturation (95°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 30 s) and a final extension step at 72°C for 5 min. A second purification with AMPure XP beads was performed. Sequencing of the V3/V4 region of the 16S rRNA gene was performed on the Illumina MiSeq platform of Institut Pasteur (Paris, France) following the instructions of “16S Metagenomic Sequencing Library Preparation” (ref: 15044223 Rev.B).

Sequencing Data Processing and Quality-Control Filtering

Paired-end sequenced reads were assembled using FLASH (Magoc and Salzberg, 2011) with default parameters. Only assembled reads with a length >400 bases were retained for analysis. Sequencing adaptors were removed using cutadapt (Martin, 2011). Reads were trimmed using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Finally, assembled reads with a quality score <28 in more than 95% of the sequence were discarded. The number of assembled and QC-filtered reads per sample ranged from 3,608 to 260,786 for rectal microbial profiles and 1,666–440,707 for vaginal microbial profiles. The number of assembled and QC-filtered reads per sample ranged from 7 to 188 for room control microbial profiles.

OTU Identification, Taxonomic Assignment, and Statistical Analyses

Microbiota profiles were analyzed using QIIME (version 1.9.1) (Caporaso et al., 2010b). Rectal and vaginal microbiota analyses were performed separately and independently for each animal. OTU picking was performed using the uclust algorithm (Edgar, 2010) with identity set at 97%. Representative sets of sequences were aligned with the PyNAST algorithm (Caporaso et al., 2010a). Taxonomic assignments were performed using the RDP classifier (Wang et al., 2007) trained on the Greengenes database (Desantis et al., 2006). Taxa with abundance $<1\%$ in all animals were filtered out and aggregated to provide a complete view of the rectal and vaginal compartments. Alpha diversity was calculated based on the Simpson diversity index. Statistical analyses to identify associations between progesterone level and taxon relative abundances were performed using the MetagenomeSeq's fitZIG algorithm (Paulson et al., 2013). Species-level assignments for *Lactobacillus* associated reads was performed using BLAST (Boratyn et al., 2013). Jensen-Shannon divergences were computed using the philentropy R package.

Graphical and Multidimensional Scaling Representations

Graphical representations of relative taxonomy abundance were generated using GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com), Tableau Software (version 10, Seattle Washington USA), and R software (<https://www.r-project.org/>). The tree representation showing the taxa commonly or specifically found

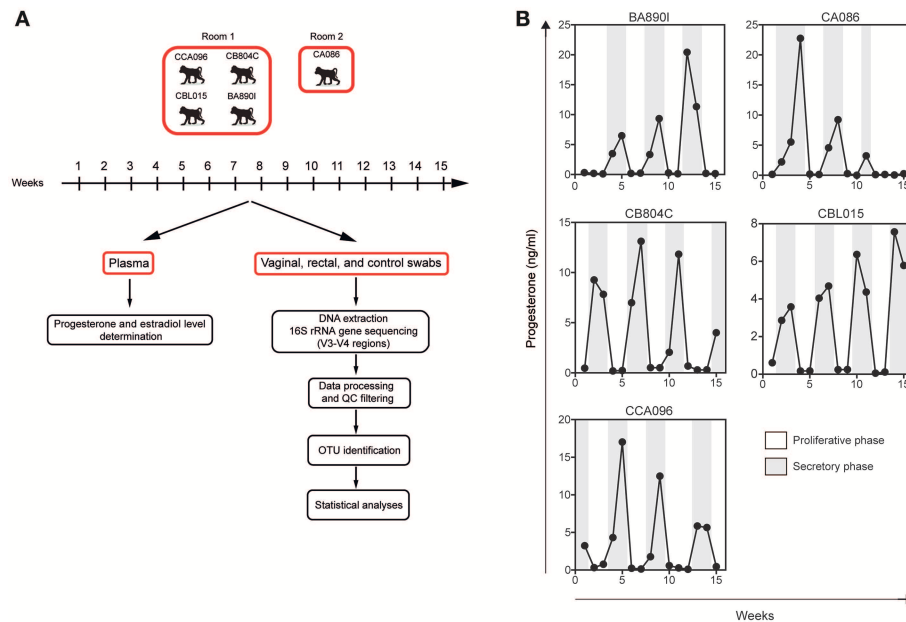


FIGURE 1 | Experimental design and individual progesterone levels. **(A)** Five cynomolgus macaques were included in this study to characterize their vaginal and rectal microbiota. Blood, rectal, and vaginal swabs were sampled weekly during a period of 15 weeks. Four animals were housed in the same room and one animal in another. Room controls (air swab) were performed each week to check for possible contamination or other technical biases. After DNA extraction from the swab material, the bacterial 16S rRNA gene was sequenced using primers targeting the V3 and V4 regions. Sequenced reads were preprocessed and analyzed with QIIME for OTU detection and taxonomic assignment. Statistical analyses were then performed to identify relevant taxa. **(B)** Progesterone levels (black line) were determined in the plasma of the animals once a week for 15 weeks.

in the rectum and vaginal tissues was constructed as the union of the two taxonomic trees generated by QIIME. Multidimensional scaling (MDS) representations were generated based on the SVD-MDS algorithm (Becavin et al., 2011). Distances between microbial profiles were computed as Euclidian distances between their relative abundances of identified taxa. The Kruskal Stress indicated in each MDS representation corresponds to the percentage of information lost in the dimensionality reduction process.

RESULTS

Rectal and Vaginal Microbiota Are Highly Diverse With Greater Variability Between Individuals in the Vaginal Compartment

Five cynomolgus macaques were included in this study to characterize their vaginal and rectal microbiota. Sample collection was performed once a week for 15 weeks (Figure 1A), corresponding to approximately three menstrual cycles. Plasma levels of progesterone (Figure 1B) were determined weekly for the five animals.

We first aimed to identify the taxa present in the rectal and vaginal microbiota of the five cynomolgus macaques, regardless of their kinetics, and quantify their relative abundance. We identified taxa present in the rectum and vagina for each animal and then combined OTU having the same taxonomic level for

each compartment to account for potential individual variability. Only taxa identified with an abundance >1% in at least one animal and one timepoint were considered.

We identified taxa from 12 phyla in rectal samples and nine phyla in vaginal samples (Figures 2A,B). *Firmicutes* was the major phylum detected in the rectum of all the animals, whereas *Bacteroidetes* was the major phylum in the vagina. This was followed by *Bacteroidetes* in the rectum and *Firmicutes* and *Fusobacteria* in the vagina. In the rectum, *Fusobacteria* represented <1% of the bacteria. *Proteobacteria* were detected in both the rectum and vagina at >1% abundance, together with *Spirochaetes* in the rectum and *Actinobacteria* in the vagina.

Individually, the most representative phyla in the rectum were, in order of decreasing abundance: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Spirochaetes*, except for one animal (CB804C) that had a higher abundance of *Spirochaetes* than *Proteobacteria* (Supplementary Table 1). In the vagina, more than 90% of the bacteria were comprised of *Bacteroidetes*, *Firmicutes*, and *Fusobacteria*, but not always in the same order of abundance, except for animal CB804C (Supplementary Table 2). CB804C vaginal samples had a greater abundance of *Actinobacteria* than the other animals. CBL015 had a higher and CCA096 a lower abundance of *Fusobacteria*. *Actinobacteria* and *Proteobacteria* were also present in all the animals.

We identified 31 genera in cynomolgus macaque rectal samples (Figure 2C and Supplementary Table 3), and when the

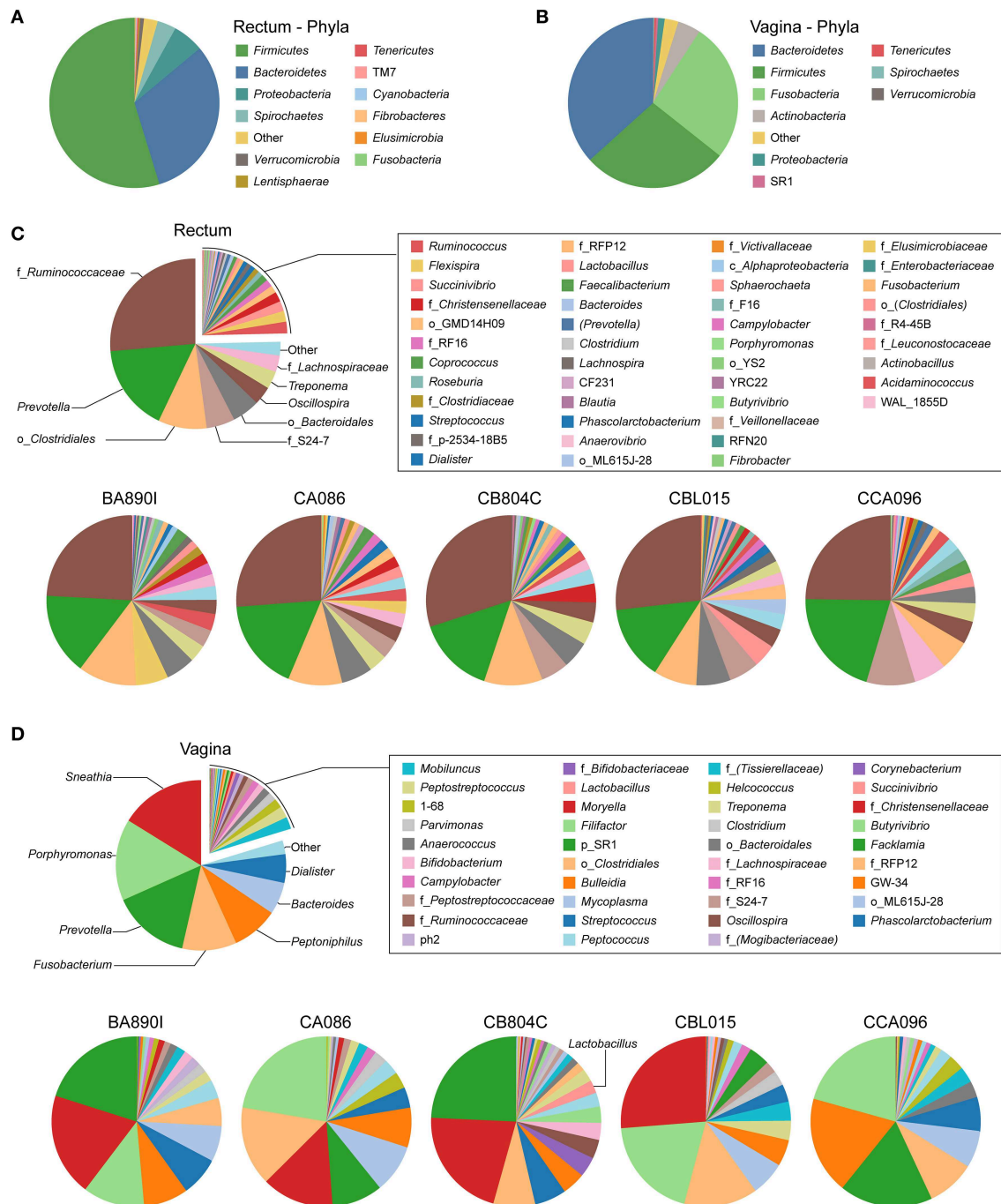


FIGURE 2 | Relative abundance of identified taxa in rectal and vaginal samples. Analyses of relative taxa abundance were performed independently for rectal and vaginal samples. The means of the relative taxa abundances of the five animals are displayed using pie chart representations (**A,B** and top panels of **C,D**). The means of the relative taxa abundances for each animal are also represented (bottom panels of **C,D**). The most abundant taxa are indicated on the chart while other taxa are indicated in the legend. For each taxon, the name of the genus is indicated, when possible. In other cases, the family (f_), order (o_), class (c_), or phylum (p_) of the taxa are indicated.

genus or subsequent taxa could not be assigned, we identified 15 families, six orders, and one class. Among all the animals, seven genera, five families, and three orders had an abundance >1% (**Table 2**). Thirteen of the 15 identified taxa with an

abundance >1% in the entire cynomolgus macaque population were identified in all animals and three (*f_Ruminococcaceae*, *Prevotella*, and *o_Clostridiales*) were identified in all rectal samples (**Table 2**).

TABLE 2 | Most abundant taxa identified in the macaque rectal microbiota.

Taxa	% of sequences ^b	Present in (%) ^a	
		Samples	Animals
f_Ruminococcaceae	26.3	100	100
Prevotella	16.5	100	100
o_Clostridiales	9.2	100	100
f_S24-7	5.4	93	100
o_Bacteroidales	5.3	99	100
Oscillospira	3.5	99	100
Treponema	3.4	96	100
f_Lachnospiraceae	3.1	88	100
Ruminococcus	2.2	83	100
Flexispira	2.1	36	80
Succinivibrio	1.9	49	100
f_Christensenellaceae	1.9	60	100
o_GMD14H09	1.4	56	80
f_RF16	1.3	51	100
Coproccoccus	1.3	48	100
Other	2.6	99	100

^aPresent at ≥1%.^bAverage number of sequences in the 75 samples from the five animals.

The most abundant taxa in the rectum (Figure 2C, lower pie charts) were, in order of decreasing abundance: f_Ruminococcaceae (min 24.1%–max 30.0%), Prevotella (14.1–20.6%), and o_Clostridiales (5.8–11.1%), except for one animal (CCA096). For CCA096, f_S24-7 (9.3%) and f_Lachnospiraceae (6.2%) were more abundant than o_Clostridiales (5.8%).

We identified 32 genera in cynomolgus macaque vaginal samples (Figure 2D and Supplementary Table 4), and when the genus or subsequent taxa could not be assigned, we identified 10 families, three orders, and one phylum. Among all animals, 14 genera and one family had an abundance >1% (Table 3). Eleven of the 15 identified taxa with an abundance >1% in the entire population were identified in all animals, and none were identified in all vaginal samples (Table 3).

The most representative genera in the vagina (Figure 2D, lower pie charts) were different for each animal. For example, Sneathia was an abundant genus in four animals (mean ranging from 13.7 to 26.2%) but was not present in animal CCA096. Peptoniphilus (18.6%) was abundant in CCA096 but present at relative abundances ranging between 4.4 and 8.5% in the other animals. Porphyromonas was the most abundant genera in two animals (>20% in CA086 and CCA096), whereas it was only present at an abundance of 3.1% in CB804C. Similarly, Prevotella, predominated in the vaginal microbiota in two animals (>20% in BA890I and CB804C) but was only present at abundances of 2 and 9.7% in two others (CBL015 and CA086, respectively). Peptostreptococcus, Mobiluncus, Anaerococcus, 1–68, and Campylobacter were present in all animals at abundances ranging from 3.6 to 0.5%. Non-identified taxa were also present in the vagina of all animals from an abundance of 1.7–3.6%.

TABLE 3 | Most abundant taxa identified in the macaque vaginal microbiota.

Taxa	% of sequences ^b	Present in (%) ^a	
		Samples	Animals
Sneathia	16.2	77	80
Porphyromonas	15.5	97	100
Prevotella	14.8	87	100
Fusobacterium	10.3	83	100
Peptoniphilus	8.8	99	100
Bacteroides	6.1	61	100
Dialister	5.5	95	100
Mobiluncus	2.3	69	100
Peptostreptococcus	2.2	79	100
1–68	1.8	55	100
Parvimonas	1.6	59	80
Anaerococcus	1.4	39	100
Bifidobacterium	1.2	28	80
Campylobacter	1.2	44	100
f_Peptostreptococcaceae	1.1	32	80
Other	2.6	96	100

^aPresent at ≥1%.^bAverage number of sequences in the 75 samples from the five animals.**TABLE 4 |** Species inference for Lactobacillus sequences in CB804C vaginal samples.

Lactobacillus species	Number of aligned reads	% of aligned reads
L. crispatus	49	52.1
L. helveticus	22	23.4
L. acidophilus	8	8.5
L. murinus	4	4.3
L. reuteri	4	4.3
L. delbrueckii	2	2.1
Unknown	2	2.1
L. apodemi	1	1.1
L. johnsonii	1	1.1
L. nagelii	1	1.1

CB804C had the highest number of identified taxa (34) and was also the only one to have detectable Lactobacillus in its vaginal microbiota (mean abundance of 2.46%) (Figure 2D, lower pie charts). Lactobacillus was present in all 15 collected vaginal samples but varied in abundance from 0.06 to 10.54%. The QIIME analysis was unable to determine the specific species based on the GreenGene database. Further analysis, using RefSeq database (Pruitt et al., 2007) identified the closest Lactobacillus species, in terms of sequence homology to be closely related to Lactobacillus crispatus (52.13%), Lactobacillus helveticus (23.40%), and Lactobacillus acidophilus (8.51%) (Table 4).

Overall, we identified a large set of taxa in the cynomolgus macaque rectal and vaginal microbiota. Taxa differed in terms of abundance between the five animals with greater variability in relative abundance and composition in the vaginal microbiota.

The Vaginal Macaque Microbiota Is More Heterogeneous Than the Rectal Microbiota

We next compared the macaque rectal and vaginal microbiota and assessed their similarity and specificity. We created a tree representation (Figure 3A) to compare the taxonomic composition of the macaques' rectal and vaginal microbiota. Taxa are reported and classified with their associated phylum, class, order, family, and genus for clarity. Among the 31 genera identified in rectal samples, 17 (55%) were specific to this tissue. Among the 32 genera identified in vaginal samples, 18 (56%) were specific to this tissue. A total of 14 genera were found both in the rectal and vaginal samples.

We created a MDS representation (Figure 3B) to compare the rectal and vaginal microbial profiles in terms of taxa composition at the genus level. Rectal and vaginal samples were well-segregated. The variability of microbiota composition in the vagina was higher than that of rectum. We qualified sample variabilities by inter-class similarity (IC), which was 0.7334 for the rectum and 2.0351 for the vagina. Overall, vaginal and rectal samples were different in terms of relative taxa abundance, with higher taxonomic composition variability in vaginal samples.

Our analyses revealed that vaginal and rectal microbiota share about half of the detected taxa at the genus level. The cynomolgus macaque vaginal microbiota showed higher variability between animals in both taxa composition and relative abundance.

Vaginal Cynomolgus Macaque Microbiota Display Similar Composition and Abundance to Woman Vaginal Microbiota Belonging to CST-IV-A

We compared the cynomolgus macaque vaginal microbial profiles with those of humans using a previously published dataset of human vaginal samples collected cross-sectionally (Ravel et al., 2010). This dataset consists of samples from 376 patients assigned to five community state types (CST) designated CST I, II, III, IV-A, IV-B, and CST V. The MDS analysis clearly showed that the cynomolgus macaque vaginal microbiota is most similar, in terms of taxonomic composition and relative abundance, to a subset of women's vaginal microbiota belonging to CST IV-A (Figure 3C).

The Kinetics of Rectal Microbiota Profiles Are Constant and Similar Between Animals, but the Kinetics of Vaginal Microbiota Profiles Are Highly Variable and Animal-Specific

We next displayed the kinetics of the taxonomic profiles in both rectal and vaginal samples collected within animals over 15 weeks and quantified their variability at each timepoint for each macaque.

We generated streamgraph representations for both the rectal (Figure 4A) and vaginal (Figure 4B) microbiota of each macaque to visualize the kinetics of taxonomic profiles. The kinetics were more stable for the rectal microbiota (mean of all 5 animals

Jensen-Shannon divergence medians across all time points = 0.0796) than those of the vagina (0.1394). The rank of the most abundant taxa throughout the study was consistent among the different animals in the rectum but was not in the vagina. Several taxa, such as *Lactobacillus*, were not detectable at all timepoints in the vagina.

For the rectum, the number of taxa per timepoint was between 15 and 25, without major differences between timepoints or animals (Supplementary Table 5). For the vagina, the number of taxa per timepoint varied between seven and twenty, with differences between timepoints and animals (Supplementary Table 6).

We quantified the change in alpha diversity, calculated using the Simpson diversity index, for each animal between consecutive timepoints (Figure 4C). The mean alpha diversity was 0.8957 (standard deviation = 0.0465) for the rectal samples whereas the mean alpha diversity was 0.8279 (standard deviation = 0.0529) for the vaginal samples, indicating that the change in diversity is higher in the vagina. The alpha diversity of vaginal samples peaked at various timepoints and was particularly apparent for some animals (e.g., animal CB804C).

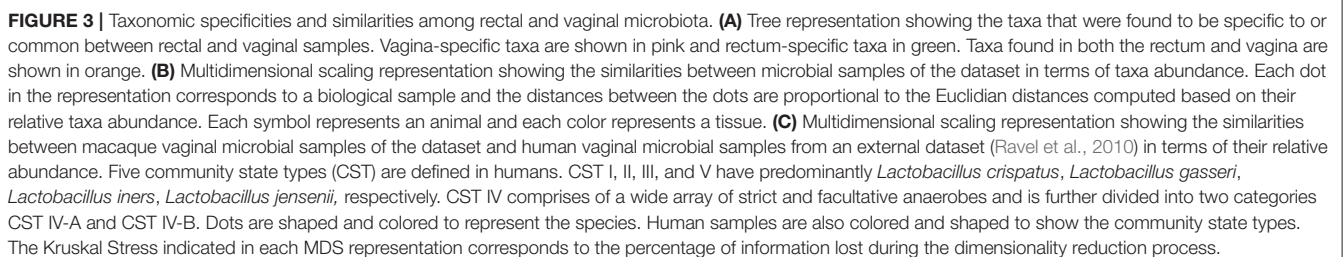
These analyses show different kinetics of taxonomic profiles over 15 weeks in both the rectal and vaginal microbiota. In the rectum, similar kinetics were observed between animals, whereas in the vagina, longitudinal profiles were diverse and clearly different between animals. These observations were supported by alpha diversity changes computed between each timepoint for each animal.

Hormonal Cycles of Female Macaques Influence the Vaginal Microbiota

We then explored whether the lack of stability in vaginal microbiota profiles in cynomolgus macaques is associated with the hormonal cycle. Hormonal cycles were defined by progesterone plasma levels, measured weekly for each animal. This analysis was performed separately for each animal to account for individual variability.

Microbial profiles were classified into two groups, representing low (proliferative phase) or high (secretory phase) progesterone levels. For each animal, we compared the relative abundance of the 47 taxa identified in the vaginal microbiota between the two groups of samples (high vs. low progesterone levels). This differential analysis identified 12 taxa (26% of vaginal taxa) that were differentially abundant in at least one animal according to the progesterone level (Figure 5A), consisting of *Mobiluncus*, *Bacteroides*, *Prevotella*, *Lactobacillus*, 1–68, *Helcococcus*, *Peptoniphilus*, ph2, *Peptococcus*, *f_Peptostreptococcaceae*, *Peptostreptococcus*, and *Fusobacterium*.

For example, *f_Peptostreptococcaceae* was associated with hormonal cycling in animal BA890I, as well as *Fusobacterium*, *Lactobacillus*, and *Peptoniphilus* in animal CB804C (Figure 5B). *Peptostreptococcaceae* was statistically associated ($p = 0.0209$) with the hormonal cycle in animal BA890I, with lower relative abundance in the secretory phase (low progesterone levels). *Fusobacterium* was statistically associated ($p = 0.0033$) with the hormonal cycle in animal CB804C, with a lower abundance in



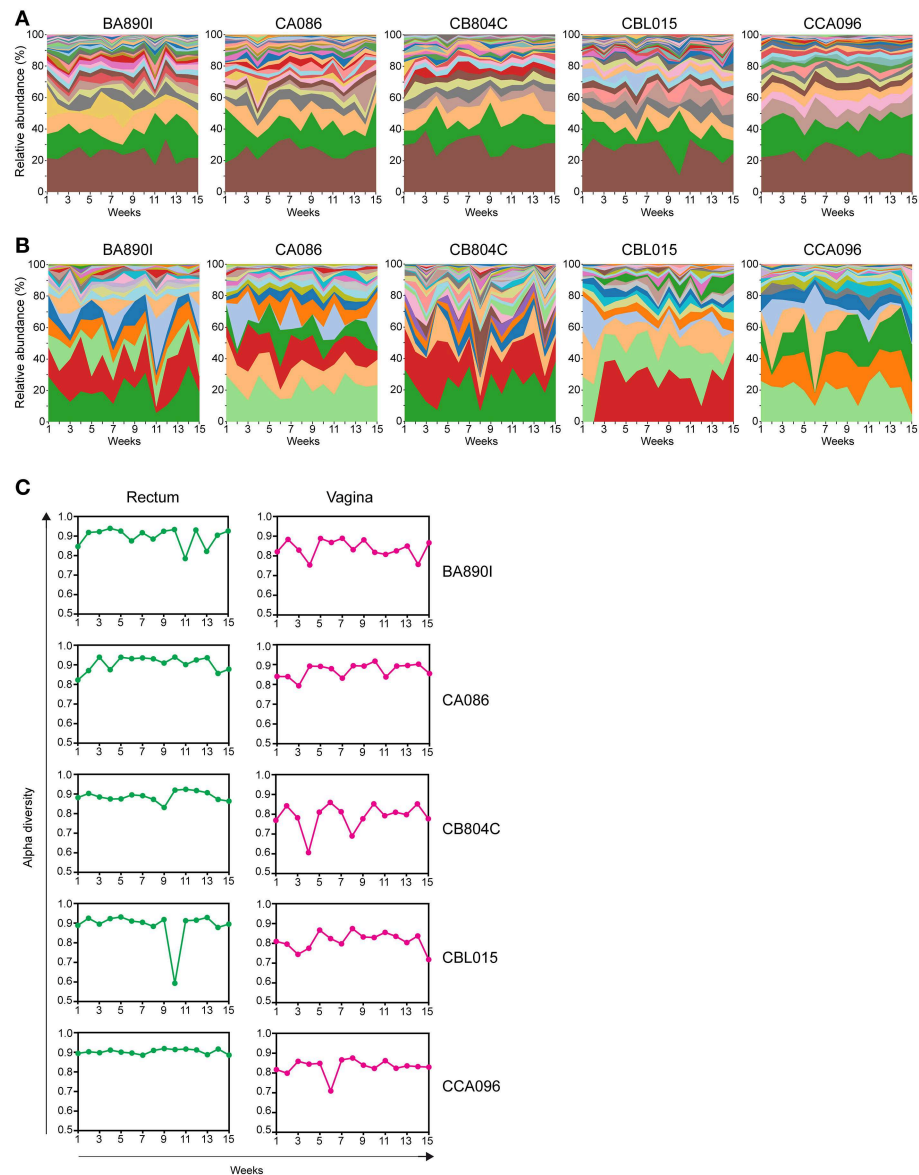


FIGURE 4 | Kinetics of relative taxa abundance. The kinetics of the relative abundance for all identified taxa are shown for each animal, using stream graph representations for rectal (A) and vaginal samples (B) during the 15 weeks of follow-up. (C) The alpha-diversities were computed for each animal and at each time point and are represented for the rectum and vagina. Alpha-diversities were calculated based on the Simpson diversity index (1-dominance).

the proliferative phase (high progesterone levels). Conversely *Lactobacillus* ($p = 0.0058$) and *Peptoniphilus* ($p = 0.0301$) in animal CB804C had statistically higher abundance during the secretory phase. Overall, 11 taxa were more abundant during the secretory phase, whereas eight were more abundant in the proliferative phase (Figure 5B and Supplementary Figure 1). In summary, approximately one-quarter of detected vaginal taxa were associated with hormonal cycling.

Thus, time in the menstrual cycle is associated with differential relative abundance of vaginal microbial taxa in macaques. Approximately, a quarter of all identified vaginal taxa had abundances associated with phases of the menstrual cycle in

each animal. This proportion dropped to 9% when considering the entire cohort, highlighting considerable individual variability in the bacterial composition and abundance in the cynomolgus macaque vaginal microbiota.

DISCUSSION

We describe here the composition and dynamics of the rectal and vaginal microbiota in female cynomolgus macaques over 15 weeks capturing at least three menstrual cycles. This is the first time that such a longitudinal study has been reported comparing

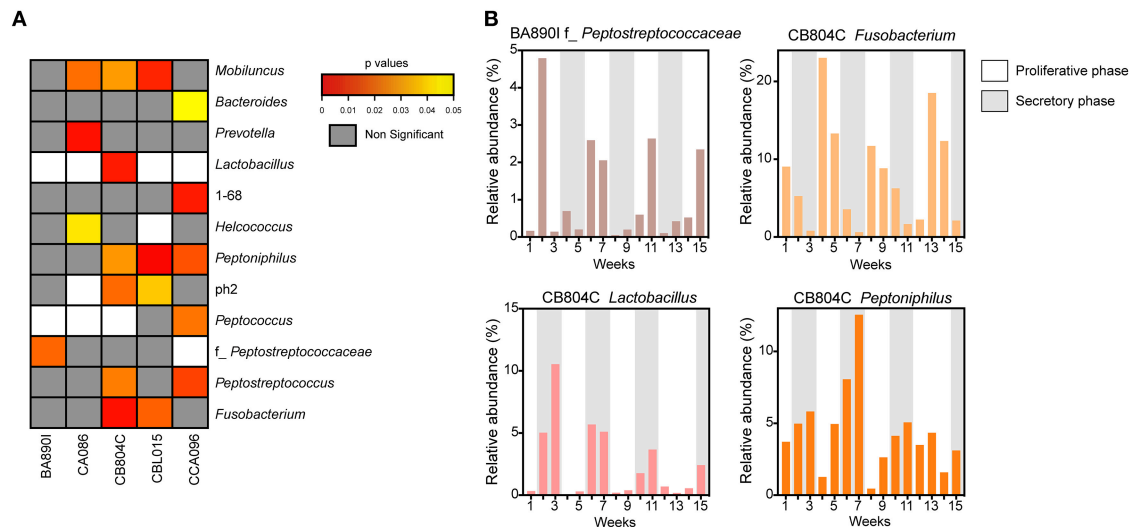


FIGURE 5 | Associations between progesterone levels and taxa abundance in vaginal samples. **(A)** Heatmap representation showing the *p*-values for the association for each taxon, for each animal. The heatmap was restricted to the set of taxa found to be significantly different in abundance between samples taken when progesterone levels were high vs. when they were low. White rectangles represent situations in which differential analyses were not possible because the relative abundance was too low. For each taxon, the name of the genus or family (f_) is indicated. **(B)** Examples of relative taxa abundance and progesterone levels for four significant taxa in animals BA890I and CB804C. The histograms indicate the relative taxa abundance. Progesterone levels are indicated by the gray (≥ 1 ng/ml) or white (< 1 ng/ml) rectangles.

the microbiota composition of both body sites in the same animals and assessing the hormonal impact.

The major phyla found in the rectum of the cynomolgus macaques are consistent with previous reports on the fecal microbiota of cynomolgus macaques (Seekatz et al., 2013; Cui et al., 2018; Li et al., 2018). The composition of the cynomolgus rectal microbiota is similar to that of high diversity community type II, previously characterized by higher relative abundance of taxa of both *Firmicutes* (*Ruminococcaceae*) and *Bacteroidetes* (*Prevotella*) and lower abundance of *Lactobacillus* (Seekatz et al., 2013). *Firmicutes* and *Bacteroidetes* are also the major phyla found in rectal swabs or feces in humans. The cynomolgus rectal microbiota is somewhat similar to that of enterotype 2 described in humans (Wu et al., 2011), in which *Prevotella* is dominant rather than *Bacteroides*. This enterotype is associated with a low fat, high fiber dietary regimen. X. Li et al. reported that the gut microbiome of cynomolgus macaques is more similar to that of human than those of pig and mouse at the gene level and that the gut microbiota of cynomolgus macaques fed with a high fat, low fiber diet became more similar to the gut microbiota of humans (Li et al., 2018). We did not detect *Actinobacteria*, which is one of the major constituents of the human gastrointestinal tract microbiota (Zoetendal et al., 2008), in the cynomolgus rectal microbiota but rather *Spirochaetes*. Such a lack of rectal *Actinobacteria* does not appear to be related to sample collection or detection issues as *Actinobacteria* were observed in vaginal samples.

The vaginal cynomolgus macaque microbiota is composed of a large variety of anaerobic gram-negative bacteria and unlike humans, is not dominated by *Lactobacillus*. The abundance of

Lactobacillus (mostly *L. crispatus*) was $> 1\%$ for only one female macaque in the study. *Lactobacillus* was present in some of the samples in the four other females, but at an abundance of $< 0.6\%$ (data not filtered, not shown). *L. amylovorus* has been identified at low levels and not in all samples in pigtailed macaques (Spear et al., 2012). In rhesus macaques, previous reports have described vaginal microbiota with few *Lactobacillus* and when present the species was *L. johnsonii* (Yu et al., 2009; Spear et al., 2010). In humans, the most prevalent species are *L. iners*, *L. crispatus*, *L. jensenii*, and *L. gasseri* (Spear et al., 2011; Ma et al., 2012).

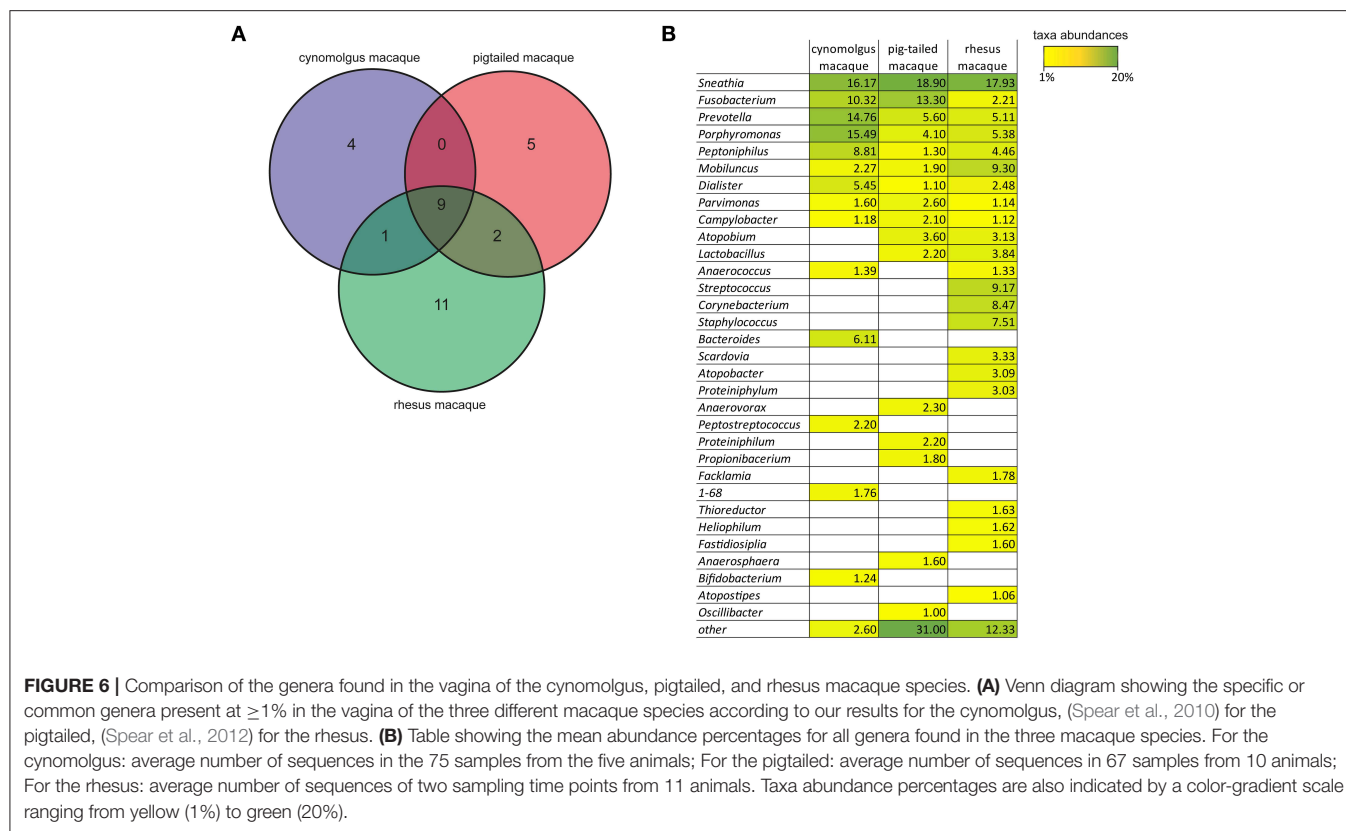
It has been previously shown that both Nugent scores and pH values increase as the proportion of non-*Lactobacillus* spp. increases. We did not measure vaginal pH nor determine the Nugent score for the five female cynomolgus macaques included in our study. However, we had the opportunity to measure the vaginal pH and to determine the Nugent scores of female cynomolgus macaques included in parallel studies and housed in the same animal facility. We thus measured the vaginal pH at one timepoint in 12 female cynomolgus macaques, and it ranges between 5.5 and 7.5, with a mean of 6.75. We also measured the vaginal pH of three other females once a week for 1 month. The values were relatively stable over time, ranging from 6.25 to 7.5, and were associated with a Nugent score of 8 for all timepoints. These high pH measures are certainly associated with a lack of copious amount lactic acid produced by *Lactobacillus* spp. when present in high abundance, as lactic acid, with a pKa of 3.86, is a major driver of the low vaginal pH (< 4.5) in human. These data combined with our MDS representation clearly show that the vaginal microbial communities in cynomolgus macaques are very close to that of CST IV-A in human: a

highly diverse state comprising numerous strict and facultative anaerobes, also associated with elevated Nugent scores and pH (Ravel et al., 2010). This profile has been shown to be strongly associated with increased risk of HIV-1 and other STIs. Most of the vaginal bacterial species of the cynomolgus macaques (such as *Sneathia*, *Porphyromonas*, and *Prevotella*) are also found in women with symptomatic or asymptomatic BV a condition more common in Black and Hispanic women (Ravel et al., 2010; Kenyon and Osbak, 2015). In contrast, *Gardnerella vaginalis*, another species commonly found in women with BV was not detected in cynomolgus macaques. *G. vaginalis* has been found in pigtailed macaques in a few samples at low levels (Spear et al., 2012). The low level of *Lactobacillus* and the polymicrobial composition of the vaginal microbiota have also been reported in rhesus (Spear et al., 2010) and pigtailed (Spear et al., 2012) macaques. The Venn diagram on **Figure 6A** shows the main genera that are common or specific to the three macaque species. Nine genera are common to the three species with different abundance percentages but *Sneathia* is the most abundant in all (**Figure 6B**). Three genera are shared by two species and four are cynomolgus specific, five pigtailed specific and 11 rhesus specific. To date, humans are the only mammals to have vaginal microbiota often dominated by *Lactobacillus* spp., and a very low pH (≤ 4.5). The growth of genital *Lactobacillus* spp. is postulated to depend on epithelial cell-produced glycogen (Mirmonsef et al., 2014). It is hypothesized that degradation products of glycogen by human α -amylases provide a selective nutritional advantage to *Lactobacillus* spp., which in turn produce high amount of lactic acid (Boskey et al., 1999; Miller et al., 2016). Elevated estrogen levels promote thickening of the epithelium and the production and accumulation of glycogen in the epithelium (Mirmonsef et al., 2014). Free glycogen levels are significantly negatively associated with both vaginal pH and progesterone in women (Mirmonsef et al., 2016). Humans have higher vaginal concentrations of glycogen than other mammals (Miller et al., 2016) and α -amylase is expressed in the vaginal epithelium (Mirmonsef et al., 2014). The levels of lactic acid and glycogen in the genital fluids of rhesus and pigtailed macaques are lower than those in women (Mirmonsef et al., 2012). Unfortunately, no measures of glycogen, α -amylase activity, and lactic acid levels in the vaginal tract of cynomolgus macaques are available. It has been postulated that high estrogen levels, high *Lactobacillus* spp. relative abundance, and low vaginal pH are difficult to detect in NHP, as they do not cycle continuously (Miller et al., 2016). However, female cynomolgus macaques have an ovarian cycle similar to that of women. Miller et al. reported that mammals other than humans that exhibit continuous cycling have lower vaginal pH during high-estrogen phases, but they never reach the low pH observed in humans. Ravel et al. postulated that vaginal microbiota depleted of *Lactobacillus* spp., could also be considered normal in the absence of sign and symptoms, as beneficial functions may be provided by several types of bacterial composition (Ma et al., 2012). However, this normal state might not be optimal and appear to still carry some risks if exposure to STI agents but evidence supporting this hypothesis are still lacking. On the other hand, it has been shown that genital proinflammatory cytokine levels strongly

correlate with diverse community types (Anahtar et al., 2015) and that diverse cervicovaginal bacterial communities are associated with increased HIV-1 acquisition (Gosmann et al., 2017). Furthermore, certain cervicovaginal communities are associated with decreased antiretroviral concentrations in the female genital tract (Donahue Carlson et al., 2017), *G. vaginalis* and other anaerobic bacteria metabolisms significantly deplete tenofovir (Klatt et al., 2017). *G. vaginalis* is poorly or not detectable in the vaginal microbiota of the three macaque species that are the most commonly used to study HIV-1 infection but other anaerobes such as *Prevotella* (**Figure 6B**) that can also lead to tenofovir depletion are present. This vaginal environment of macaque species should such be consider when analyzing studies on STI acquisition and prevention in these models. Each macaque species has its own advantages and disadvantages. But in term of vaginal microbiota, even if they have their own specificities, they all have a polymicrobial composition with highly diverse communities comprising numerous strict and facultative anaerobes. The differences between the three macaque species are mostly: (i) in term of ovarian cycle as mentioned above; (ii) in term of SIV/SHIV pathogenesis (Ten Haaf et al., 2001; Favre et al., 2009; Antony and Macdonald, 2015); (iii) the susceptibility to infection/coinfection with human pathogens; and (iv) the availability and cost of each model in the different parts of the world.

The inter-individual compositional similarities and stability of the cynomolgus macaque rectal and vaginal microbiota were different. The microbiota composition of the rectum was quite similar between animals, whereas that of the vagina was distinct for each animal. The five animals had the same dietary regimen and were of the same origin, which can explain the low inter-individual variability in the rectum. The bacterial composition of the vagina is influenced by several parameters such, as hormone levels, environmental factors, diet, genetics, sexual behavior, and STIs. The five female macaques included in this study were first or second generation and may have been exposed to different environmental factors.

Diversity was higher in the rectum than in the vagina, and was still associated with higher stability. In the vagina, this lack of stability, with rapid changes, has previously been observed in some women, and the cause of this lack of resilience remains unknown (Gajer et al., 2012). We demonstrated that temporal changes in vaginal composition are statistically associated with progesterone levels, in contrast to those of the rectum microbiota, which were not. However, for each animal, the number and type of vaginal bacteria differentially abundant according to progesterone level were different, which could be explained by the high inter-individual compositional differences observed. Interestingly, some taxa such as *Mobiluncus* or *Peptostreptococcus*, were associated with progesterone levels in some animals but were found in lower abundance in others. Factors not captured in this study and not associated with hormonal cycling may influence the composition and abundance of taxa in the cynomolgus macaque vaginal microbiota. In our study, we observed high relative abundance of *Lactobacillus* spp. in one animal to be associated with high level of progesterone. However, high levels of progesterone were concomitant with



lower level of estradiol and followed the major peak of estradiol (Supplementary Figure 2). Thus, it is likely that, similarly to human, in this animal, high levels of progesterone are driving the higher relative abundance of *Lactobacillus* spp.

A better understanding of the relationship between bacterial composition and dynamics, and the influence of the hormonal cycle will help to improve our understanding of the role of vaginal microbiota in STI susceptibility or resistance using animal models such as the cynomolgus macaque. Longitudinal analysis of the vaginal and rectal microbiota in non-human-primate models are necessary for this purpose. Further investigations in macaque models are necessary to understand whether the rectal or vaginal microbiota affect susceptibility to infections and local immune response. Ultimately, access to a well-characterized cynomolgus macaque model in which the vaginal microbiota could be manipulated (local antibiotic treatments and/or local administration of specific bacterial strains) to mimic that of human, will help us to decipher the role of the vaginal microbiota play in the protection against STIs in human.

DATA AVAILABILITY

The datasets generated for this study can be found in SRA database (Leinonen et al., 2011), PRJNA497378. All raw sequenced paired-end reads, assembled reads, scripts used for the data analysis, and complementary visualization charts of taxa abundances are available on the IDMIT data dissemination platform through the link <http://data.idmitcenter.fr/cynobiota/>.

ETHICS STATEMENT

Treatment of (NHP) at the CEA complied with French national regulations (CEA authorization A 92-032-02), the Standards for Human Care and Use of Laboratory Animals (OLAW Assurance number #A5826-01), and European Directive 2010/63 (recommendation #9). Experiments were supervised by veterinarians in charge of the animal facility. This study was approved and accredited by the Comité d'Ethique en Expérimentation Animale du CEA (A16-048), and the French Research Ministry.

AUTHOR CONTRIBUTIONS

VC, RL, RM, and EM: study conception and design. M-TN, NT, CC, RM, and EM: acquisition of data. M-TN, NT, JR, RM, and EM: analysis and interpretation of data. M-TN, NT, CA, RM, and EM: drafting of manuscript. M-TN, NT, FB, JR, RL, and EM: critical revisions.

FUNDING

This work was funded by the French Agence Nationale de Recherches sur le Sida et les Hépatites Virales (ANRS). This work was supported by the French government Programme d'Investissements d'Avenir (PIA) under Grant ANR-11-INBS-0008 funding the Infectious Disease Models and Innovative Therapies (IDMIT, Fontenay-aux-Roses, France) infrastructure

and the Grant ANR-17-E15-0020-03 funding the ACROBAT project. JR was supported by the National Institute for Allergy and Infectious Diseases of the National Institutes of Health under award number U19AI084044. NT was supported by fellowships from the ANRS. FB was supported by fellowships from the ANRS, Sidaction, and the Institut Carnot Pasteur Microbes and Santé.

ACKNOWLEDGMENTS

The authors would like to thank Thierry Pedron and Azadeh Saffarian from the Institut Pasteur in Paris for their valuable

contributions and advice concerning this study. The authors also would like to thank the Biomix Pole of the CITECH structure at the Institut Pasteur in Paris for the sequencing services and all members of the ASW and L2I groups of the IDMIT infrastructure.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2019.00188/full#supplementary-material>

REFERENCES

- Alfonso, K. J., Avena, L. E., Worwa, G., Carrion, R., and Griffiths, A. (2017). Development of a lethal intranasal exposure model of ebola virus in the cynomolgus macaque. *Viruses* 9:E319. doi: 10.3390/v9110319
- Anahtar, M. N., Byrne, E. H., Doherty, K. E., Bowman, B. A., Yamamoto, H. S., Soumillon, M., et al. (2015). Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* 42, 965–976. doi: 10.1016/j.immuni.2015.04.019
- Antonio, M. A., Hawes, S. E., and Hillier, S. L. (1999). The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J. Infect. Dis.* 180, 1950–1956. doi: 10.1086/315109
- Antony, J. M., and Macdonald, K. S. (2015). A critical analysis of the cynomolgus macaque, *Macaca fascicularis*, as a model to test HIV-1/SIV vaccine efficacy. *Vaccine* 33, 3073–3083. doi: 10.1016/j.vaccine.2014.12.004
- Becavin, C., Tchitchek, N., Mints-Eya, C., Lesne, A., and Benecke, A. (2011). Improving the efficiency of multidimensional scaling in the analysis of high-dimensional data using singular value decomposition. *Bioinformatics* 27, 1413–1421. doi: 10.1093/bioinformatics/btr143
- Boratyn, G. M., Camacho, C., Cooper, P. S., Coulouris, G., Fong, A., Ma, N., et al. (2013). BLAST: a more efficient report with usability improvements. *Nucleic Acids Res.* 41, W29–33. doi: 10.1093/nar/gkt282
- Borgdorff, H., Armstrong, S. D., Tytgat, H. L., Xia, D., Ndayisaba, G. F., Wastling, J. M., et al. (2016a). Unique insights in the cervicovaginal *Lactobacillus iners* and *L. crispatus* proteomes and their associations with microbiota dysbiosis. *PLoS ONE* 11:e0150767. doi: 10.1371/journal.pone.0150767
- Borgdorff, H., Gautam, R., Armstrong, S. D., Xia, D., Ndayisaba, G. F., Van Teijlingen, N. H., et al. (2016b). Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier. *Mucosal Immunol.* 9, 621–633. doi: 10.1038/mi.2015.86
- Boskey, E. R., Telsch, K. M., Whaley, K. J., Moench, T. R., and Cone, R. A. (1999). Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. *Infect. Immun.* 67, 5170–5175.
- Braundmeier, A. G., Lenz, K. M., Inman, K. S., Chia, N., Jeraldo, P., Walther-Antonio, M. R., et al. (2015). Individualized medicine and the microbiome in reproductive tract. *Front. Physiol.* 6:97. doi: 10.3389/fphys.2015.00097
- Caporaso, J. G., Bittinger, K., Bushman, F. D., Desantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303
- Clemente, J. C., Ursell, L. K., Parfrey, L. W., and Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell* 148, 1258–1270. doi: 10.1016/j.cell.2012.01.035
- Cribby, S., Taylor, M., and Reid, G. (2008). Vaginal microbiota and the use of probiotics. *Interdiscip. Perspect. Infect. Dis.* 2008:256490. doi: 10.1155/2008/256490
- Cui, Y. F., Wang, F. J., Yu, L., Ye, H. H., and Yang, G. B. (2018). Metagenomic comparison of the rectal microbiota between rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*). *Zool. Res.* 40, 89–93. doi: 10.24272/j.issn.2095-8137.2018.061
- Desantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05
- Donahue Carlson, R., Sheth, A. N., Read, T. D., Frisch, M. B., Mehta, C. C., Martin, A., et al. (2017). The female genital tract microbiome is associated with vaginal antiretroviral drug concentrations in human immunodeficiency virus-infected women on antiretroviral therapy. *J. Infect. Dis.* 216, 990–999. doi: 10.1093/infdis/jix420
- Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461
- Eschenbach, D. A., Davick, P. R., Williams, B. L., Klebanoff, S. J., Young-Smith, K., Critchlow, C. M., et al. (1989). Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. *J. Clin. Microbiol.* 27, 251–256.
- Favre, D., Lederer, S., Kanwar, B., Ma, Z. M., Proll, S., Kasakow, Z., et al. (2009). Critical loss of the balance between Th17 and T regulatory cell populations in pathogenic SIV infection. *PLoS Pathog.* 5:e1000295. doi: 10.1371/journal.ppat.1000295
- Ferreira, V. H., Kafka, J. K., and Kaushic, C. (2014). Influence of common mucosal co-factors on HIV infection in the female genital tract. *Am. J. Reprod. Immunol.* 71, 543–554. doi: 10.1111/aji.12221
- Fredricks, D. N., Fiedler, T. L., and Marrazzo, J. M. (2005). Molecular identification of bacteria associated with bacterial vaginosis. *N. Engl. J. Med.* 353, 1899–1911. doi: 10.1056/NEJMoa043802
- Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schutte, U. M., Zhong, X., et al. (2012). Temporal dynamics of the human vaginal microbiota. *Sci. Transl. Med.* 4, 132–152. doi: 10.1126/scitranslmed.3003605
- Ganesh, B. P., and Versalovic, J. (2015). Luminal conversion and immunoregulation by probiotics. *Front. Pharmacol.* 6:269. doi: 10.3389/fphar.2015.00269
- Giorgi, A., Torriani, S., Dellaglio, F., Stola, E., and Bernuzzi, L. (1987). Identification of vaginal lactobacilli from asymptomatic women. *Microbiologica* 10, 377–384.
- Gosmann, C., Anahtar, M. N., Handley, S. A., Farcasanu, M., Abu-Ali, G., Bowman, B. A., et al. (2017). *Lactobacillus*-deficient cervicovaginal bacterial communities are associated with increased HIV acquisition in young South African women. *Immunity* 46, 29–37. doi: 10.1016/j.immuni.2016.12.013
- Homayouni, A., Ziyadi, S., Mohammad-Alizadeh-Charandabi, S., Ghalibaf, M., Mortazavian, A. M., and Mehrabany, E. V. (2014). Effects of probiotics on the recurrence of bacterial vaginosis: a review. *J. Low. Genit. Tract Dis.* 18, 79–86. doi: 10.1097/LGT.0b013e31829156ec
- Kenyon, C., and Osbak, K. (2015). Sexual networks, HIV, race and bacterial vaginosis. *AIDS* 29, 641–642. doi: 10.1097/QAD.0000000000000566
- Klatt, N. R., Cheu, R., Birse, K., Zevin, A. S., Perner, M., Noël-Romas, L., et al. (2017). Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* 356, 938–945. doi: 10.1126/science.aai9383
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for

- classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808
- Landman, C., and Quevrain, E. (2016). Gut microbiota: description, role and pathophysiologic implications. *Rev. Med. Interne* 37, 418–423. doi: 10.1016/j.revmed.2015.12.012
- Leinonen, R., Sugawara, H., Shumway, M., and International Nucleotide Sequence Database C. (2011). The sequence read archive. *Nucleic Acids Res.* 39, D19–21. doi: 10.1093/nar/gkq1019
- Li, X., Liang, S., Xia, Z., Qu, J., Liu, H., Liu, C., et al. (2018). Establishment of a *Macaca fascicularis* gut microbiome gene catalog and comparison with the human, pig, and mouse gut microbiomes. *Gigascience* 7, 1–10. doi: 10.1093/gigascience/giy100
- Ma, B., Forney, L. J., and Ravel, J. (2012). Vaginal microbiome: rethinking health and disease. *Annu. Rev. Microbiol.* 66, 371–389. doi: 10.1146/annurev-micro-092611-150157
- Magoc, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507
- Manuzak, J. A., Hensley-McBain, T., Zevin, A. S., Miller, C., Cubas, R., Agricola, B., et al. (2016). Enhancement of microbiota in healthy macaques results in beneficial modulation of mucosal and systemic immune function. *J. Immunol.* 196, 2401–2409. doi: 10.4049/jimmunol.1502470
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 17, 10–12. doi: 10.14806/ej.17.1.200
- Miller, E. A., Beasley, D. E., Dunn, R. R., and Archie, E. A. (2016). Lactobacilli dominance and vaginal pH: why is the human vaginal microbiome unique? *Front. Microbiol.* 7:1936. doi: 10.3389/fmicb.2016.01936
- Mirmonsef, P., Gilbert, D., Veazey, R. S., Wang, J., Kendrick, S. R., and Spear, G. T. (2012). A comparison of lower genital tract glycogen and lactic acid levels in women and macaques: implications for HIV and SIV susceptibility. *AIDS Res. Hum. Retroviruses* 28, 76–81. doi: 10.1089/aid.2011.0071
- Mirmonsef, P., Hotton, A. L., Gilbert, D., Burgad, D., Landay, A., Weber, K. M., et al. (2014). Free glycogen in vaginal fluids is associated with *Lactobacillus* colonization and low vaginal pH. *PLoS ONE* 9:e102467. doi: 10.1371/journal.pone.0102467
- Mirmonsef, P., Hotton, A. L., Gilbert, D., Gioia, C. J., Maric, D., Hope, T. J., et al. (2016). Glycogen levels in undiluted genital fluid and their relationship to vaginal pH, estrogen, and progesterone. *PLoS ONE* 11:e0153553. doi: 10.1371/journal.pone.0153553
- Paulson, J. N., Stine, O. C., Bravo, H. C., and Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods* 10, 1200–1202. doi: 10.1038/nmeth.2658
- Petrova, M. I., Mathys, L., Lebeer, S., Noppen, S., Van Damme, E. J., Tanaka, H., et al. (2013). Inhibition of infection and transmission of HIV-1 and lack of significant impact on the vaginal commensal lactobacilli by carbohydrate-binding agents. *J. Antimicrob. Chemother.* 68, 2026–2037. doi: 10.1093/jac/dkt152
- Pruitt, K. D., Tatusova, T., and Maglott, D. R. (2007). NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res.* 35, D61–65. doi: 10.1093/nar/gkl842
- Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S. K., Mcculle, S. L., et al. (2010). Vaginal microbiome of reproductive-age women. *PNAS* 108, 4680–4687. doi: 10.1073/pnas.1002611107
- Seekatz, A. M., Panda, A., Rasko, D. A., Toapanta, F. R., Eloee-Fadros, E. A., Khan, A. Q., et al. (2013). Differential response of the cynomolgus macaque gut microbiota to *Shigella* infection. *PLoS ONE* 8:e64212. doi: 10.1371/journal.pone.0064212
- Sharpe, S. A., White, A. D., Sibley, L., Gleeson, F., Hall, G. A., Basaraba, R. J., et al. (2017). An aerosol challenge model of tuberculosis in Mauritian cynomolgus macaques. *PLoS ONE* 12:e0171906. doi: 10.1371/journal.pone.0171906
- Spear, G. T., Gilbert, D., Landay, A. L., Zariffard, R., French, A. L., Patel, P., et al. (2011). Pyrosequencing of the genital microbiotas of HIV-seropositive and -seronegative women reveals *Lactobacillus iners* as the predominant *Lactobacillus* species. *Appl. Environ. Microbiol.* 77, 378–381. doi: 10.1128/AEM.00973-10
- Spear, G. T., Gilbert, D., Sikaroodi, M., Doyle, L., Green, L., Gillevet, P. M., et al. (2010). Identification of rhesus macaque genital microbiota by 16S pyrosequencing shows similarities to human bacterial vaginosis: implications for use as an animal model for HIV vaginal infection. *AIDS Res. Hum. Retroviruses* 26, 193–200. doi: 10.1089/aid.2009.0166
- Spear, G. T., Kersh, E., Guenther, P., Vishwanathan, S. A., Gilbert, D., Zariffard, M. R., et al. (2012). Longitudinal assessment of pigtailed macaque lower genital tract microbiota by pyrosequencing reveals dissimilarity to the genital microbiota of healthy humans. *AIDS Res. Hum. Retroviruses* 28, 1244–1249. doi: 10.1089/aid.2011.0382
- Tachedjian, G., O'hanlon, D. E., and Ravel, J. (2018). The implausible “in vivo” role of hydrogen peroxide as an antimicrobial factor produced by vaginal microbiota. *Microbiome* 6:29. doi: 10.1186/s40168-018-0418-3
- Ten Haaf, P., Almond, N., Biberfeld, G., Cafaro, A., Cranage, M., Ensoli, B., et al. (2001). Comparison of early plasma RNA loads in different macaque species and the impact of different routes of exposure on SIV/SHIV infection. *J. Med. Primatol.* 30, 207–214. doi: 10.1034/j.1600-0684.2001.d01-54.x
- Vujkovic-Cvijin, I., Dunham, R. M., Iwai, S., Maher, M. C., Albright, R. G., Broadhurst, M. J., et al. (2013). Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. *Sci. Transl. Med.* 5:193ra191. doi: 10.1126/scitranslmed.3006438
- Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07
- Weinbauer, G. F., Niehoff, M., Niehaus, M., Srivastav, S., Fuchs, A., Van Esch, E., et al. (2008). Physiology and endocrinology of the ovarian cycle in macaques. *Toxicol. Pathol.* 36, 7S–23S. doi: 10.1177/0192623308327412
- Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., et al. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science* 334, 105–108. doi: 10.1126/science.1208344
- Yu, R. R., Cheng, A. T., Lagenaur, L. A., Huang, W., Weiss, D. E., Treece, J., et al. (2009). A Chinese rhesus macaque (*Macaca mulatta*) model for vaginal *Lactobacillus* colonization and live microbicide development. *J. Med. Primatol.* 38, 125–136. doi: 10.1111/j.1600-0684.2008.00316.x
- Ziklo, N., Huston, W. M., Taing, K., Katouli, M., and Timms, P. (2016). In vitro rescue of genital strains of *Chlamydia trachomatis* from interferon-gamma and tryptophan depletion with indole-positive, but not indole-negative *Prevotella* spp. *BMC Microbiol.* 16:286. doi: 10.1186/s12866-016-0903-4
- Zoetendal, E. G., Rajilic-Stojanovic, M., and De Vos, W. M. (2008). High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut* 57, 1605–1615. doi: 10.1136/gut.2007.133603

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Nugeyre, Tchitche, Adapen, Cannou, Contreras, Benjelloun, Ravel, Le Grand, Marlin and Menu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Dynamics of Vaginal and Rectal Microbiota over Several Menstrual Cycles in Female Cynomolgus Macaques

Supplemental Materials

Marie-Thérèse Nugeyre^{1,2,*}, Nicolas Tchitchek^{1,*}, Cindy Adapen¹, Claude Cannou^{1,2}, Vanessa Contreras¹, Fahd Benjelloun^{1,2}, Jacques Ravel³, Roger Le Grand¹, Romain Marlin¹, and Elisabeth Menu^{1,2,¶}

¹ CEA, Université Paris-Sud, Inserm, U1184 « Immunology of viral infections and autoimmune diseases » (IMVA), IDMIT Department, IBFJ, Fontenay-Aux-Roses, France

² MISTIC group, Department of Virology, Institut Pasteur, Paris, France

³ Institute for Genome Sciences and Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, USA.

* These two authors equally contributed to the work.

¶ Correspondence to Dr. Elisabeth Menu, E-mail: elisabeth.menu@cea.fr

Supplementary Table 1 – Relative abundances of microbial taxa in macaque rectal samples at the phylum level.

Phylum	Animals					
	ALL	BA890I	CA086	CB804C	CBL015	CCA096
Firmicutes	54.73	54.63	54.85	58.82	51.75	53.58
Bacteroidetes	31.34	28.93	31.40	26.72	34.30	35.34
Fusobacteria	0.07		0.17		0.21	
Proteobacteria	5.92	8.78	5.38	3.03	7.91	4.50
Other	2.62	2.65	2.26	2.80	2.97	2.42
Actinobacteria						
Spirochaetes	3.57	3.30	3.48	5.35	2.23	3.50
Verrucomicrobia	0.72	0.99	0.98	1.01	0.63	
Tenericutes	0.22		0.64	0.47		
SR1						
Lentisphaerae	0.27		0.38	0.34		0.65
TM7	0.20			0.98		
Cyanobacteria	0.14	0.72				
Fibrobacteres	0.10			0.49		
Elusimicrobia	0.09		0.46			

Supplementary Table 2 – Relative abundances of microbial taxa in macaque vaginal samples at the phylum level.

Phylum	VAG					
	ALL	BA890I	CA086	CB804C	CBL015	CCA096
Firmicutes	27.65	27.45	22.59	27.80	21.76	38.65
Bacteroidetes	36.68	38.70	41.25	29.93	28.19	45.32
Fusobacteria	26.50	24.94	28.80	29.32	40.24	9.19
Proteobacteria	1.25	0.84	1.77	0.62	2.06	0.96
Other	2.60	3.55	2.97	2.66	1.69	2.15
Actinobacteria	4.35	3.83	1.74	8.32	4.13	3.73
Spirochaetes	0.19		0.35	0.59		
Verrucomicrobia	0.04			0.19		
Tenericutes	0.36	0.69	0.53	0.57		
SR1	0.39				1.93	

Supplementary Table 3 – Relative abundances of microbial taxa in macaque rectal samples.

phylum	class	order	family	genus	BA890I	CA086	CB804C	CBL015	CCA096	all samples
Bacteroidetes	Bacteroidia	Bacteroidales			5.58	5.93	5.16	6.51	3.07	5.25
Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	[Prevotella]	0.44	0.76	0.00	0.80	1.16	0.63
Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	CF231	0.00	1.06	0.00	0.74	0.00	0.36
Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	YRC22	0.00	0.71	0.00	0.00	0.00	0.14
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	0.00	0.00	0.00	2.88	0.38	0.65
Bacteroidetes	Bacteroidia	Bacteroidales	p-2534-18B5		1.35	0.00	0.32	2.13	0.27	0.81
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Porphyromonas	0.43	0.27	0.00	0.00	0.22	0.18
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	15.58	17.45	14.83	14.14	20.62	16.52
Bacteroidetes	Bacteroidia	Bacteroidales	RF16		2.24	1.75	1.09	1.47	0.35	1.38
Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		3.30	3.47	5.32	5.62	9.28	5.40
Cyanobacteria	4C0d-2	YS2			0.72	0.00	0.00	0.00	0.00	0.14
Elusimicrobia	Elusimicrobia	Elusimicrobiales	Elusimicrobiaceae		0.00	0.46	0.00	0.00	0.00	0.09
Fibrobacteres	Fibrobacteria	Fibrobacterales	Fibrobacteraceae	Fibrobacter	0.00	0.00	0.49	0.00	0.00	0.10
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.00	2.10	0.00	0.92	0.30	0.66
Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae		0.31	0.00	0.00	0.00	0.00	0.06
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.30	1.93	0.00	1.64	0.44	0.86
Firmicutes	Clostridia	Clostridiales			11.10	9.92	11.11	8.19	5.75	9.21
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	WAL_1855D	0.18	0.00	0.00	0.00	0.00	0.04
Firmicutes	Clostridia	Clostridiales	Christensenellaceae		2.03	2.02	3.59	1.07	0.67	1.88
Firmicutes	Clostridia	Clostridiales	Clostridiaceae		1.59	0.98	0.63	0.44	0.92	0.91
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	0.56	0.64	0.37	0.61	0.00	0.44
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		2.27	2.67	2.06	2.36	6.17	3.11
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	0.56	0.00	0.56	0.50	0.00	0.33
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.00	0.00	0.64	0.00	0.00	0.13
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	1.14	1.13	0.62	1.01	2.59	1.30
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospira	0.31	0.00	0.34	0.41	1.00	0.41
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	0.98	0.00	0.00	1.13	2.53	0.93
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		24.13	26.20	30.02	26.81	24.77	26.39
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium	1.09	1.07	1.09	0.00	0.00	0.65
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	2.71	2.94	3.83	3.68	4.34	3.50
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	3.21	2.38	1.93	1.15	2.28	2.19
Firmicutes	Clostridia	Clostridiales	Veillonellaceae		0.17	0.00	0.00	0.00	0.39	0.11
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Acidaminococcus	0.00	0.00	0.00	0.20	0.00	0.04
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Anaerovibrio	0.49	0.00	0.00	0.47	0.48	0.29
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Dialister	0.99	0.49	1.14	0.45	0.96	0.81
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	0.00	0.00	0.88	0.70	0.00	0.32
Firmicutes	Clostridia	[Clostridiales]			0.00	0.37	0.00	0.00	0.00	0.07
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	RFN20	0.52	0.00	0.00	0.00	0.00	0.10
Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	0.00	0.17	0.00	0.21	0.00	0.07
Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae		0.00	0.38	0.00	0.00	0.65	0.21
Lentisphaerae	[Lentisphaeria]	Z20	R4-45B		0.00	0.00	0.34	0.00	0.00	0.07
Proteobacteria	Alphaproteobacteria				1.02	0.00	0.00	0.00	0.00	0.20
Proteobacteria	Deltaproteobacteria	GMD14H09			0.00	2.01	0.90	2.81	1.42	1.43
Proteobacteria	Epsilonproteobacteria	Campylobacteriales	Campylobacteraceae	Campylobacter	0.20	0.00	0.74	0.00	0.00	0.19
Proteobacteria	Epsilonproteobacteria	Campylobacteriales	Helicobacteraceae	Flexispira	6.17	2.40	1.39	0.31	0.00	2.05
Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Succinivibrio	1.38	0.97	0.00	4.41	2.83	1.92
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		0.00	0.00	0.00	0.38	0.00	0.08
Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Actinobacillus	0.00	0.00	0.00	0.00	0.26	0.05
Spirochaetes	Spirochaetes	Sphaerochaetales	Sphaerochaetaceae	Sphaerochaeta	0.00	0.00	1.02	0.00	0.00	0.20
Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema	3.30	3.48	4.33	2.23	3.50	3.37
Tenericutes	RF3	ML615J-28			0.00	0.64	0.47	0.00	0.00	0.22
TM7	TM7-3	CW040	F16		0.00	0.00	0.98	0.00	0.00	0.20
Verrucomicrobia	Verruco-5	WCHB1-41	RFP12		0.99	0.98	1.01	0.63	0.00	0.72
Other	Other	Other	Other	Other	2.65	2.26	2.80	2.97	2.42	2.62

Supplementary Table 4 – Relative abundances of microbial taxa in macaque vaginal samples.

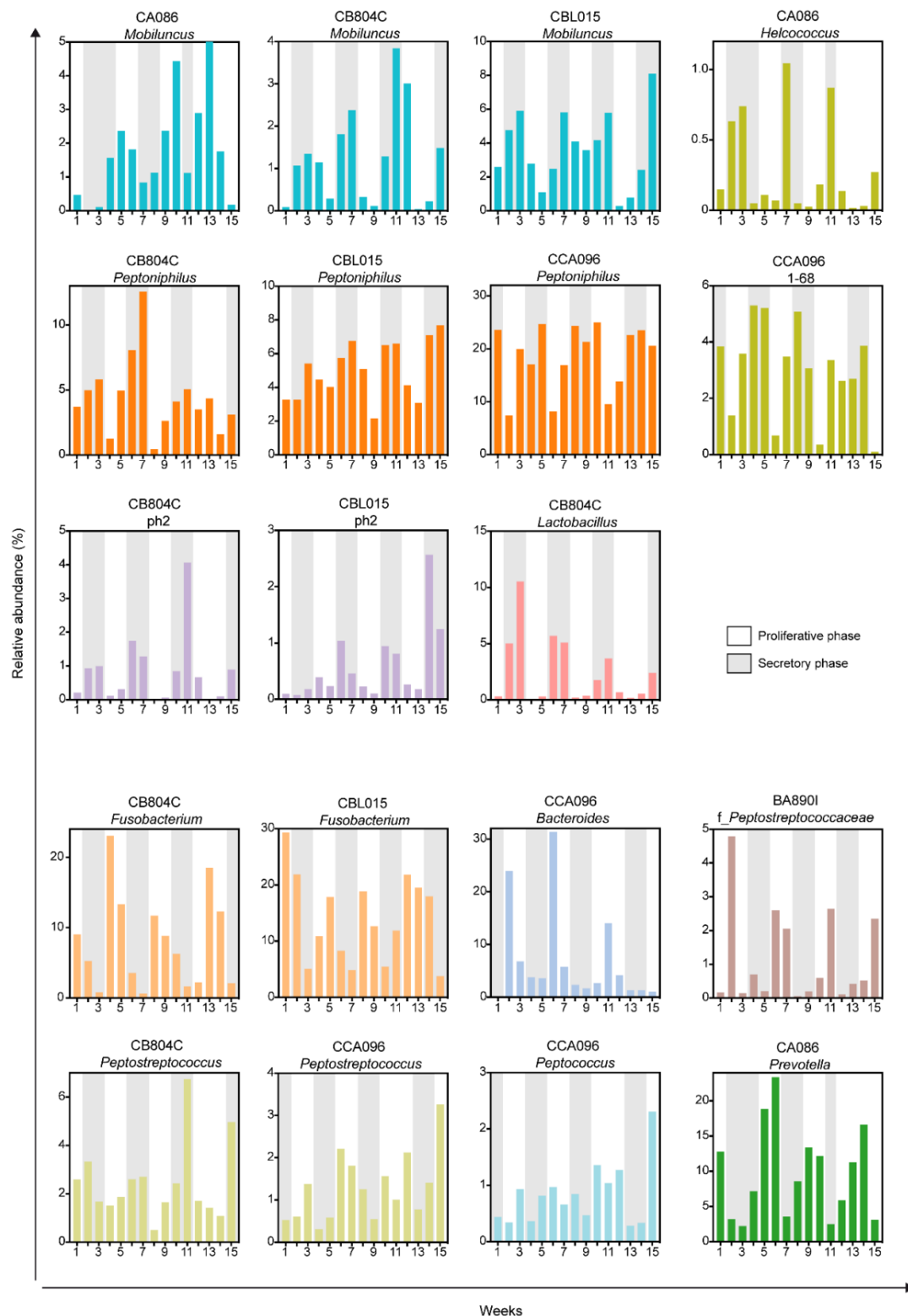
phylum	class	order	family	genus	BA8901	CA086	CB804C	CBL015	CCA096	all samples
Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Mobiluncus	1.66	1.74	1.23	3.64	3.08	2.27
Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	0.38	0.00	0.00	0.00	0.00	0.08
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae		0.00	0.00	3.84	0.00	0.00	0.77
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	1.79	0.00	3.25	0.49	0.65	1.24
Bacteroidetes	Bacteroidia	Bacteroidales			0.00	0.00	0.69	0.00	0.00	0.14
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	6.89	9.26	0.91	6.52	6.94	6.11
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Porphyromonas	11.71	22.34	3.11	19.64	20.66	15.49
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	20.10	9.65	24.29	2.02	17.72	14.76
Bacteroidetes	Bacteroidia	Bacteroidales	RF16		0.00	0.00	0.48	0.00	0.00	0.10
Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		0.00	0.00	0.46	0.00	0.00	0.09
Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia	0.28	0.00	0.00	0.00	0.00	0.06
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.00	0.00	2.46	0.00	0.00	0.49
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0.00	0.00	0.54	0.19	0.64	0.27
Firmicutes	Clostridia	Clostridiales			0.00	0.00	1.65	0.00	0.21	0.37
Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]		0.00	0.39	0.00	0.00	0.00	0.08
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]		0.00	0.00	0.00	0.00	0.96	0.19
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	1-68	0.94	3.14	0.61	1.13	2.97	1.76
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Anaerococcus	1.40	0.49	1.30	0.67	3.08	1.39
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	GW-34	0.00	0.00	0.00	0.18	0.00	0.04
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Helcococcus	0.13	0.29	0.19	0.00	0.32	0.19
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Parvimonas	1.82	2.51	0.87	2.81	0.00	1.60
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Peptoniphilus	8.50	7.58	4.41	5.02	18.56	8.81
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	ph2	1.80	0.00	0.82	0.59	0.75	0.79
Firmicutes	Clostridia	Clostridiales	Christensenellaceae		0.00	0.00	0.33	0.00	0.00	0.07
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	0.00	0.00	0.00	0.00	0.72	0.14
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		0.00	0.00	0.27	0.30	0.00	0.11
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.00	0.30	0.00	0.00	0.00	0.06
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Moryella	1.15	1.03	0.00	0.00	0.00	0.44
Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Peptococcus	0.00	0.00	0.00	0.39	0.83	0.24
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae		1.17	1.33	0.88	2.26	0.00	1.13
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Filifactor	0.54	0.00	0.81	0.00	0.75	0.42
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	1.94	1.61	2.45	3.73	1.29	2.20
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		0.00	0.00	3.59	0.65	0.17	0.88
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	0.00	0.00	0.43	0.00	0.00	0.09
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Dialister	7.34	3.92	6.04	3.33	6.60	5.45
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	0.00	0.00	0.15	0.00	0.00	0.03
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Bulleidia	0.45	0.00	0.00	0.50	0.82	0.35
Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	Fusobacterium	5.36	15.11	7.95	14.00	9.19	10.32
Fusobacteria	Fusobacteria	Fusobacteriales	Leptotrichiaceae	Sneathia	19.57	13.69	21.37	26.24	0.00	16.17
Proteobacteria	Epsilonproteobacteria	Campylobacteriales	Campylobacteraceae	Campylobacter	0.84	1.77	0.62	1.82	0.86	1.18
Proteobacteria	ammaproteobacteria	Aeromonadales	Succinivibrionaceae	Succinivibrio	0.00	0.00	0.00	0.24	0.10	0.07
Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema	0.00	0.35	0.59	0.00	0.00	0.19
SR1					0.00	0.00	0.00	1.93	0.00	0.39
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	Mycoplasma	0.69	0.53	0.40	0.00	0.00	0.32
Tenericutes	RF3	ML615J-28			0.00	0.00	0.17	0.00	0.00	0.03
Verrucomicrobia	Verruco-5	WCHB1-41	RFP12		0.00	0.00	0.19	0.00	0.00	0.04
Other	Other	Other	Other	Other	3.55	2.97	2.66	1.69	2.15	2.60

Supplementary Table 5 – Numbers of identified taxa in macaque rectal samples ($\geq 1\%$ of sequences)

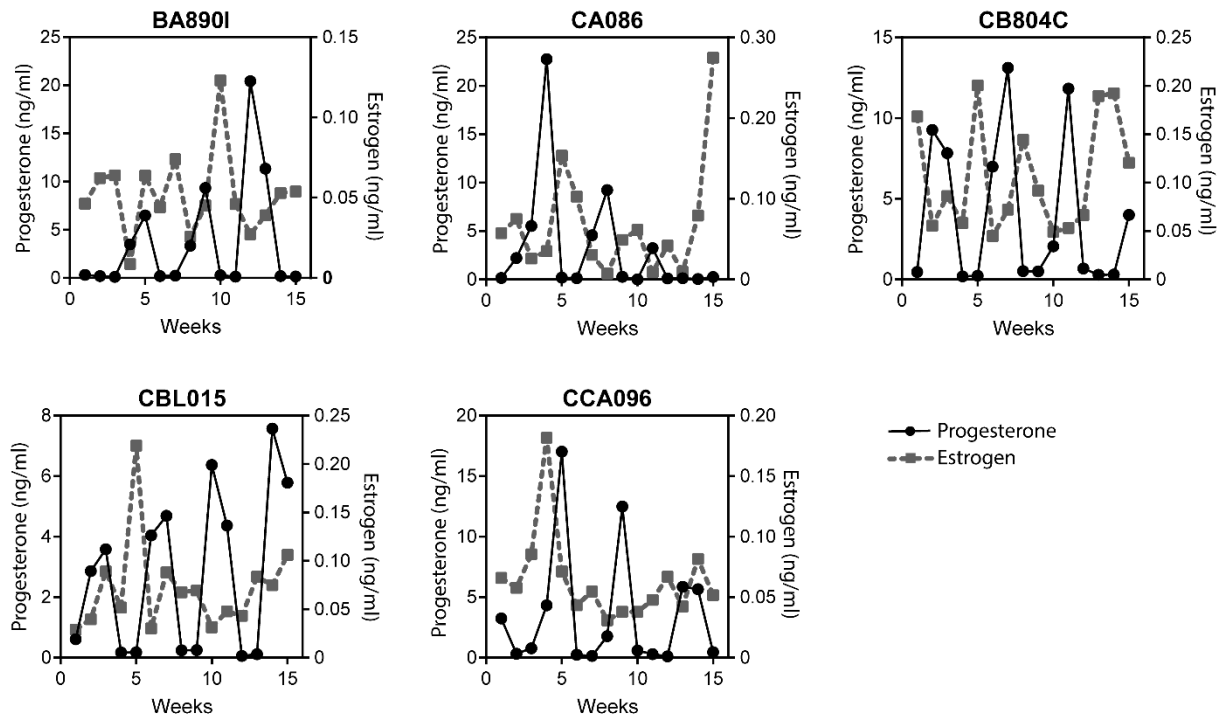
Week	BA890I	CA086	CB804C	CBL015	CCA096
1	17	19	17	18	20
2	18	17	16	15	15
3	25	16	15	18	17
4	18	18	15	14	15
5	19	17	16	17	18
6	17	18	13	19	15
7	17	16	13	18	17
8	16	18	15	15	14
9	19	18	16	18	15
10	15	17	18	15	15
11	20	18	15	17	19
12	15	21	15	17	16
13	20	20	12	18	16
14	18	14	14	17	19
15	20	14	15	14	15

Supplementary Table 6 – Numbers of identified taxa in macaque vaginal samples ($\geq 1\%$ of sequences)

Week	BA890I	CA086	CB804C	CBL015	CCA096
1	11	16	11	11	10
2	17	15	14	13	12
3	11	10	19	14	12
4	15	13	10	13	9
5	13	11	12	13	10
6	13	14	16	14	7
7	15	11	11	14	12
8	14	13	16	15	14
9	10	11	17	12	10
10	10	15	20	15	12
11	16	13	16	13	13
12	13	12	13	13	13
13	11	12	10	12	10
14	12	15	19	16	10
15	14	14	15	12	14



Supplementary Figure 1. Kinetic profiles of vaginal microbial taxa found to be statistically associated with progesterone levels. The relative taxa abundance and progesterone levels are shown for individual animals for 13 of the 15 taxa found to be statistically different between samples taken when progesterone levels were high (≥ 1 ng/ml, grey rectangles) versus those taken when they were low level (< 1 ng/ml, white rectangles). The histograms indicate the relative taxa abundance.



Supplementary Figure 2. Progesterone and estradiol concentrations. The concentrations of progesterone and estradiol were measured each week during the study and are shown for each animal.

Second article: Local innate markers and vaginal microbiota composition are influenced by hormonal cycle phases

Cindy Adapen, Louis Réot, Natalia Nunez, Claude Cannou, Romain Marlin, Julien Lemaitre, Léo d'Agata, Emmanuel Gilson, Eric Ginoux, Roger Le Grand, Marie-Thérèse Nugeyre, Elisabeth Menu

Manuscript submitted in a peer-reviewed international journal, september 2021.

I. Introduction and objectives

The vaginal microenvironment in women is composed of factors essential to restrain pathogen invasion. Several of these factors including soluble factors (cytokines/chemokines or antimicrobial peptides), vaginal microbiota composition, and immune cells are subject to hormonal fluctuation within the FRT [174, 222, 318]. Those factors by modulating the local inflammation are determinant in STI susceptibility. For instance, an increase bacterial diversity in the vaginal microbiota has been associated to increase production of inflammatory cytokines therefore to a higher susceptibility to infection such as HIV-1 or *Chlamydia trachomatis* [191, 192]. Neutrophils including their interaction with the vaginal microbiota have been poorly studied in the FRT even though they have been described in several STI to be a determinant factor either for clearing pathogen or pathogenesis [46, 159]. Female cynomolgus macaques are an appropriate model to study STI thanks to the similarities observed between them and women in terms of FRT morphology, endocrine system and menstrual cycle [298–300]. We have previously shown that their vaginal microbiota is highly diverse and the composition is similar to women suffering from bacterial vaginosis. Furthermore, we have described variations of the vaginal microbiota composition induced by hormonal secretion [317].

To evaluate the consequences of this environment on the local inflammation, we analysed in parallel the vaginal microbiota composition, the neutrophil populations and the cytokine/chemokine profiles in nine female cynomolgus macaques.

We set up a longitudinal study covering three menstrual cycles. Throughout this study, several samples were collected once a week, including cervicovaginal swabs, wecks and cytobrushes to monitor respectively the evolution of the vaginal microbiota composition, cytokine/chemokine concentration and neutrophil phenotype.

II. Data summary

We have demonstrated significant differences in terms of cytokine presence and concentration between blood and cervicovaginal fluids. Cytokines and chemokines observed in cervicovaginal but not in the blood were subject to hormonal variation, with a peak expression of several soluble factors during menstruation. Neutrophil subsets in the blood and cervicovaginal cytobrushes were different. Blood neutrophils were dominated by one mature subset expressing CD11b, CD101 and high expression of CD32a and CD62L whereas three main population of neutrophils in cervicovaginal cytobrushes were present: CD11b^{high} CD101⁺ CD10⁺ CD32a⁺, CD11b^{high} CD101⁺ CD10⁻ CD32a⁺ and CD11b^{low} CD101⁻ CD10⁻ CD32⁻. All of them had a low expression of CD62L. During menstruation, a subset of neutrophils was increased in cervicovaginal cytobrushes, which is mature and activated. Moreover, this subset expressed high level of CD62L, which is normally, not express in the tissue but highly expressed in the blood. Concerning the vaginal microbiota, three main phyla were observed: *Firmicutes*, *Bacteroidota*, *Actinobacteriota*. Various bacterial taxa were observed to be differentially abundant according to hormonal phases. Nine taxa were increased during low progesterone compared to high progesterone. Six taxa were more abundant during menstruation compared to high progesterone. When analysing together, by an automatic hierarchical clustering, the factors (cytokines, neutrophils, bacterial taxa) that were differentially expressed according to hormonal phases we observed that many samples collected during menstruation were clustering together. Those samples

exhibited high expression of cytokines and CD11b^{high} CD101⁺ CD10⁺ CD32a⁺ expressing CD62L. Some animals clustered together. Two animal samples concentrated in one group with high expression of cytokines. In contrast, two other animal samples concentrated in another group with low expression of cytokines.

Those data highlight a variation of cytokines, vaginal microbiota and neutrophil populations according to hormonal phases in female cynomolgus macaques. Menstruation were associated with an increased inflammation translated into high expression of cytokines and increase number of mature neutrophils expressing CD62L.

To conclude, this study point out a strong need for appropriate sampling schedule and longitudinal analysis in research programs including female animals and/or women based cohorts. Indeed, hormonal fluctuations should be taken into account when studying mucosal immunity in the female reproductive tract otherwise misinterpretations of the results might be made.

III. Article

Local innate markers and vaginal microbiota composition are influenced by hormonal cycle phases

Cindy Adapen¹, Louis Réot¹, Natalia Nunez², Claude Cannou^{1,3}, Romain Marlin¹, Julien Lemaitre¹, Léo d'Agata², Emmanuel Gilson², Eric Ginoux², Roger Le Grand¹, Marie-Thérèse Nugeyre^{1,3}, Elisabeth Menu^{1,3}

¹*Université Paris-Saclay, Inserm, CEA, Center for Immunology of Viral, Auto-immune, Hematological and Bacterial diseases (IMVA-HB/IDMIT), Fontenay-aux-Roses, France*

²*Life&Soft, 92260 Fontenay-aux-Roses, France*

³*MISTIC group, department of Virology, Institut Pasteur, Paris, France*

ABSTRACT

Background

The female reproductive tract (FRT) mucosa is the first line of defence against sexually transmitted infection (STI). FRT environmental factors, including immune cell composition and vaginal microbiota, interact with each other to modulate STI susceptibility. Moreover, menstrual cycle induces important modifications within the FRT mucosa and impacts these factors. Menstrual cycle and FRT morphology are similar in women and cynomolgus macaques. The cynomolgus macaque vaginal microbiota is highly diverse and associated to the dysbiotic vaginal microbiota observed in women. Cynomolgus macaques are used as a study model for STI pathogenesis and prophylaxis, however, the impact of menstrual cycle on immune markers and vaginal microbiota in female cynomolgus macaques is unknown. A longitudinal study covering three menstrual cycles was thus performed in cynomolgus macaques. The evolution of the vaginal microbiota composition and inflammation (cytokine/chemokine profile and neutrophil phenotype) in the FRT and in the blood was determined throughout the menstrual cycle.

Results

Cervicovaginal cytokine and chemokine concentrations were impacted by the menstrual cycle with a peak of production during menstruation. Three main cervicovaginal neutrophil subpopulations were observed: CD11b^{high} CD101⁺ CD10⁺ CD32a⁺, CD11b^{high} CD101⁺ CD10⁻ CD32a⁺ and CD11b^{low} CD101⁻ CD10⁻ CD32⁻. These populations had variable expression during the menstrual cycle. During menstruation, a subset of neutrophils was increased: CD11b^{high} CD101⁺ CD10⁺ CD32a⁺ expressing a higher level of CD62L. In the vaginal microbiota, various bacterial taxa are differentially abundant according to menstrual cycle phases. The compilation of the factors that vary according to hormonal phases highlights a clustering of samples collected during menstruation. These samples were characterized by a high concentration of cytokines and an increased number of the neutrophil subpopulation: CD11b^{high} CD101⁺ CD10⁺ CD32a⁺ CD62L⁺.

Conclusions

A significant impact of menstruation on the local environment (cytokine production, neutrophil phenotype and vaginal microbiota composition) was demonstrated in female cynomolgus macaques. Menstruation trigger an increase production of cytokines and a recruitment of neutrophils characterised as mature/activated that originate from the blood within the FRT. These results supported the need for the monitoring of the menstrual cycle and longitudinal sampling schedule for further studies in female animals and/or women focusing on the mucosal FRT environment.

Keywords (3 to 10 words): Inflammation, vaginal microbiota, menstrual cycle, female reproductive tract, blood.

INTRODUCTION

Heterosexual transmission from male to female is one of the major route of sexually transmitted infections (STI) and occurs mainly through the female reproductive tract (FRT). The FRT is composed of a wide array of environmental factors such as mucus layer, secretory IgA/IgG, vaginal microbiota, local immune cells, epithelium barrier and soluble factors such as antimicrobial peptides or cytokines and chemokines [1]. All of these factors interact with each other to modulate the susceptibility to pathogen invasion. These environmental factors are impacted by hormonal variations, which are involved in vaginal microbiota composition modifications but also in the regulation of innate and adaptive immune responses within the FRT and in peripheral blood [1–4]. Menstrual cycle in women is composed of four phases: menstruation, follicular phase (*i. e.* proliferative phase), ovulation phase, and luteal phase (*i. e.* secretory phase). Two groups of hormones regulate the menstrual cycle: pituitary hormones (including follicle stimulating and luteinizing hormones) and ovarian hormones (including estradiol and progesterone). Menstrual bleeding is induced by a drop in progesterone and estradiol concentrations, triggered by the absence of fertilization. Then starts the secretory phase characterized by an increase of estradiol produced by mature follicle in the ovary. Estradiol concentration will peak one to two days before ovulation. Subsequently, the proliferative phase begin characterized by an increased production of progesterone [5]. Hormone production has been described to induce vaginal microbiota composition changes in women. At the puberty, increased level of estradiol drastically changes the vaginal microbiota by shifting from a diverse microbiota rich in anerobic bacteria to a *Lactobacillus* species (*spp.*) dominant microbiota [6, 7]. In addition, the vaginal microbiota composition has also been described to influence local immune responses. Indeed, a *Lactobacillus spp.* dominated microbiota (Community state type (CST) I, II, V) induces a low inflammation and a lower susceptibility against STI acquisition [8, 9]. On the contrary, a diverse microbiota composed of anaerobic bacteria such as *Prevotella*, *Dialister*, *Atopium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia* and *Mobiluncus* with a low abundance of *Lactobacillus spp.* (CST-IV) has been linked to an increased

local inflammation. This clinical condition is called bacterial vaginosis (BV). This inflammation is mediated by an increased expression of pro-inflammatory cytokines such as IL-1, IL-8, IL-12, IL-18, TNF α , IFN γ and a higher frequency of activated CD4+ T cells and Th17 cells [10]. Very little is known about the relationship between neutrophils and the vaginal microbiota. Neutrophils are essential in antimicrobial immunity but a sustained presence of neutrophils within the vagina may lead to tissue damage and inflammation as seen in several STIs [11, 12]. More informations are available on the interaction of the gut microbiota and neutrophils. The gut microbiota is essential for neutrophil production and priming, in return neutrophils are essential for the containment of the microbiota [13–15]. Hensley *et al.* have shown that an altered ratio of *Lactobacillus/Prevotella* is responsible of an increased survival of neutrophils in colorectal biopsies of HIV-1 infected ART treated patients [16].

In our study, we focused on the characterization of FRT inflammation during the menstrual cycle and the relationship with local microbiota changes in cynomolgus macaques used as a model for the study of human STI.

The FRT of cynomolgus macaques is similar to the one of women in terms morphology, endocrine system and menstrual cycle, making them an appropriate study model to analyze the effect of hormonal variation on immune markers [17–19]. We have previously shown that they have a highly diverse vaginal microbiota composed mainly of anaerobic bacteria such as *Sneathia*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Peptoniphilus*, *Bacteroides Dialister* and few *Lactobacillus spp.*. This composition is similar to the one of women belonging to CST IV [20]. Similar to women, the abundance of certain bacterial genus within the vaginal microbiota of females macaques are influenced by hormonal change [20]. These variations observed in the FRT might influence the level of the local inflammation and subsequently the susceptibility to STIs.

To characterize the modifications of the FRT inflammation during the menstrual cycle, we performed a longitudinal study in 9 female cynomolgus macaques. Inflammation was evaluated by measuring cytokine/chemokine concentration and by characterizing

neutrophil subpopulations in parallel with the determination of the vaginal microbiota composition. Studying the interactions between the vaginal microbiota and inflammatory markers during different phases of the menstrual cycle is essential to understand the impact of those factors on STI susceptibility and beyond.

MATERIAL AND METHODS

Ethics statement

Nine sexually mature adult female cynomolgus macaques (*Macaca fascicularis*), aged 7 to 9 years old and originating from Mauritian AAALAC certified breeding centers were included in this study. All animals were housed in IDMIT facilities (CEA, Fontenay-aux-roses), under BSL-2 containment (Animal facility authorization #D92-032-02, Préfecture des Hauts de Seine, France) and in compliance with European Directive 2010/63/EU, the French regulations and the Standards for Human Care and Use of Laboratory Animals, of the Office for Laboratory Animal Welfare (OLAW, assurance number #A5826-01, US). This study was approved and accredited by the institutional ethical committee "Comité d'Ethique en Expérimentation Animale du Commissariat à l'Energie Atomique et aux Energies Alternatives" (CEtEA #44) under statement number A18-083. The study was authorized by the "Research, Innovation and Education Ministry" under registration number APAFIS#20692-2019051709424034v1. The nine animals were housed in two different rooms in social groups of 5/6 animals under controlled conditions of humidity, temperature and light (12h light/dark cycles). The animals were fed once or twice a day with commercial monkey chow and fruits. Water was available *ad libitum*. They were provided with environmental enrichment including toys and novel food under the supervision of the CEA Animal Welfare Officer.

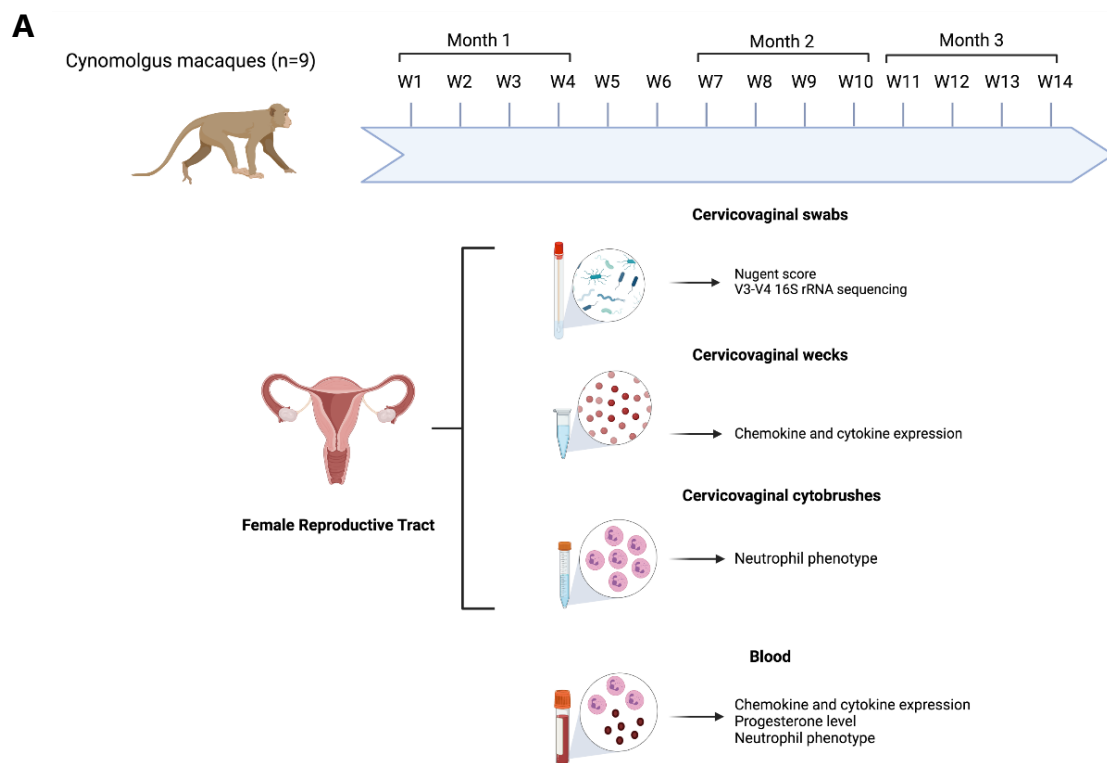
Experimental design and sample collection

Nine female cynomolgus macaques were studied during three months. Age, weight and haplotype are summarized in table 1. Sample collection was performed once a week during 12 weeks representing approximately three menstrual cycles.

Experimental design is summarized in Figure 1A. Sampling order was as followed: Weck-Cel Spear during blood withdrawal, cervicovaginal swabs and cervicovaginal cytobrushes. Cervicovaginal fluids were collected with a Weck-Cel Spear (Medtronic) placed in the vaginal vault for 2 min. Secretions were recovered from the spears by adding 600 μ L of elution buffer (PBS, NaCl 0.25 M and protease inhibitor mixture; Merck Millipore, Fontenay-sous-Bois, France) and centrifuged at 13 000 g for 20 min. Secretions were then aliquoted and stored at -80 °C before cytokine/chemokine quantification. Blood was collected and used for Complete Blood Count (CBC), then plasma was collected, aliquoted and stored at -80 °C for cytokine and progesterone quantification. Vaginal samples for microbiota analysis were collected with nylon flocked swabs (ESWABR1, Copan Diagnostics Inc., Murrieta, CA, USA) inserted in the vaginal vault and turned four to five times before storing in amies liquid. Swabs were then aliquoted and either used for Nugent score or stored frozen at -80°C until DNA extraction. Room control samples (air swabs) were done at the last time point. Cervicovaginal cells were collected using two successive cytobrushes (VWR; Belgium) inserted in the vaginal cavity and turned 4 to 5 times. After collection, the cytobrushes were put in a 15mL tube containing 5mL of RPMI with 10% Fetal Calf Serum (PAA The cell culture company, ref A15-102, lot: A10210-2737) and 5% Penicilin/streptomycin/neomycin (PSN). The samples were kept on ice before processing.

Table 1: Informations on the female cynomolgus macaques included in the study.

Animal	Date birth	Age	Weight (mean \pm SD)	Haplotype
BA738J	2011-11-24	8.6	6.62 \pm 0.06	H6 H6
BA885J	2011-12-27	8.5	4.62 \pm 0.08	H5 H3
BB172H	2011-12-26	8.5	3.80 \pm 0.12	Rec H2H5 Rec H3H1
BZ833	2010-11-05	9.6	5.42 \pm 0.05	H1 H1
CA086	2010-11-16	9.6	4.05 \pm 0.10	H2 H1
CB804C	2012-02-05	8.3	3.76 \pm 0.14	H1 Rec-H1H5H3
CBL015	2012-12-02	7.6	6.49 \pm 0.09	H3 H1
CCA096	2013-01-19	7.4	5.29 \pm 0.12	Rec H6-H11 Rec H2-H6
CCB062	2013-02-05	7.4	5.90 \pm 0.07	Rec H3-H2 Rec H5-H2



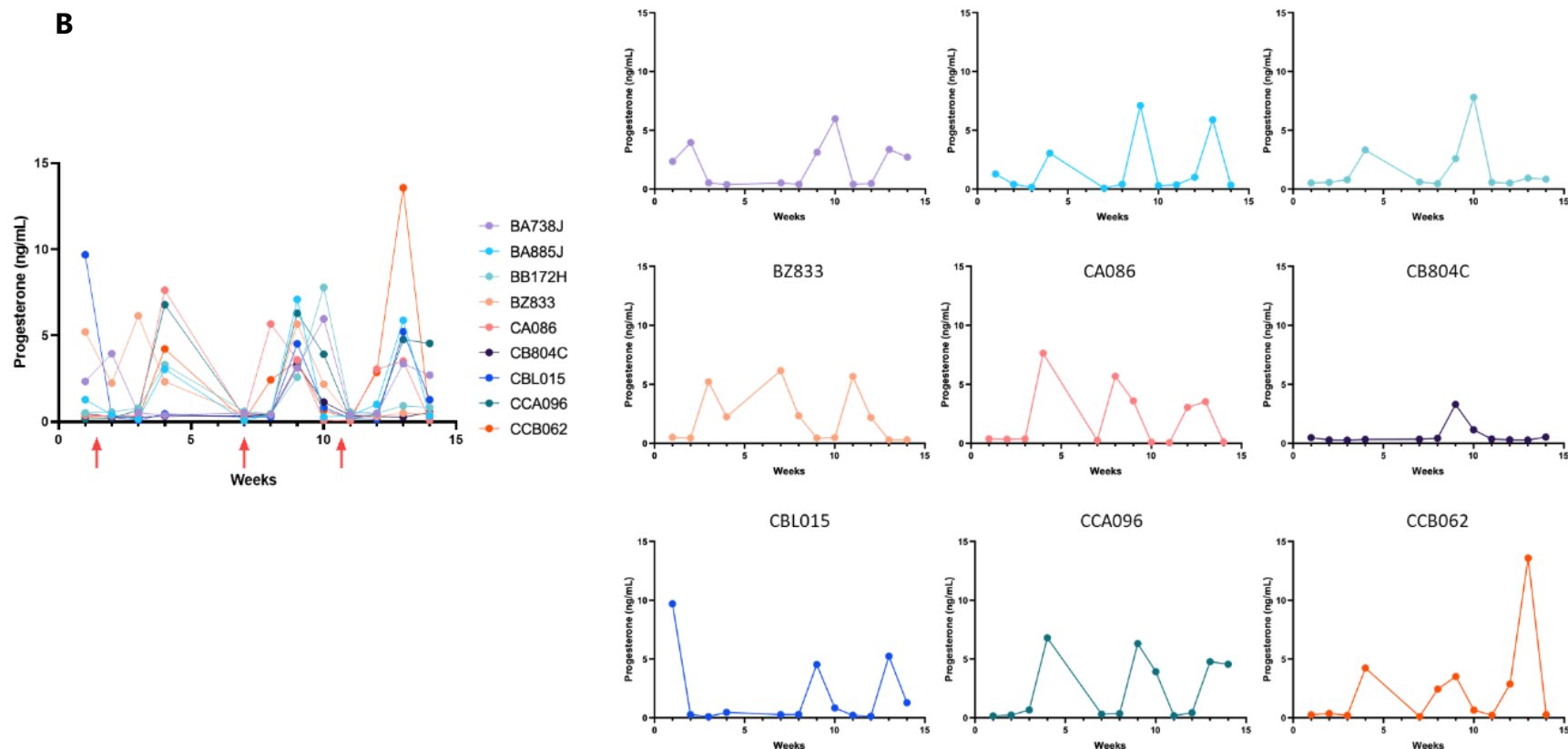


Figure 1: Experimental design and progesterone level. (A) Nine female cynomolgus macaques were included in the study. Blood, cervicovaginal fluids (Weck-cel®) and swabs as well as cervicovaginal cells were collected once a week during three months. Samples were not collected during weeks 5 and 6. Created in BioRender.com. (B) Progesterone concentrations were quantified in plasma once a week in all individuals. The graphical representation of progesterone concentration including all animals was obtained using interpolated and aligned data (left). On the contrary, graphical representation of progesterone concentration in each individual was obtained using the raw data (right). Red arrows represent menstruation.

Progesterone quantification

Progesterone level was determined each week in peripheral blood plasma samples by ELISA (IBL international; Germany) according to manufacturer's instructions [Fig1B]. Progesterone groups were determined as follow: high progesterone ($>1,4\text{ng/mL}$), low progesterone ($<1,4\text{ng/mL}$) and menstruation (bleeding and/or low progesterone level after a peak of progesterone). In order to estimate the displacement between the animals menstrual cycle (though progesterone levels), we used the popular methodology for time series alignment : cross-correlation maximum. Computation were carried by Python's package SciPy [21]. The optimal lag is derived as the one that maximizes the cross-correlation, which is a similarity measure between time-series as a function of the lag.

Cytokine and chemokine quantification

Pro- and anti-inflammatory cytokines as well as chemokines were quantified in cervicovaginal fluids and plasma by 23plex assay for the detection of: G-CSF, GM-CSF, IFN γ , IL-1 β , IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23(p40), IL-13, IL-15, IL-17A, CCL2, CCL3, CCL4, sCD40L, TGF α , TNF α , VEGF, IL-18 (NHP cytokine magnetic bead panel kit; Merck Millipore; Germany), according to manufacturer's instructions.

Cytospin and blood smear

Cytospin was performed at each time point on cervicovaginal cell suspension. Briefly, between 10^4 and 10^5 cells were seeded and spun at 72xg during one minute. Whole blood smear was also done at each time point. Dry cytospins and whole blood smears were then fixed with Labofix Q path (VWR Avantor; Belgium) and a May-Grunwald Giemsa staining were performed (RAL diagnostics; France). Slides were observed with a Eclipse 80i microscope (NIKON). Image were acquired with a 60X objective using a high-definition cooled color digital camera (DXM1200C; NIKON).

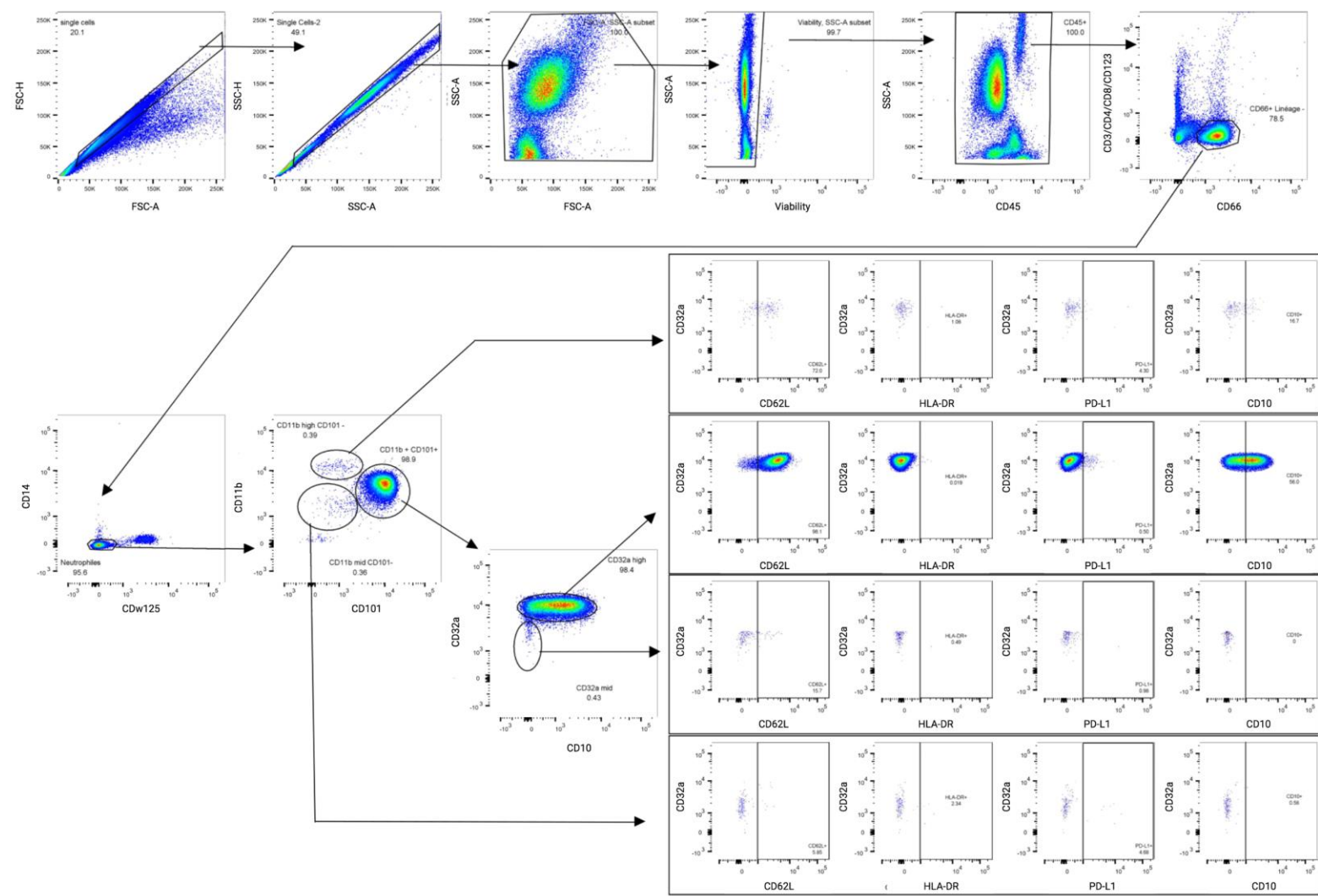
Neutrophil phenotyping

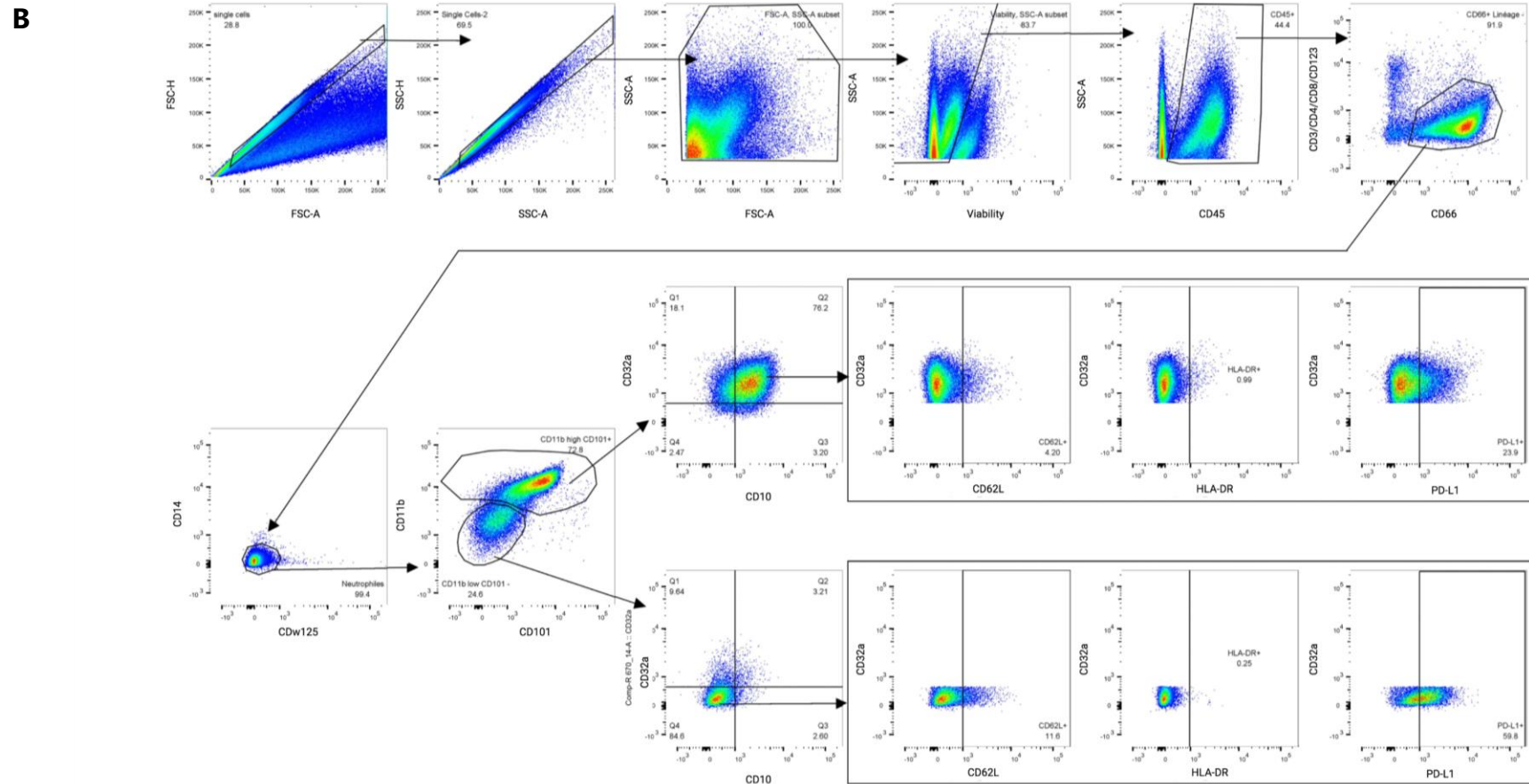
Neutrophil populations were analyzed in whole blood and cervicovaginal cells. Cervicovaginal cells were filtered with 35µm filter (Corning Falcon ; USA). Then, cervicovaginal cells and whole blood were incubated with the antibodies listed in table 2, washed and fixed with FACS lysing buffer (BD, Biosciences) or BD cell Fix solution (BD, Biosciences). A fourteen color panel, containing neutrophil surface markers of maturation and activation, were used. Phenotyping was performed on a Fortessa (BD, Biosciences) with DIVA (BD) and FlowJo (Tristar, USA) software. The gating strategy in cervicovaginal cytobrushes is described in the supplementary figure 1.

Table 2: Antibodies used to characterized neutrophil subpopulations.

Antibody	Label	Reference	Manufacturer
Bluevid	BUV736	L23105	Lifetechnologies
CD64	BUV737	612776	BD
CD11b	FITC	130-110-552	Milteny
CD45	Viogreen	130-177-193	Milteny
CD3	BV650	563916	BD
CD8	BV650	563821	BD
CD20	BV650	563780	BD
CD123	BV650	563405	BD
CD62L	BV711	565040	BD
CD14	Vioblue	130-110-524	Milteny
CD10	PercP-Cy5,5	312216	Biolegend
CDw125	PE	130-110-544	Milteny
PD-L1	PE-Dazzle594	329732	Biolegend
CD101	PE-Vio770	130-115-832	Milteny
CD32a	AF647	60012	Stemcell
HLA-DR	AF700	307626	Biolegend
CD66	APC-Vio770	130-119-847	Milteny

A





Supplementary figure 1: FACS gating strategy for neutrophil phenotyping in blood samples (A) and cervicovaginal cytobrushes (B) of one representative animal.

Nugent score determination

Vaginal fluids obtained by swabs were spread on a slide, fixed with heat (50 °C) and 100 % ethanol before Gram staining (RAL diagnostics; France). Slides were then observed with a 100X oil immersion lens using an Axioplan 2 microscope (Zeiss). Gram positive rod shaped bacteria as well as non-rod shaped bacteria were counted in 10 random fields for each slide. The Nugent score was determined by adding the score of non-rod and rod shaped bacteria [Table 3]. A score between 7-10 indicates bacterial vaginosis, score 4 to 6 indicates an intermediate flora and score 0 to 3 indicates a normal flora [22].

Table 3: Nugent score determination.

Score	<i>Lactobacillus</i> (number of bacteria/field)	Anaerobic bacteria (number of bacteria/field)	<i>Mobiluncus</i> (number of bacteria/field)
0	>30	0	0
1	5 to 30	<1	<1 to 4
2	1 to 4	1 to 4	5 to >30
3	<1	5 to 30	
4	0	>30	

DNA extraction and 16S rRNA gene sequencing

DNA from vaginal fluids was extracted using PowerFecal DNA Pro isolation kit (Qiagen; Germany) following manufacturer's instructions. DNA was quantified with Qubit 4 using the high sensitive DNA kit (Life technologies; USA). PCR and sequencing of the V3-V4 region of the 16S rRNA gene was performed at @BRIDGe platform (GABI, INRA, AgroParisTech, Paris-Saclay University). The V3-V4 hyper-variable regions of the 16S rRNA gene were amplified from the DNA extracts during the first PCR step using universal primers 5' CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG and 5' GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT which are fusion primers [23]. The PCR reaction was carried out in a T100 Thermal cycler (Biorad, USA) as follows: an initial denaturation step (94°C 10 min) was followed by 30 cycles of amplification (94°C for 1 min, 68° C for 1 min and 72°c for 1 min) and a final elongation

step at 72°C for 10 min. Sample multiplexing was performed by adding tailor-made 6 bp unique indexes during the second PCR step at the same time as the second part of the P5/P7 adapters to obtain primers 5' AATGATACGGCGACCACCGAGATCTACACT-CTTCCCTACACGAC and reverse primer 5' CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTGACT-GGAGTTCAGACGTGT. The PCR reaction was carried out on a T100 Thermal cycler with an initial denaturation step (94°C for 10 min), 12 cycles of amplification (94°C for 1 min, 65°C for 1 min and 72°C for 1 min) and a final elongation step at 72°C for 10 min. All libraries were pooled with equal amounts in order to generate equivalent number of raw reads for each library. The pool, at a final concentration between 5 and 20 nM, was used for sequencing. The PhiX Control v3 (Illumina, USA) was added to the pool at 15% of the final concentration as described in the Illumina procedure. 600 µl of this pool and PhiX mixture were loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions using MiSeq Reagent Kit v3. FastQ files were generated at the end of the run (MiSeq Reporter software, Illumina, USA) to perform the quality control. The quality of the run was checked internally using PhiX Control and then each paired-end sequence was assigned to its sample using the multiplexing index.

Sequencing data processing and taxonomic assignation

Illumina sequences were processed using FROGS pipeline (Find Rapidly OTU with Galaxy Solution) [24]. Bacterial 16S rRNA paired-end reads were merged with a maximum of 0.1 maximum rate of mismatch in the overlap region using Vsearch [25]. Each of the samples was unique time point so, after dereplication, the clusterisation step ran with an aggregation distance equal to 1 (maximum number of differences between all of our sequences) and thus denoising was not needed. Chimeras were removed using also Vsearch and kept more of 93 % of the total sequence abundance. Sequences were then filtered to keep at least 0.0005% of all sequences and phiX databank was equally applied to eliminate Illumina contaminants. Finally, taxonomic affiliation was performed using SILVA 138 pintail 100 database. Alpha diversity was

calculated using Shannon diversity index. Beta diversity was calculated using weighted UniFrac distance method.

Statistical analysis

Heatmap representing fold change of cytokine concentration in cervicovaginal fluids or peripheral blood plasma was obtained using Tableau software (Seattle, USA). Ordinary one way ANOVA test was used to compare fold change of cytokine concentration to total fold change mean as a reference. GraphPad prism software version 9 for windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was used for graphical representation of the vaginal microbiota composition (pie-charts), cytokine concentration, percentage of neutrophil subpopulations and bacterial abundance according to progesterone level. Significant differences between groups were confirmed using either a paired T-test or Kruskal-Wallis test with p values adjustment with Dunn's test. MetagenomeSeq's fitZIG algorithm [26] was used to determine differentially abundant bacteria according to menstrual phases. Significant differentially abundant cytokines, neutrophils and microbiome data were merged into one global heatmap using R (version 4.1.0) with pheatmap package (version 1.0.12). Data were normalized and a fold change was calculated using as the reference the mean value for a variable in all samples. To deal with extreme values, a log₁₀ transformation was applied directly on the fold change. Samples were clustered with a hierarchical clustering approach available in pheatmap function. Annotation colors were added to visually assess if samples are clustered by animal or by progesterone level. Finally, absent measurements in the data were flagged as non determined (ND) and values with abundance at zero were colored in grey.

RESULTS:**Cervicovaginal cytokines vary according to hormonal phases**

To monitor local and systemic inflammation, cytokines and chemokines were measured in cervicovaginal fluids and peripheral blood plasma using a multiplex technique.

Peripheral blood samples

For all time points, cytokines were quantified in the plasma for each female and the mean fold change calculated based on the total mean value was represented for cytokines that are relevant for the study [Fig2A]. The concentration of G-CSF, IFN γ , IL-2, IL-8, IL-10, IL-12/23, CCL2, CCL3, CCL4, sCD40L, TGF α , TNF α and VEGF varied among females [Fig2A]. For instance, CBL015 had a higher plasmatic concentration of IL-2 and IFN γ compared to other females whereas BA738J had a higher plasmatic concentration of G-CSF, IL-12/23 (p40) and IL-2. We observed that cytokine concentration such as G-CSF, IL-8, IL12/23 (p40), CCL2 or sCD40L fluctuated according to time [Fig2B]. To further analyse if the variations were due to hormonal cycle, all the samples were divided into three groups based on their progesterone level and clinical observations (high or low progesterone level and menstruation). There were no significant alterations of cytokine concentrations in the plasma due to hormonal cycle [Supplementary Fig2].

A

[Mean value]

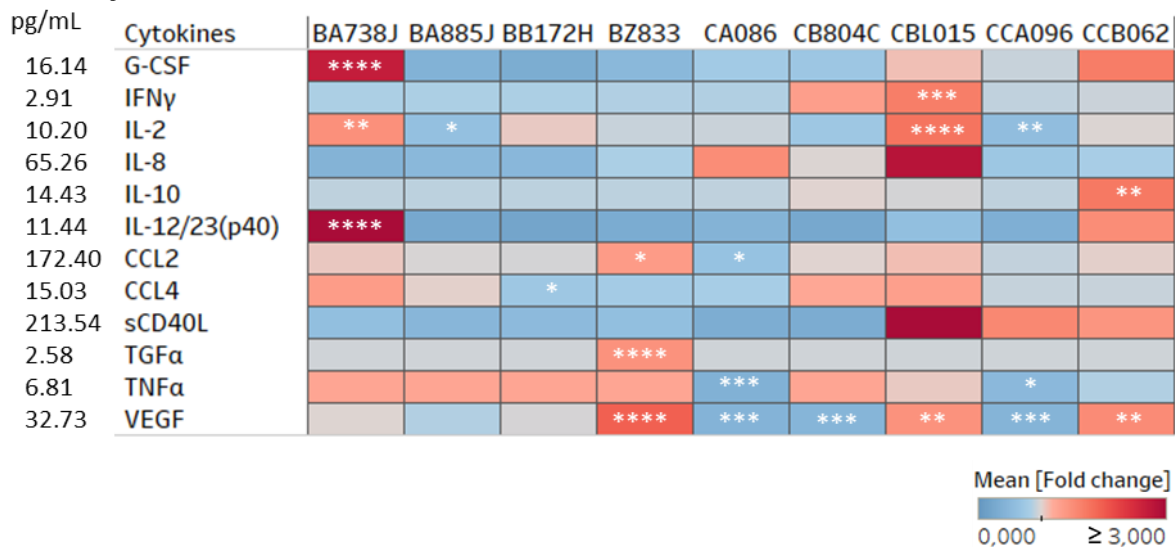
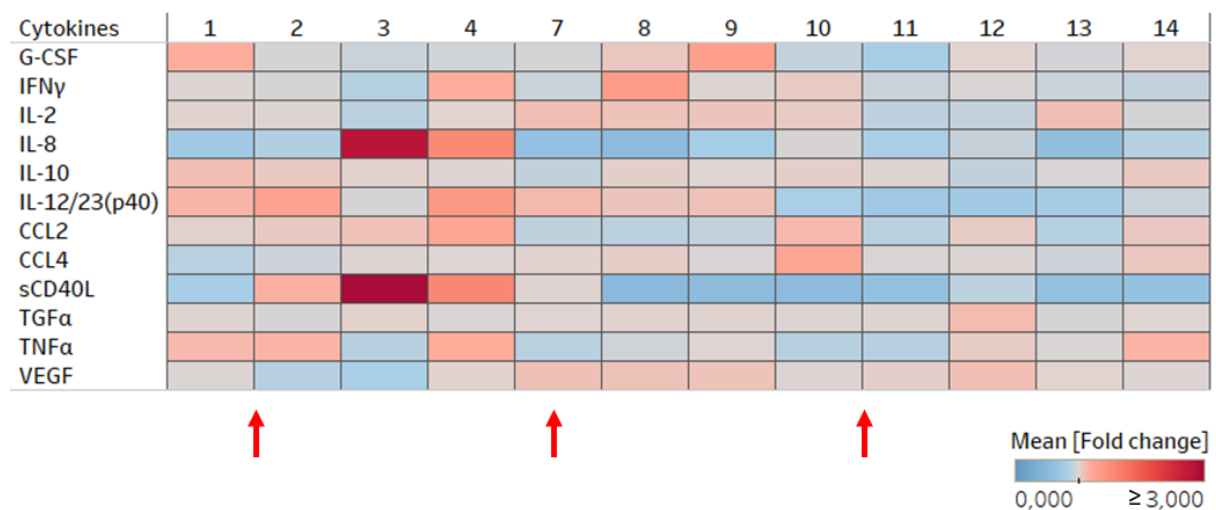
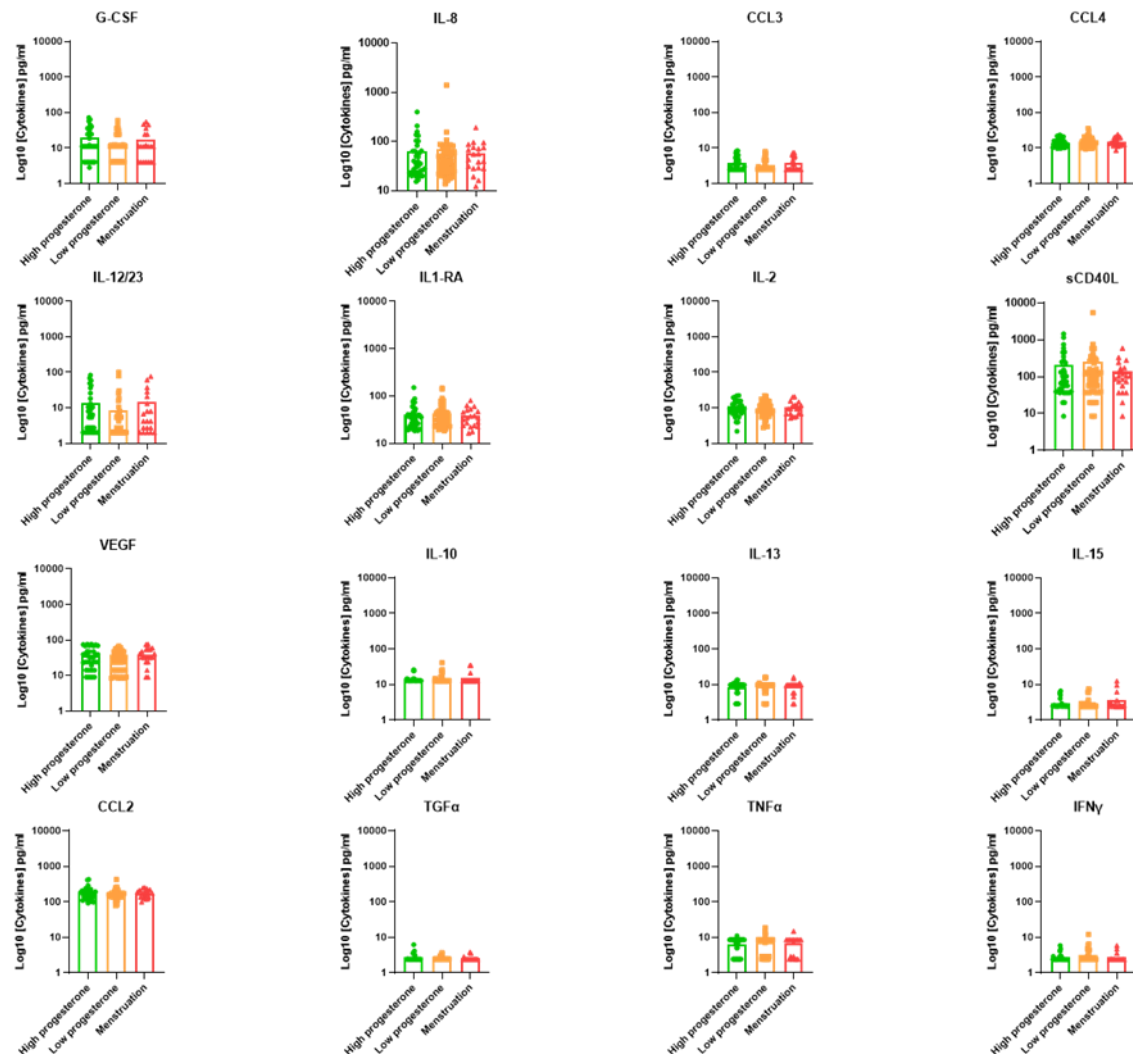
**B**

Figure 2: Cytokine and chemokine expression in the plasma of female cynomolgus macaques (n=9). (A) Heatmap representing the mean fold change expression of cytokines and chemokines in the plasma in each animal (n=9). The fold change was calculated based on the mean expression of each cytokine/chemokine for all females and time points. A one way ANOVA test was performed to compare total fold change to each animal fold change values for each cytokine. Asterisks indicate p values considered statistically significant (*p ≤ 0.05 , **p ≤ 0.01 , ***p ≤ 0.001 , ****p ≤ 0.0001). (B) Heat map representing the mean fold change expression of cytokines and chemokines in the plasma in all animals according to time (n=9). The fold change was calculated based on the mean expression of each cytokine/chemokine in each female. Red arrows represent menstruation.



Supplementary figure 2: Cytokine and chemokine expression in the plasma of female cynomolgus macaques according to hormonal cycle phases (n=9). Samples were clustered in three groups based on progesterone level or menstruation and each cytokine/chemokine concentration (pg/mL) was plotted. A Kruskal-Wallis test with the Dunn test to adjust the p value was performed.

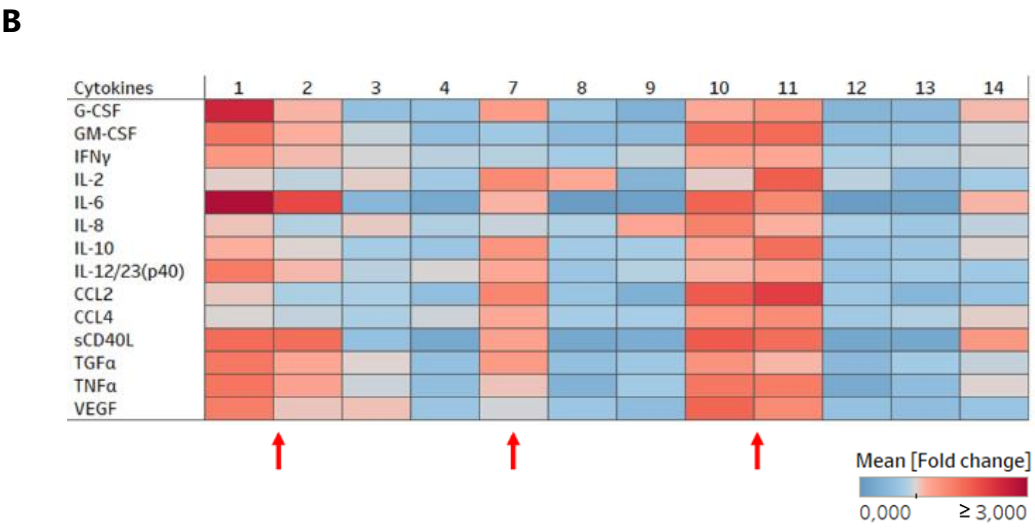
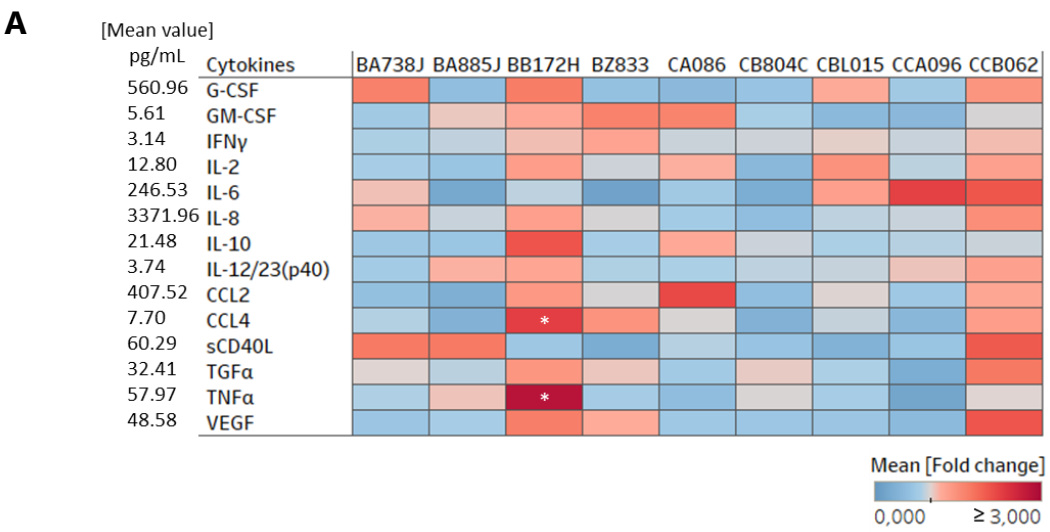
Cervicovaginal fluids

In parallel to peripheral blood plasma, the local inflammation was studied in cervicovaginal fluids. In all nine females, 23 cytokines were detected in vaginal fluids with differential concentration. However only relevant cytokines for this study were displayed on the heatmap representing the mean fold change expression relative to the total mean value of each cytokines [Fig3A]. BB172H expressed a higher level of

TNF α and CCL4 in contrast to other females [Fig3A]. As shown on the heatmap representing the kinetics of cytokine level in all animals, there was a modification of the cytokine concentrations with time [Fig3B]. Interestingly, around or during the menstruation, there was an increased production of several cytokines. To confirm the impact of menstrual cycle on cytokine concentrations, all the samples were divided into three groups based on the menstrual cycle phases (high or low progesterone level and menstruation) [Fig3C]. The analysis showed that 12 out of 23 cytokines/chemokines (CCL2, G-CSF, TNF α , GM-CSF, IFN γ , VEGF, IL-10, IL-12/23, IL-2, TGF α , IL-6 and sCD40L) were increased in cervicovaginal fluids during menstruation, compared to high progesterone. As for CCL4 and IL-8, their concentration was increased during menstruation compared to low progesterone. Moreover, differences were also observed between the low and high progesterone groups. Indeed G-CSF, IL-8 and IL-2 expression was increased in the low progesterone group, compared to high progesterone.

To observed similarities and differences of cytokine concentrations measured in the blood and in cervicovaginal fluids, cytokine concentrations between compartments were compared. In all animals, mean production of several cytokines (IL-1 β , IL-1RA, IL-5, IL-6, IL-8, IL-15, IL-18, TGF α , TNF α), growth factors (GM-CSF, G-CSF) and chemokines (CCL2, CCL3) was higher in cervicovaginal fluids compared to peripheral blood plasma. Only CCL4 was higher in peripheral blood plasma compared to cervicovaginal fluids [Supplementary Fig3]. Therefore, distinct cytokine profiles were observed in each compartment.

Altogether, these results show that, in female cynomolgus macaques, hormonal cycle impacts significantly cytokine profiles in cervicovaginal fluids but not in peripheral blood plasma.



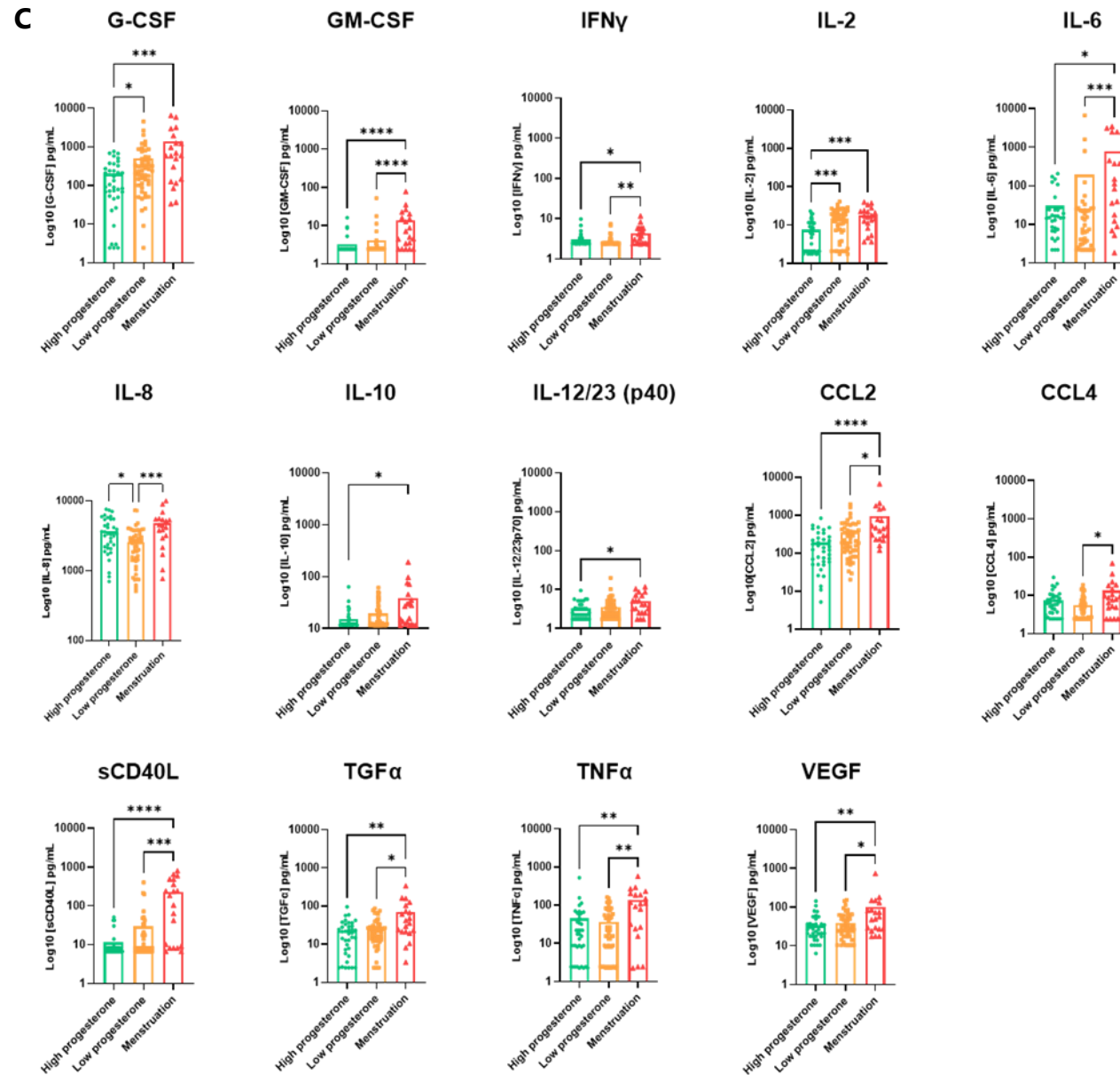
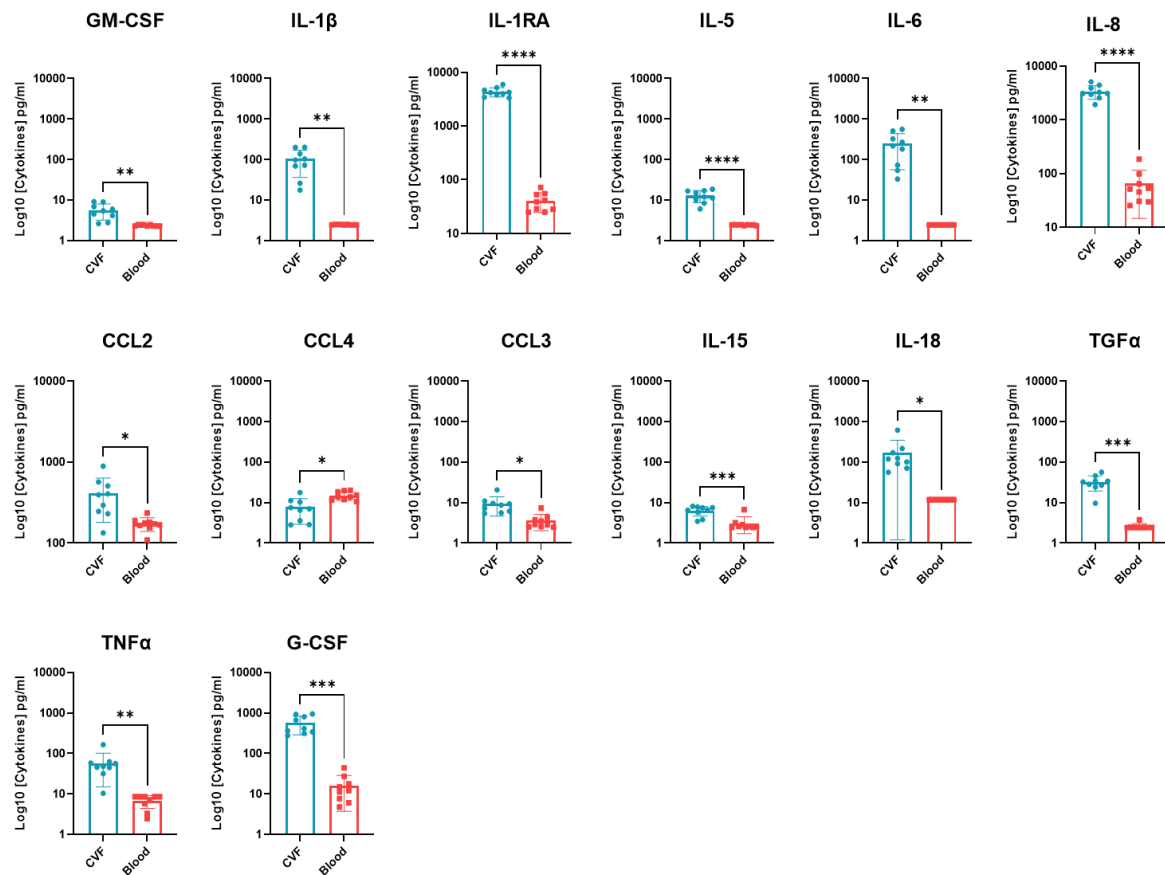


Figure 3: Cytokine and chemokine expression in cervicovaginal fluids of female cynomolgus macaques (n=9). (A) Heatmap representing the mean fold change expression of cytokines and chemokines in cervicovaginal fluids in each animal (n=9). The fold change was calculated based on the mean expression of each cytokine/chemokine for all females. A one way ANOVA test was performed to compare total fold change to each animal fold change values for each cytokine. (B) Heat map representing the mean fold change expression of cytokines and chemokines in the cervicovaginal fluids in all animals (n=9). The fold change was calculated based on the mean expression of each cytokine/chemokine in each female. Red arrows represent menstruation (C) Samples were clustered in three groups based on progesterone level or menstruation and each cytokine/chemokine concentration was plotted. A Kruskal-Wallis test with the Dunn test to adjust the p value was performed. Asterisks indicate p values considered statistically significant (*p \leq 0.05, **p \leq 0.01, ***p \leq 0.001, ****p \leq 0.0001).



Supplementary figure 3: Cytokine and chemokine expression in the plasma and cervicovaginal fluids of female cynomolgus macaques. Mean concentration of each cytokine/chemokine for each animal in plasma and cervicovaginal fluids were plotted and a paired T-test was performed to compare both compartments. Asterisks indicate p values considered statistically significant (*p \leq 0.05, **p \leq 0.01, ***p \leq 0.001, ****p \leq 0.0001).

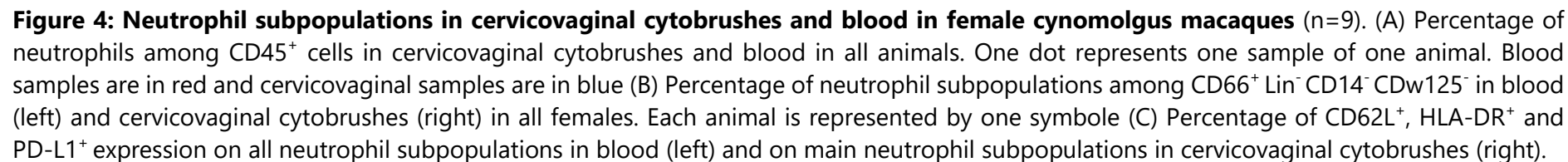
Neutrophil subpopulations in cervicovaginal cytobrushes are strongly impacted by hormonal phases

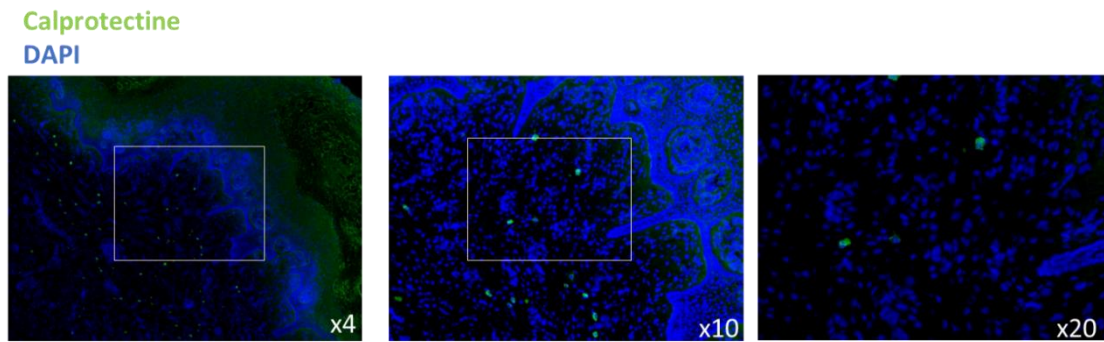
To study the presence and evolution of neutrophil subpopulations in the blood and in the cervicovaginal compartments during the menstrual cycle, vaginal cytobrushes and whole blood were collected and cells were stained with specific cell surface antibodies before flow cytometry analysis. Neutrophils were identified as CD45 positive Lineage negative (CD3, CD8, CD20, CD123, CD14, CDw125) and CD66abce positive. CD10 and CD101 were used to determine maturity. CD62L shedding and CD11b increased expression were assessed to attest the priming status of blood neutrophils [27]. CD32a, CD64, PD-L1 and HLA-DR complete the panel to study the activation of neutrophils in both compartments.

We first analyzed neutrophil populations in both compartments independently of their variation according to time. Neutrophil percentage among CD45⁺ cells (Mean \pm SD) was quite similar between vaginal cytobrushes (77.8% \pm 8.28) and blood (68.3% \pm 5.08) in all animals [Fig4A]. To study the localization of neutrophils within the mucosa, a punch of a vagina near the cervix was collected and frozen. Immunohistochemistry staining was performed and anti-calprotectin antibody was used to visualize neutrophils [Suppl figure 4]. The staining highlighted a presence of neutrophils underneath the epithelium. This suggests that part of neutrophils collected by cervicovaginal cytobrushes originates from the tissue itself while another part might come from the lumen. To study the morphology of neutrophils, blood smears as well as cytopins with the cells collected with cervicovaginal cytobrushes were performed each week. As seen on an example of a representative individual, most of the neutrophils in cervicovaginal cytobrushes were actively involved in phagocytosis, in contrast to whole blood neutrophils [Supplementary Fig5A and B]. Distinct populations were observed in both compartments. Four populations were observed in the blood (Table 4): **(A)** CD11b⁺ CD101⁺ CD32a^{high} **(B)** CD11b^{high} CD101⁻ CD32a^{high} **(C)** CD11b⁺ CD101⁺ CD32a^{mid} **(D)** CD11b^{mid} CD101⁻ CD32a^{mid} [Fig4B, left]. The population **A**

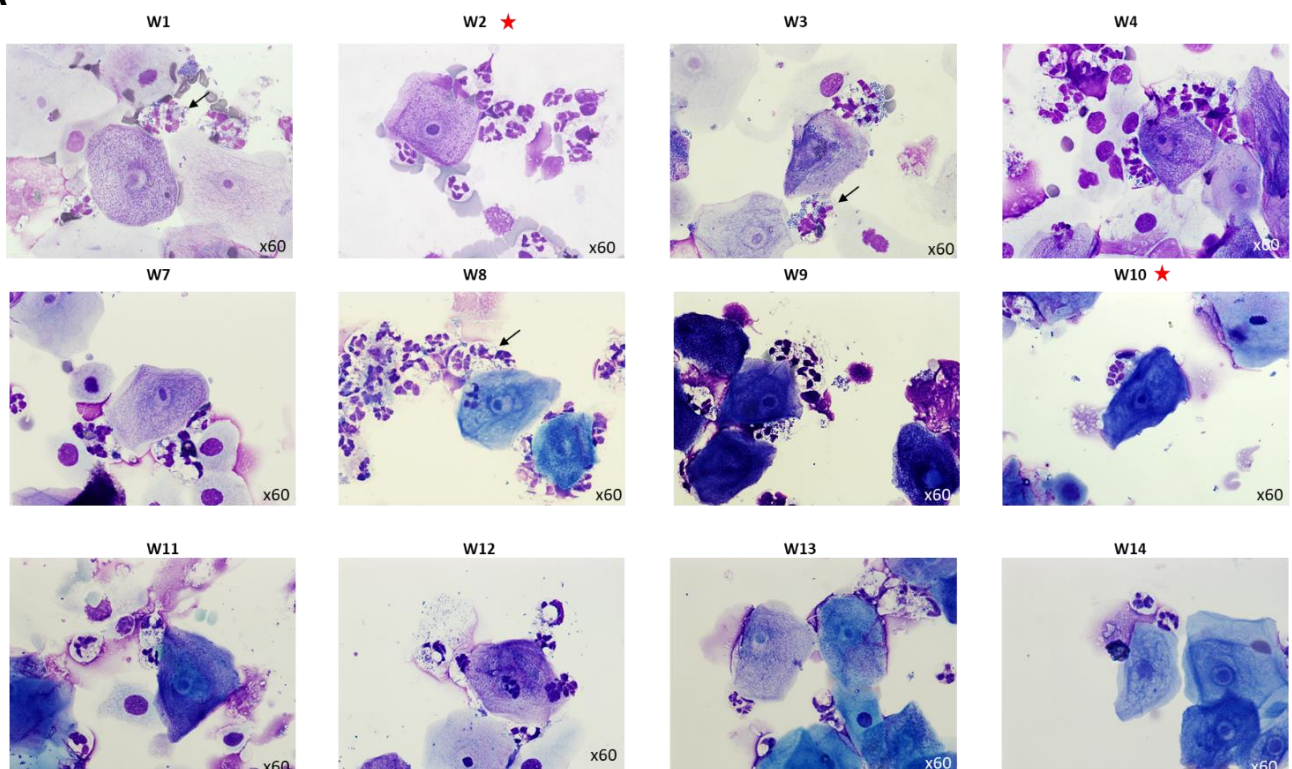
represented $97.54\% \pm 0.60$ (mean ranging from 96.7% to 98.7%). The three other populations were weakly present in the blood with $0.71\% \pm 0.34$ (mean ranging from 0.2% to 1.3%) for the population **B** and $0.35\% \pm 0.19$ for population **C** (mean ranging from 0.1% to 0.7%) and $0.14\% \pm 0.08$ for the population **D** (mean ranging from 0.06% to 0.3%). No expression of CD10 was observed on the population **C** and **D**. In contrast, the population **A** ($CD11b^+ CD101^+ CD32a^{high}$) expressed mild level of CD10 (mean ranging from 12.5% to 50%), suggesting that part of it is mature [Fig4C left].

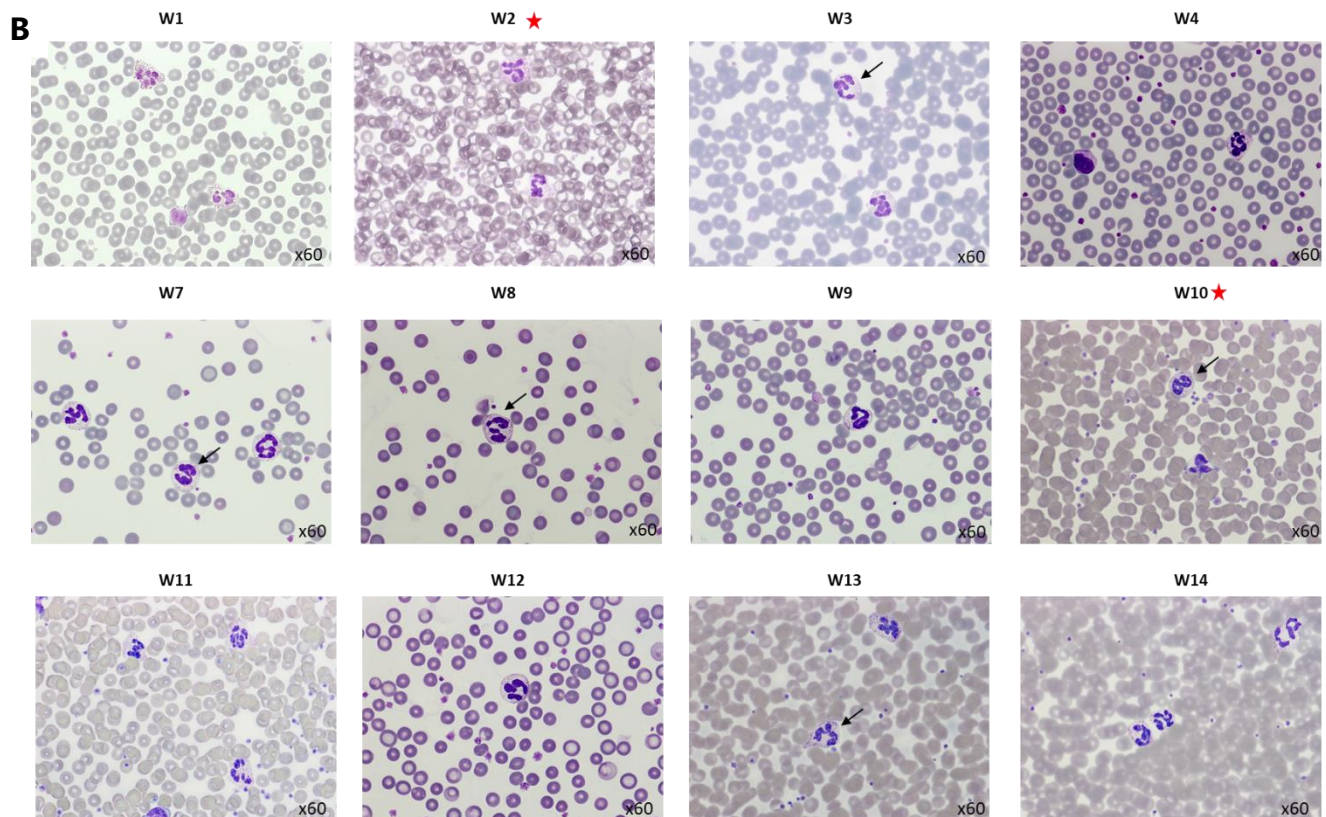
In the blood [Fig4C left], CD62L expression varied among subsets with $99.3\% \pm 0.40$ (mean ranging from 98.7% to 99.7%), $71.4\% \pm 7.14$ (mean ranging from 56.1% to 81.7%), $31.8\% \pm 13.08$ (mean ranging from 17.4% to 57.6%) and $6.9\% \pm 4.51$ (mean ranging from 2.2% to 13.4%) of neutrophils expressing CD62L from populations **A** to **D**. Strong variation of PD-L1 expression was observed among each population, indeed $23.8\% \pm 33.41$ (mean ranging from 1.8% to 82.2%), $25.2\% \pm 12.25$ (mean ranging from 4.5% to 42.9%), $10.8\% \pm 9.86$ (mean ranging from 2.3% to 26.3%) and $14.5\% \pm 13.05$ (mean ranging from 2.5% to 42.7%) of population **A** to **D** express PD-L1. Low expression of HLA-DR and CD64 was observed on neutrophils from the blood or from the cervicovaginal cytobrushes at all time points. [Fig4C left, Data not shown for CD64]. In conclusion, there was one major population in the blood characterized as $CD11b^+ CD101^+ CD32a^{high}$. This population had variable expression of CD10 suggesting the presence of both mature and immature subsets. In addition, there was a high expression of CD62L and variable expression of PD-L1 on this population. Mature and immature neutrophils expressed CD62L marker, which is decreased upon priming or tissue migration [27–29]. As for the immunosuppressive marker PD-L1, it increased expression on blood neutrophils has been detected in patients with sepsis and associated with reduce neutrophil function (phagocytosis and cytokine production) [30]. In the current study, we were not able to associate the variation of PD-L1 expression with the menstrual cycle or a specific cytokine production due to high variabilities among individuals and time points.





Supplementary figure 4: Localization of neutrophils in the vaginal mucosa. Immunohistochemistry staining of a slide obtained from a vaginal punch (upper vagina near the cervix) of one female cynomolgus macaque. Anti-Calprotectin antibody labelled with FITC (green) was used to stain neutrophils and DAPI for nucleus staining (blue).

A



Supplementary figure 5: Neutrophil morphology in the blood and cervicovaginal cytobrushes. Neutrophil staining by May-Grunwald-Giemsa of (A) blood smears and (B) cervicovaginal cells obtained from cytobrushes of one representative animal (CBL015) at all time points. Red star represents menstruation. Black arrow represents neutrophils.

CD11b⁺ CD101⁺		CD11b^{high} CD101⁻	CD11b^{mid} CD101⁻
CD32a ^{high}	CD32a ^{mid}	CD32a ^{high}	CD32a ^{mid}
Population_A	Population_C	Population_B	Population_D

Table 4: Neutrophil subpopulations characterized in peripheral blood.

Based on the expression of CD11b, CD101, CD10 and CD32, eight populations were determined in cervicovaginal cytobrushes [Table 5]. However, three main subsets were distinguished: CD11b^{high} CD101⁺ CD10⁺ CD32a⁺ (mean ranging from 18.3% to 67.1%; population 8), CD11b^{high} CD101⁺ CD10⁻ CD32a⁺ (mean ranging from 6.5% to 26.4%; population 6) and CD11b^{low} CD101⁻ CD10⁻ CD32⁻ (mean ranging from 4% to 38%; population 7) [Fig4B right]. The population 8 based on the expression of CD10, CD101

and CD32a can be assigned as a mature/activated subset of neutrophils. Mean CD62L expression was low in cervicovaginal cytobrushes with only $17\% \pm 10.77$ (mean ranging from 3.1% to 33.9%), $6.48\% \pm 3.18$ (mean ranging from 2.5% to 11.3%) and $14.49\% \pm 4.17$ (mean ranging from 7.2% to 19.4%) on population **8**, **6** and **7**. PD-L1 expression was the highest in population **7** (mean ranging from 19.5% to 46.5%) followed by the population **8** (mean ranging from 14.2% to 27.1%) then the population **6** (mean ranging from 9.6% to 25.6%) [Fig4C right].

We further studied the kinetics of the main neutrophil populations in the blood and in cervicovaginal cytobrushes. The main neutrophil subset in the blood stayed stable. However, fluctuations in the percentage of cervicovaginal neutrophil subpopulations were observed over time [Fig5A].

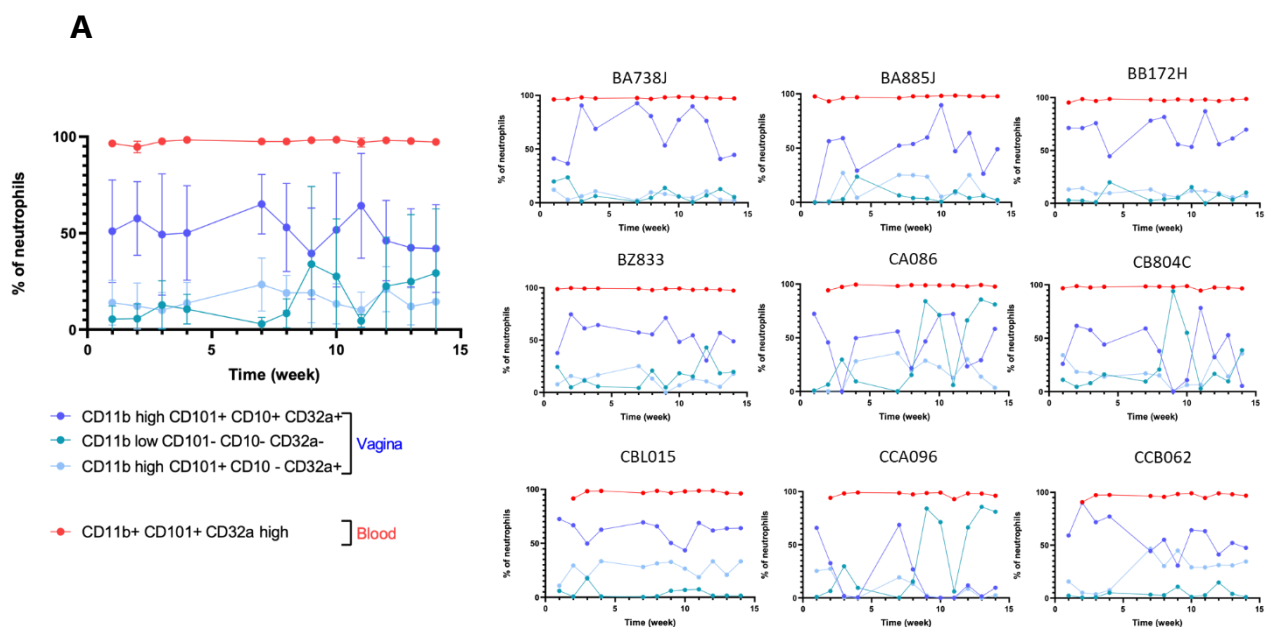
We then classified the neutrophil subpopulations in peripheral blood and cervicovaginal cytobrushes in three groups based on progesterone level and menstruation. A decrease of total neutrophils in peripheral blood was observed at menstruation compared to high progesterone. Only the population CD11b^{mid} CD101⁻ CD32a^{mid} varied with the progesterone level, peaking during menstruation [Fig5B]. On the contrary, more variations were observed in cervicovaginal neutrophils. The population 3, 4 and 7 decreased during the menstruation compared to high progesterone whereas the population 8 significantly increased during menstruation. Population 6 decreased during menstruation compared to low progesterone. The population 7 also expressed lower level of PD-L1 during menstruation. Interestingly, the population 8 expressed higher level of CD62L during menstruation compared to high and low progesterone groups. Moreover, populations **4**, **5** and **7** decreased in the low progesterone group compared to high progesterone group, whereas the population **8** increased in the low progesterone group compared to high progesterone group. Thus, these findings show an increase in neutrophil CD10 expression during menstruation and an increase in neutrophil CD32a expression during the proliferative compared to the secretory phase. Other markers were also differentially expressed in neutrophils during the menstrual cycle : the population **7** expressed lower level of PD-

L1 during menstruation and interestingly, the population **8** expressed higher level of CD62L during menstruation compared to high and low progesterone groups [Fig5C].

Altogether, these results highlight the presence of neutrophil subpopulations in cervicovaginal compartment that are impacted by the menstrual cycle in cynomolgus macaques. Menstruation induce the accumulation of mature/activated neutrophils that comes from the blood based on their high expression of CD62L that is usually decreased in the tissue [29]

Table 5: Neutrophil subpopulations characterized in cervicovaginal cytobrushes.

CD11b ^{high} CD101 ⁺				CD11b ^{low} CD101 ⁻			
CD10 ⁺		CD10 ⁻		CD10 ⁺		CD10 ⁻	
CD32a ⁺	CD32a ⁻	CD32a ⁺	CD32a ⁻	CD32a ⁺	CD32a ⁻	CD32a ⁺	CD32a ⁻
Population 8	Population 5	Population 6	Population 4	Population 2	Population 1	Population 3	Population 7



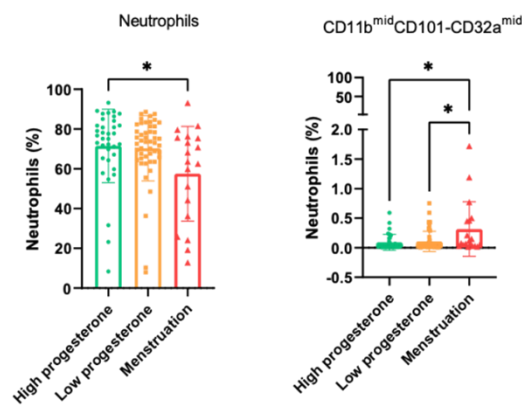
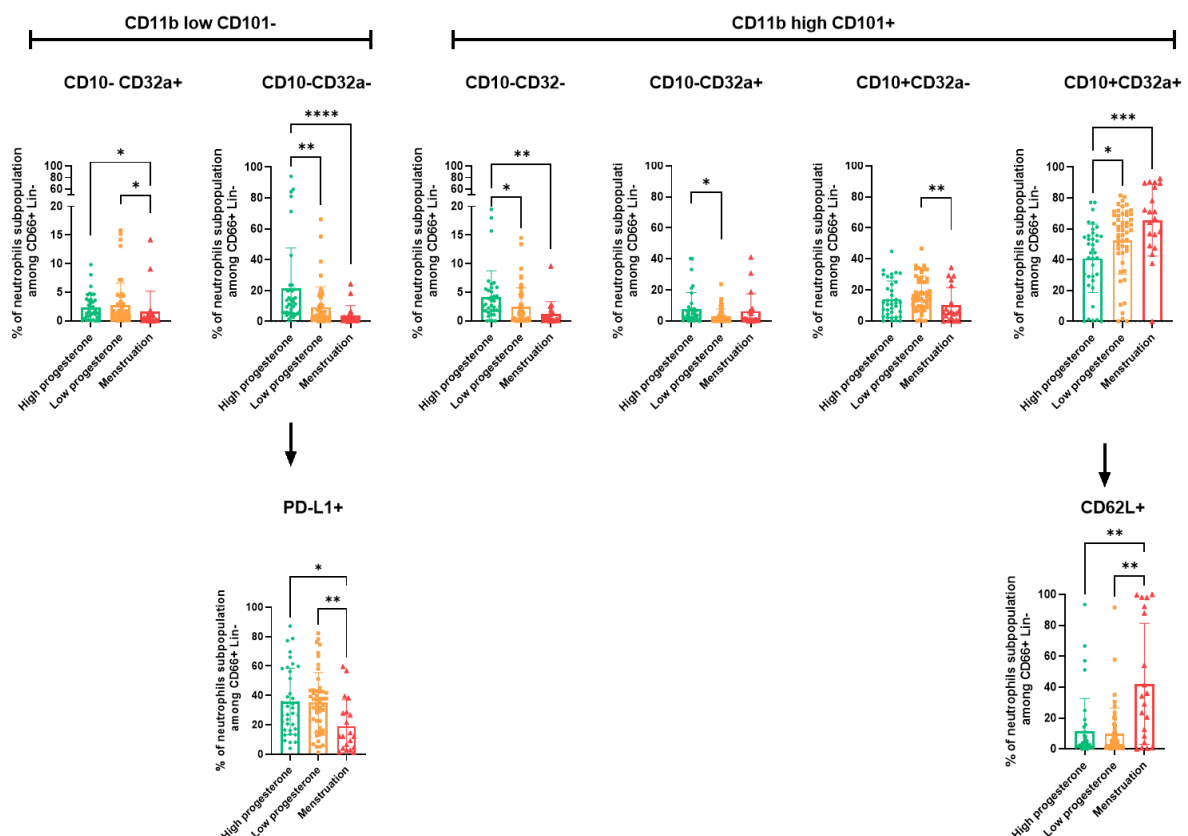
B**C**

Figure 5: Variation of neutrophil subpopulations in cervicovaginal cytobrushes and blood during the menstrual cycle. (A) Main neutrophil subpopulations among CD66⁺ Lin⁻ in both compartments according to time in all animals (mean of all animals, left) or in each animal (right). (B) and (C) Samples were clustered in three groups based on progesterone levels or menstruation and the percentage of each neutrophil subpopulations was plotted. A Kruskal-Wallis test with the Dunn test to adjust the p value was performed. Asterisks indicate p values considered statistically significant (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). Only significant results were represented. (B) in blood, (C) in cervicovaginal cytobrushes.

Vaginal microbiota composition is altered by hormonal phases

We have demonstrated that the inflammation translated into cervicovaginal cytokine concentrations and neutrophil subpopulations varied according to the menstrual cycle. Since the vaginal microbiota is involved in the regulation of the local inflammation, we characterized in parallel the composition of the microbiota to evaluate its variation during the menstrual cycle.

The composition of the microbiota in each animal was studied using two different approaches: Nugent scoring and V3-V4 16S rRNA sequencing. First, to assess whether female macaques had a diverse or a *Lactobacillus spp* dominant vaginal microbiota, Nugent score was determined for each female at each time point. In most of the females, very few variations were observed in the Nugent score, indeed, seven females displayed a Nugent score of 7 or 8 depending of the time point [Supplementary Fig6]. On the contrary, two females (CB804C and CCA096) had a variable Nugent score ranging from 5 to 8. These two females had more Gram-positive bacilli shaped bacteria that are generally associated to *Lactobacillus spp*. All females, for the majority of the time points, exhibited a diverse vaginal microbiota poor in *Lactobacillus spp*.. To deeply characterize the vaginal microbiota composition, sequencing the V3-V4 region of the 16S rRNA was performed in all the samples throughout the entire study.

We first identified bacteria present in the vaginal microbiota of each animal. Eight phyla were observed in the vaginal microbiota of those females. More than 99% of bacteria in the vaginal microbiota were part of five phyla (total abundance mean ranging from highest abundance to the lowest): the *Firmicutes* (29.7% to 64.3%), *Bacteroidota* (15.6% to 34.9%), *Fusobacteriota* (9.8% to 39.6%), *Actinobacteriota* (3.8% to 14.2%) and *Campilobacterota* (0.87 to 7.3%). The other three phyla (*Desulfobacterota*, *Proteobacteria* and *Spirochaetota*) represented less than 1% of the total abundance [Fig6A and supplementary fig7A]. Two females, CBL015 and CCA096, had a different microbiota composition observed at all taxonomic level compared to others [Fig 6]. Nine predominant families were observed in all females: *Leptotrichiaceae*, *Prevotellaceae*, *Porphyromonadaceae*, *Fusobacteriaceae*, *Bifidobacteriaceae*,

Streptococcaceae, a family part of the order *Peptostreptococcales-Tissierellales*, *Peptostreptococcaceae* and *Actinomycetaceae* [Fig6B]. Nine predominant genus were found in all the females with variable abundance (mean \pm SD) between the females: *Sneathia* (13.6% \pm 0.12), *Prevotella* (12% \pm 0.12), *Porphyromonas* (8.6% \pm 0.07), *Fusobacterium* (7.8% \pm 0.06), *Streptococcus* (7.2% \pm 0.11), *Bifidobacterium* (7% \pm 0.13), *Peptoniphilus* (5.9% \pm 0.05), *Peptostreptococcus* (4.4% \pm 0.04) and *Parvimonas* (4.3% \pm 0.03) whereas mean abundances of other genus represented 29% [Fig6C and supplementary fig7B]. *Lactobacillus spp.* were observed in all females but at really low abundance with CCA096 expressing the highest abundance (20.5% \pm 0.15) [Supplementary fig7B]. CCA096 also had more *Actinobacteriota* (40.87% \pm 0.19) related to a higher abundance of *Bifidobacterium* (37.15% \pm 0.18). As for CBL015, it had more *Firmicutes* (64.3% \pm 0.044) translating into a high abundance of *Streptococcus* (35.2% \pm 0.12). Moreover, this female had also a very low abundance of *Sneathia* (0.043% \pm 0.00) [Supplementary fig7 and fig6A and C].

To identify variation of bacterial abundance, the top 9 genus or family for all or each animal according to hormonal phases are represented [Fig7 and supplementary fig8]. Bacterial abundances and composition were modified according to hormonal groups in all animals. To study if the bacterial diversity was impacted by hormonal phases, the Shannon diversity index was calculated according to high, low progesterone or menstruation timepoints. An increased bacterial diversity (p value: 0.0065) during menstruation compared to the high progesterone group was observed [Fig8A]. To identify bacterial family and genus that were differentially abundant among progesterone groups in all animals, a statistical analysis of all families and genus relative abundance adapted to highthroughput sequencing data was performed [Fig8B and C]. This differential analysis identified 14 families differentially abundant according to hormonal groups [Fig8B]. Abundance bar plots were generated to identify increases or decreases based on hormonal groups [supplementary fig9]. Nine families (*Christensellaceae*, *Clostridiaceae*, *Corynebacteriaceae*, *Desulfovibrionaceae*, *Lachnospiraceae*, *Oscillospiraceae*, *Paludibacteraceae*, *Ruminococcaceae*,

Streptococcaceae) were and one (*Erysipelotrichaceae*) was increased at high progesterone compared to low progesterone. Six taxa (*Enterobacteriaceae*, *Lachnospiraceae*, *Oscillospiraceae*, *Succinivibrionaceae*, *Sutterellaceae*, *Tannerellaceae*) were less abundant at high progesterone compared to menstruation. Finally, *Erysipelotrichaceae* was more abundant at low progesterone compared to menstruation [Fig8B].

Eight genus: *Arcanobacterium*, *Corynebacterium*, *Fingoldia*, *Howardella*, *Mogibacterium*, *Solobacterium*, *Streptococcus* and *Sutterella* were differentially abundant in the different hormonal phases [Fig8C and Supplementary fig10]. Five genus: *Howardella*, *Solobacterium*, *Streptococcus*, *Sutterella* and *Corynebacterium* are part of the families *Lachnospiraceae*, *Erysipelotrichaceae*, *Streptococcaceae*, *Sutterellaceae* and *Corynebacteriaceae* observed to be differentially abundant according to hormonal groups.

To conclude, the results obtained attest of a vaginal microbiota composition that strongly fluctuates according to hormonal phases in all individuals. Main observations include an enrichment of specific bacterial families/genus at low progesterone and menstruation compared to high progesterone.



Supplementary figure 6: Nugent scores of each female during the three months of follow up.

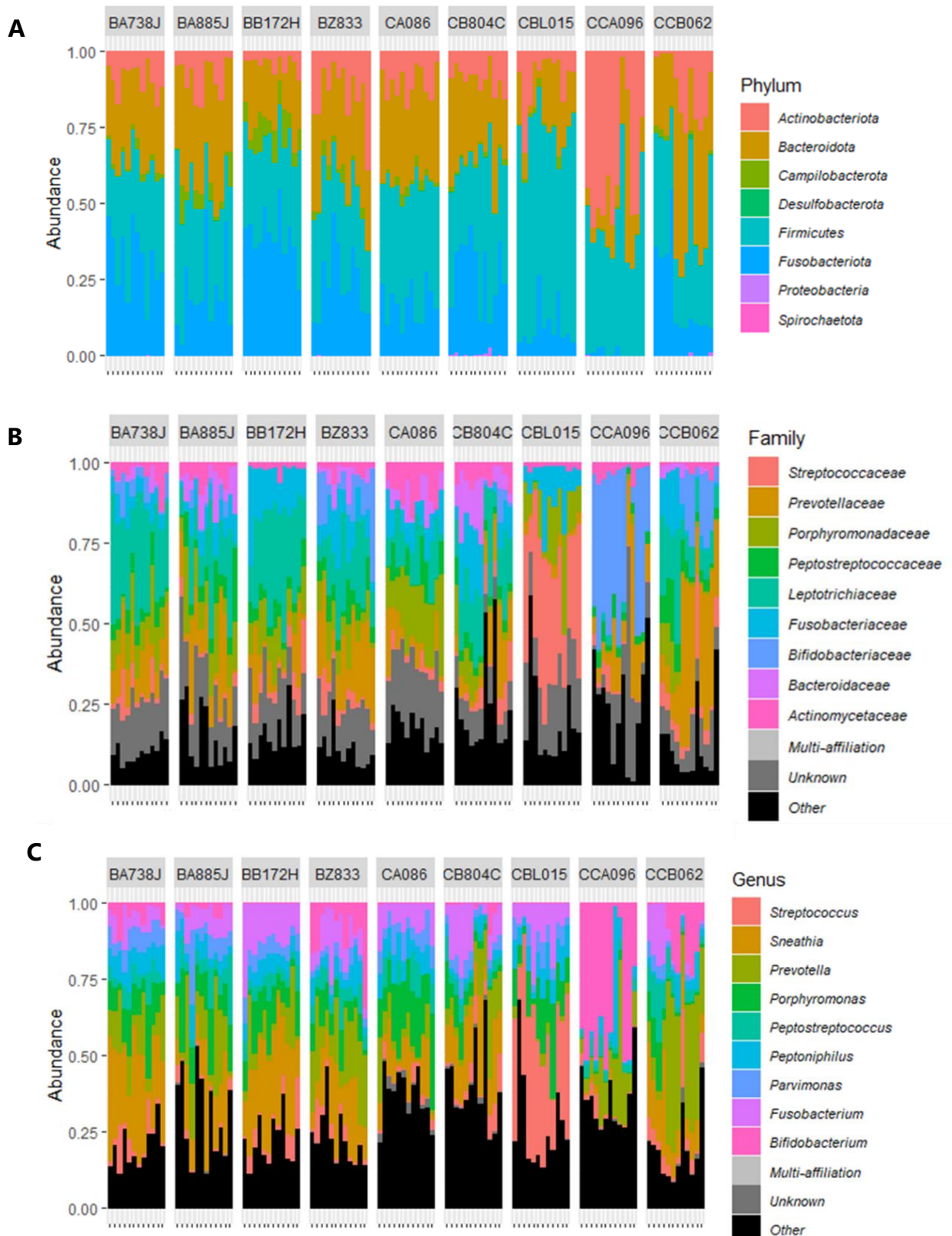
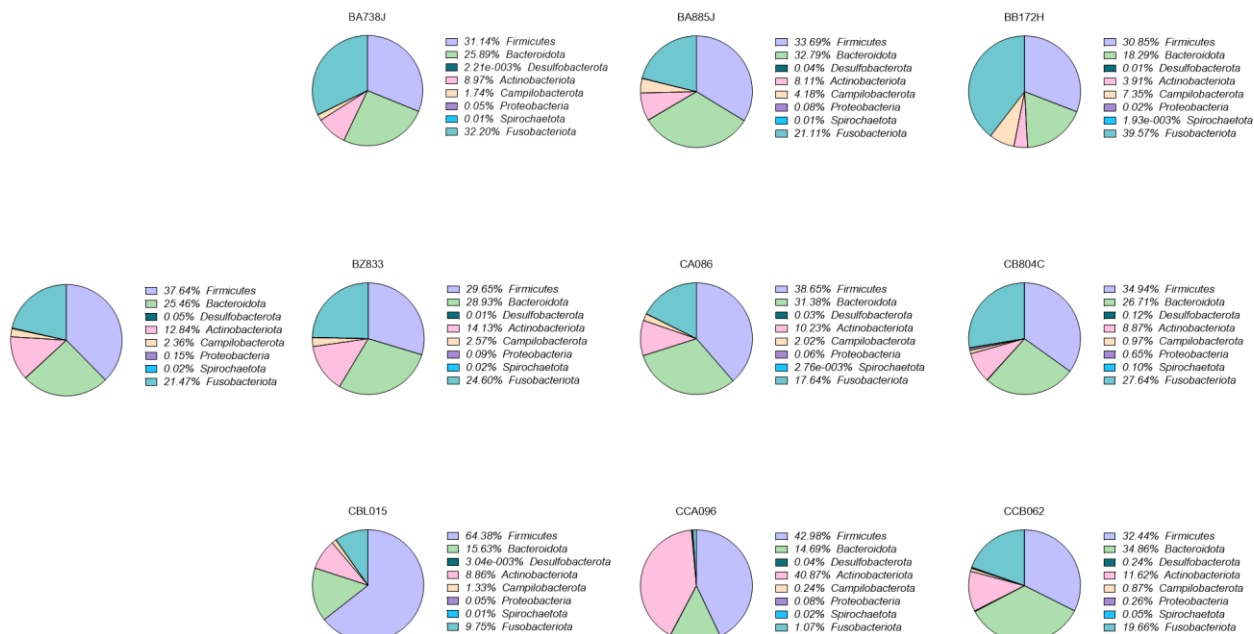
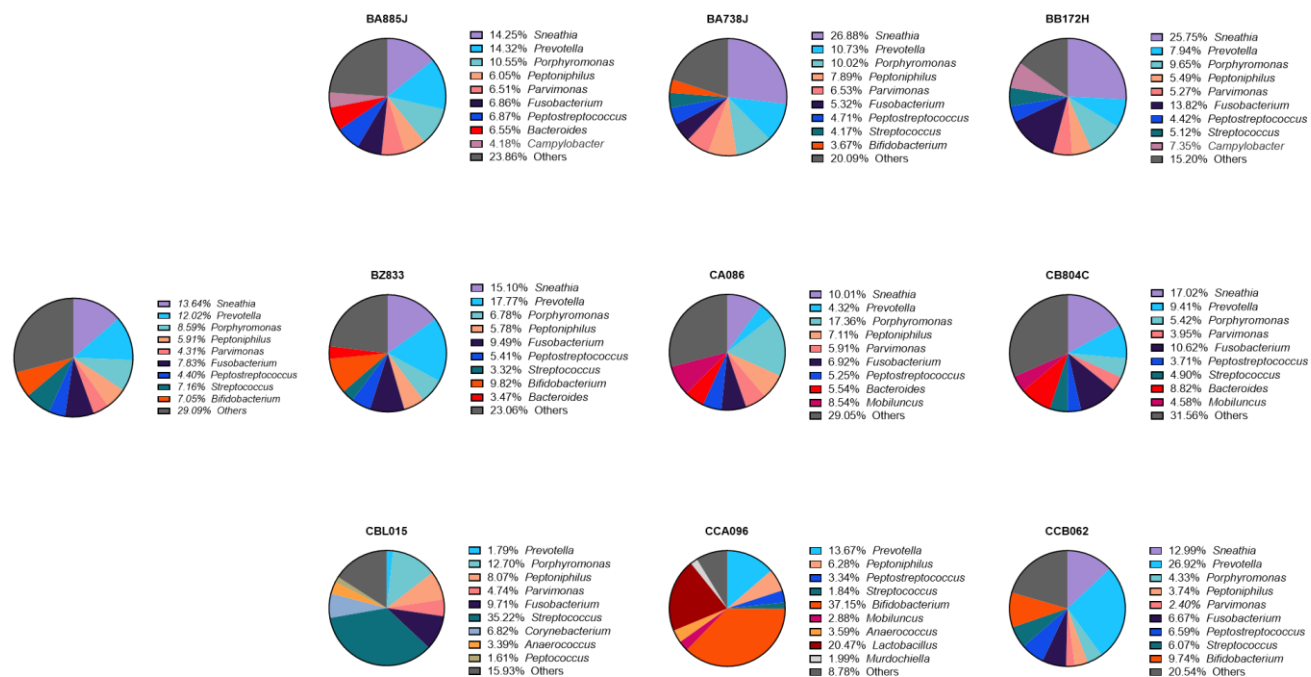


Figure 6: Kinetics of the vaginal microbiota composition of female cynomolgus macaques (n=9). (A) Relative abundance of bacterial taxa at the phylum level in each animal according to time. Relative abundance of the nine most represented genus (B) or families (C) in all females at all time points, in each female according to time. Unrepresented families and genus are in black (other).

A



B



Supplementary figure 7: Vaginal microbiota composition of the female cynomolgus macaques (n=9). Percentages of mean relative abundances of phylum (A) or top9 most represented genus (B) in all animals (left) or in each female (right) are represented in pie chart.

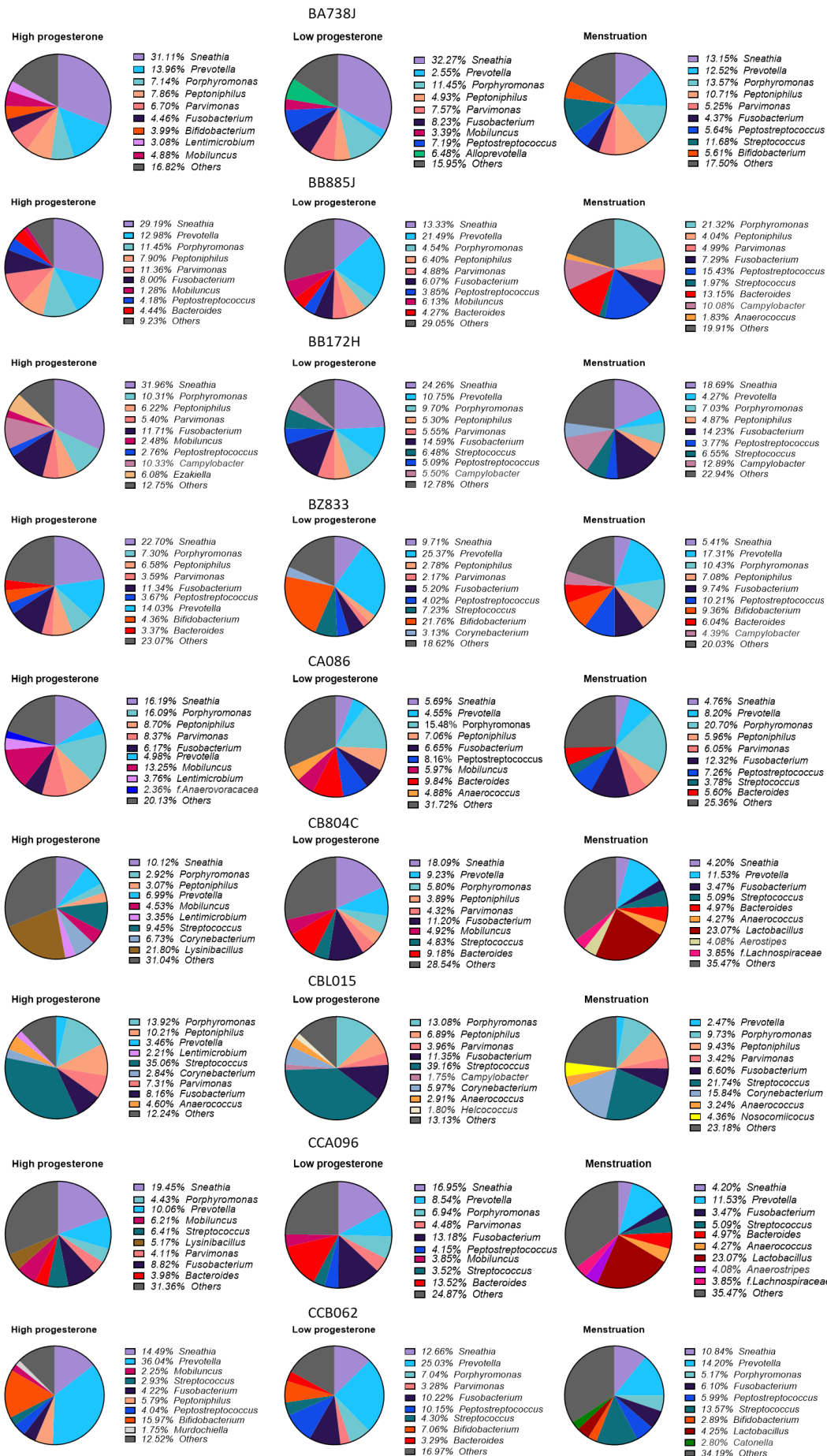
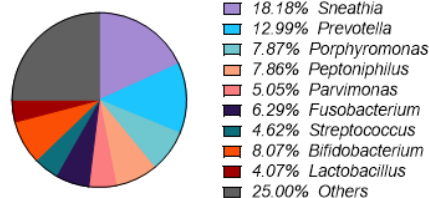
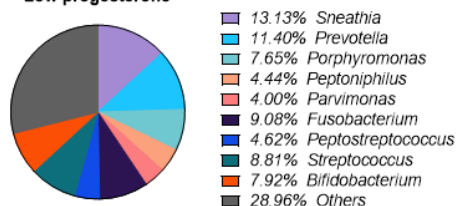
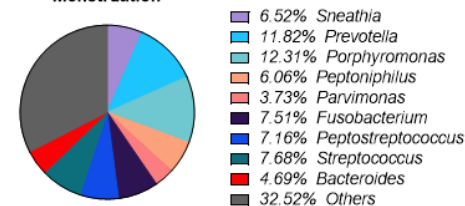
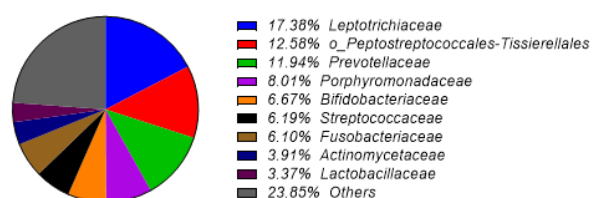
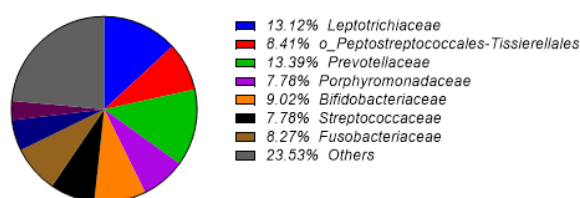
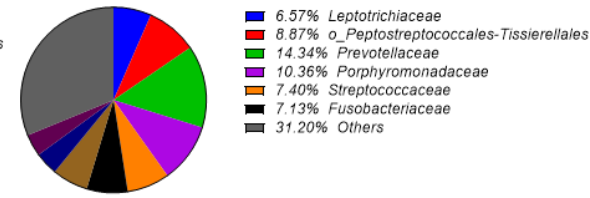


Figure 7: Variation of bacterial taxa in each phase of the menstrual cycle. Percentages of mean relative abundance of the nine most represented genus (top) in the high progesterone, low progesterone and menstruation groups is represented in pie chart for each female. Unrepresented genus are in grey (other).

GENUS**High progesterone****Low progesterone****Menstruation****FAMILY****High progesterone****Low progesterone****Menstruation**

Supplementary figure 8: Variation of bacterial taxa in each phase of the menstrual cycle. Mean relative abundance of the nine most represented genus (top) and families (below) in the high progesterone, low progesterone and menstruation groups is represented in pie chart for all females. Unrepresented genus are in grey (other).

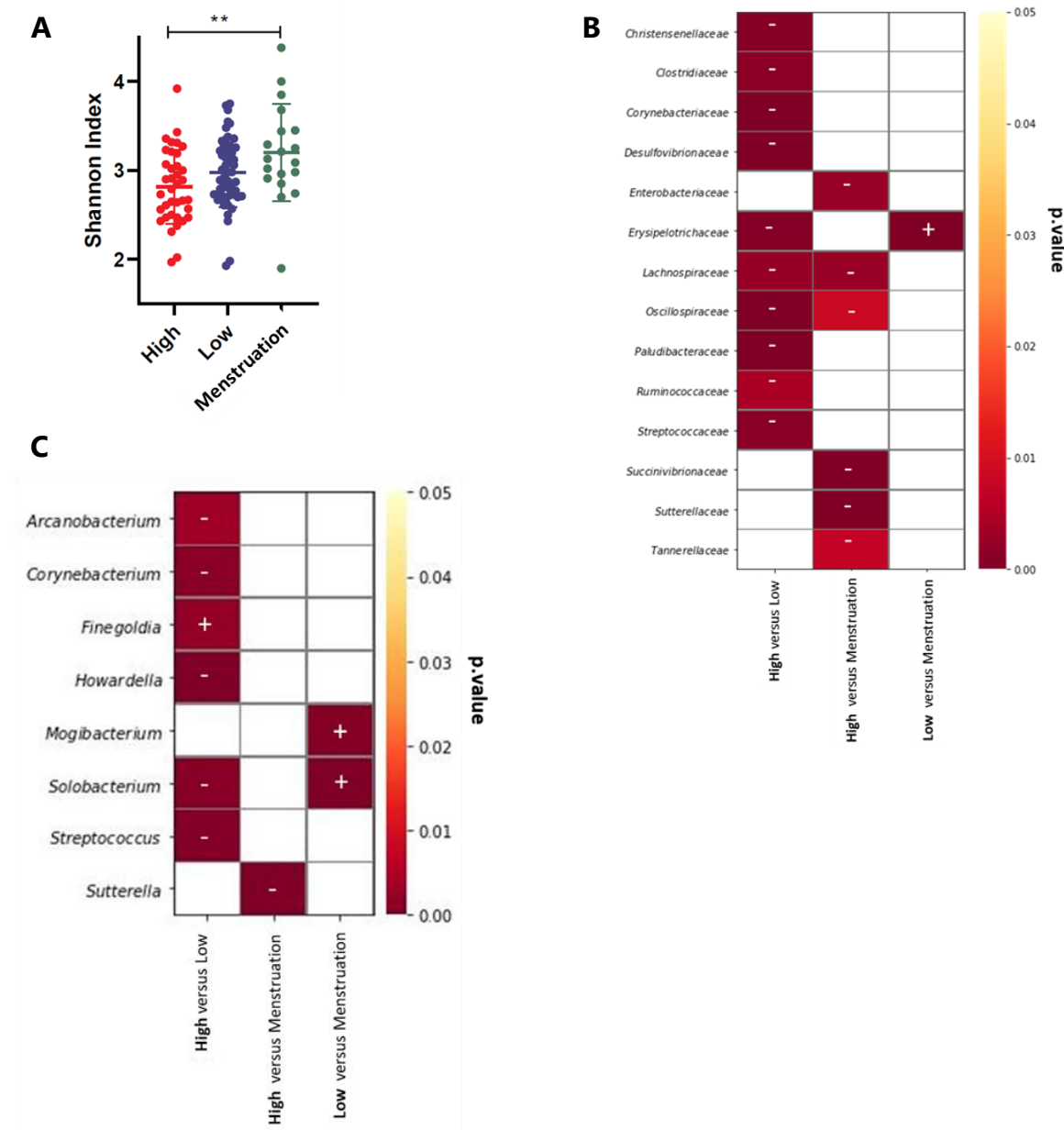
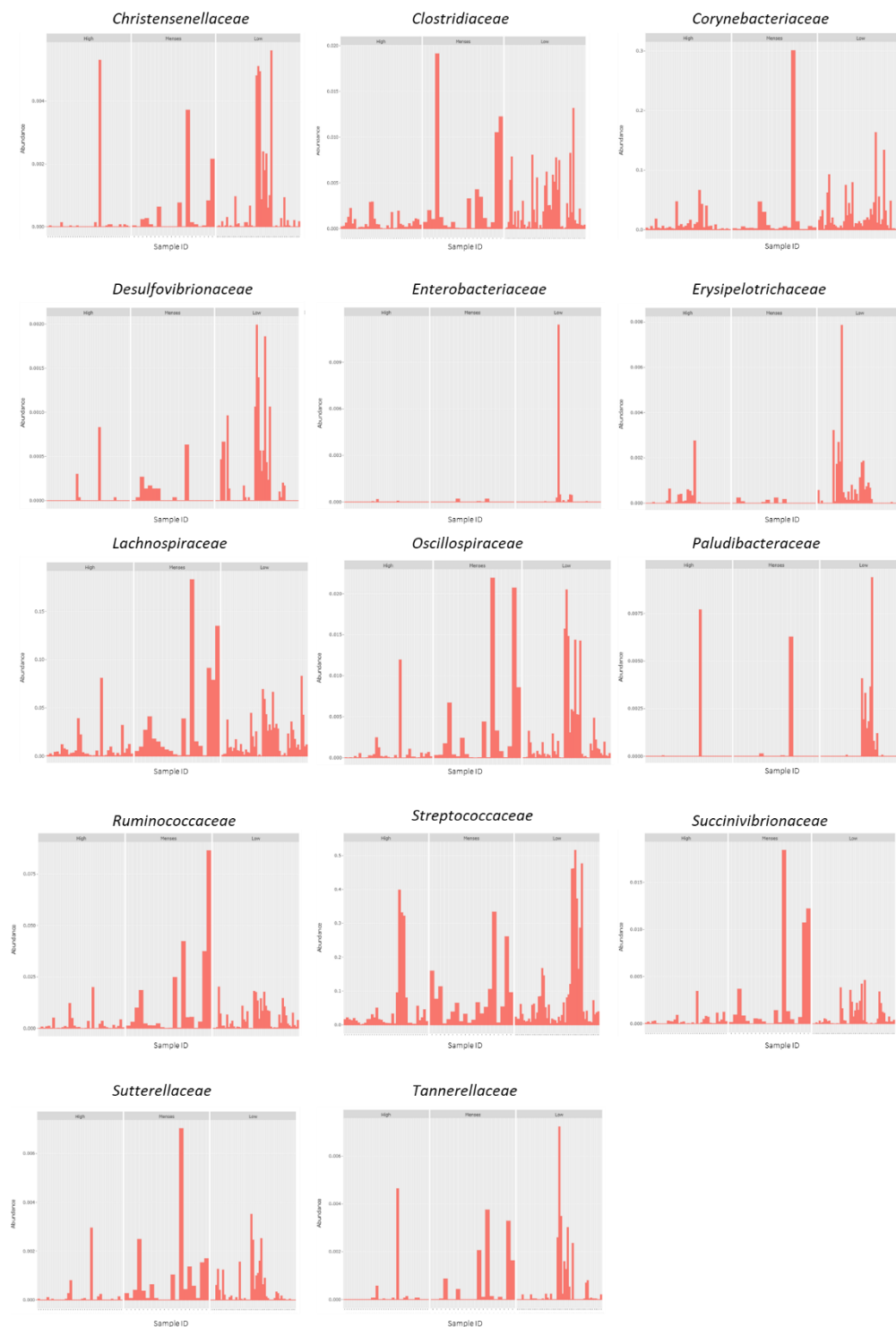
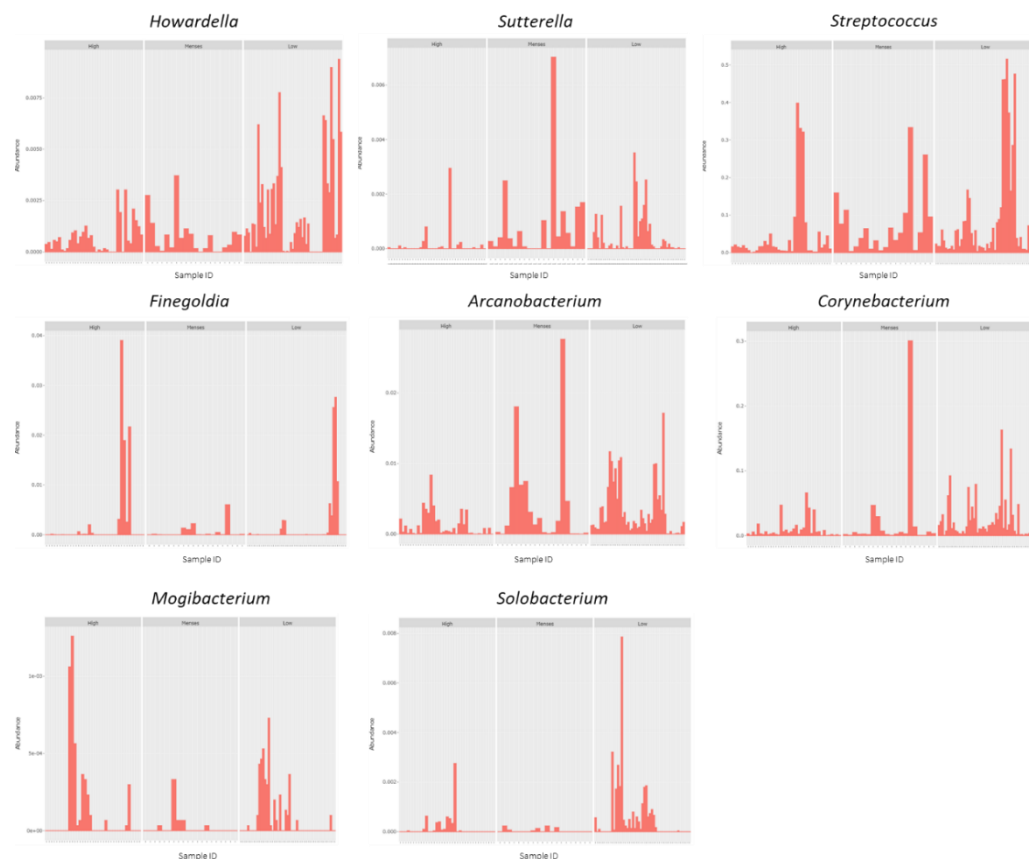


Figure 8: Vaginal microbiota diversity and differentially abundant bacterial taxa in each phase of the menstrual cycle. (A) Shannon index was calculated in each female for high progesterone, low progesterone or during menstruation and plotted together. A Kruskal-Wallis test with the Dunn test to adjust the p value was performed (** $p \leq 0.01$). Analysis were made using the FitZIG algorithm at the family (B) or genus (C) level. Heatmaps representing the p values were generated and plus and minus signs were added to visualize increase or decrease of each bacterial taxa. The sign is associated to the group in bold text. As an example: *Christensenellaceae* is decreased during high progesterone compared to low progesterone.



Supplementary figure 9: Graphical representation of relative bacterial taxa abundances according to hormonal phases at the family level. Only bacterial taxa that were differentially expressed according to hormonal phases are represented.



Supplementary figure 10: Graphical representation of relative bacterial taxa abundances according to hormonal phases at the genus level. Only bacterial taxa that were differentially expressed according to hormonal phases are represented.

Menstruation induce a higher local inflammation

To have a global overview of the association between cytokine expression, neutrophil phenotype and activation as well as bacterial abundances according to menstrual cycle phases in the vagina, a heatmap supplemented with a hierarchical clustering recapitulating all the variables was generated. This heatmap represents the log₁₀ fold change of cytokine expression, neutrophil populations and bacterial taxa expression at the family level, which were differentially expressed during the different hormonal phases [Fig9]. A hierarchical clustering separated the samples into two large groups (A, B) subdivided into four subgroups (A1, A2, B1 and B2) [Fig9].

The first large group (B) exhibited low expression of neutrophil subpopulations 3, 4 and 7 and a high expression of *Streptococcaceae* in both subgroups. An increased expression of cytokines, such as G-CSF, TNF α , TGF α , IL-6, sCD40L, as well as neutrophil subpopulation 8 expressing CD62L and *Lachnospiraceae*, was observed in the subgroup B2 compared to the subgroup B1.

Lower abundances of the family *Corynebacteriaceae* were also identified in the subgroup B2 compared to B1. The subgroup B2 was mainly composed of samples obtained during menstruation (48%), then high progesterone samples (24%), followed by low progesterone samples (27%). On the contrary, the subgroup B1 was dominated by samples obtained during low progesterone (75%), then during menstruation (15%), followed by high progesterone (10%). Two animals, CBL015 (11/12 samples) and BB172H (8/12 samples) were highly represented in the group B. In the second group (A), there was a lower expression of cytokines and a higher presence of neutrophils compared to the group B. Overall, we observed that menstruation are associated with an increase concentration of cytokines and an accumulation of a specific neutrophil population characterized as mature (CD101⁺ CD10⁺) and activated (CD32a⁺) that might originates from the blood (CD62L⁺).

Samples clusterized in the subgroup A2 had a small increased production of cytokines (TGf α and VEGF) and neutrophil subpopulations (**3**, **4**, **5**, **6**, **7** expressing PD-L1, **7** and **8**). However, a decrease in population 8 expressing CD62L was observed, compared to the subgroup A1 suggesting a lower presence of mature/activated neutrophils that originate from the blood. In the subgroup A1, low expression of neutrophil subpopulations, such as the population **8**, and an increased presence of population **7** and CD62L expression on the population **8**, were noticed which could be associated to increase presence of mature neutrophils that originate from the blood and weakly activated neutrophils (population 7). Moreover, the abundance of the family *Streptococcaceae* was lower in the subgroup A1 compared to A2. The subgroup A2 is mainly composed of samples collected at low progesterone (54%), followed by high progesterone (44%) and menstruation samples (0.2%). In the subgroup A1, 75% of the samples were obtained during high progesterone, then low progesterone and menstruation (12.5% each). A1 was dominated by the samples of one animal (CCA096). Indeed, 10 samples of CCA096 out of 12 were observed in the group A, in which 7 samples are included in the subgroup A1, suggesting strong similarities among CCA096 samples. As for CB804C, its samples were mainly affiliated with the subgroup A2 (9/12 samples) and 7 samples out of 12 were part of a smaller group.

In conclusion, samples collected during menstruation clustered together and are associated with increase inflammation (high cytokine/chemokine production and accumulation of

mature/activated neutrophils that originates from the blood). Moreover, samples retrieve during high or low progesterone were distributed in all subgroups. Finally, samples of four animals (CBL015, BB172H, CCA096, CB804C) were observed to be highly consistent among each animal. Samples from two females (CBL015, BB172H) expressed more cytokines whereas the other two have a lower cytokine expression (CCA096, CB804C) but were different in terms of neutrophil subpopulations.

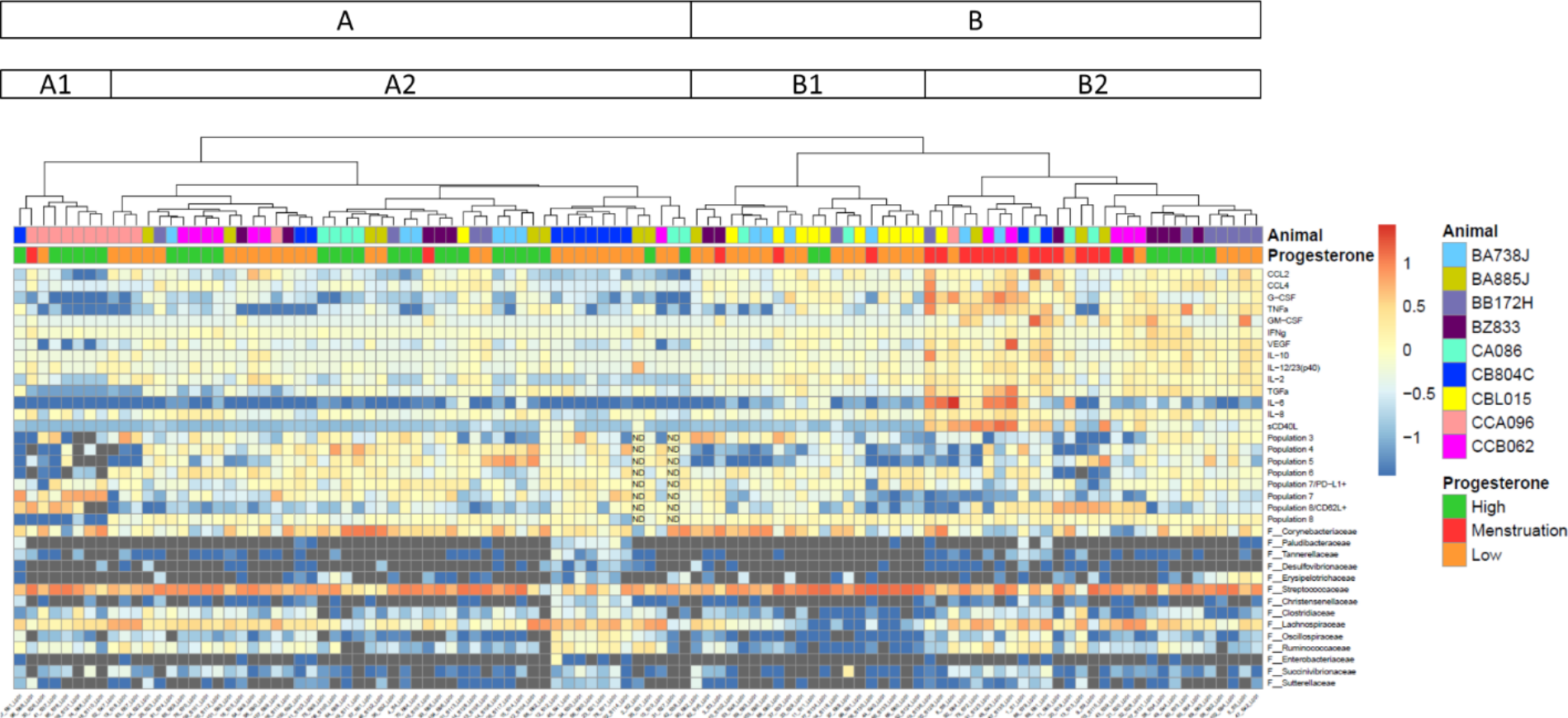


Figure 9: Heatmap representing bacterial taxa (family level), neutrophil subpopulations and cytokine/chemokine expression that varied during the hormonal cycle. The heatmap represents log10 of fold change for each parameter (cytokines, neutrophil subpopulations, bacterial taxa indicated on the right side). Increased expression is shown in red and decrease in blue. Each animal is represented with a color code as well as hormonal phases (high progesterone in green, low progesterone in orange, menstruation in red). Hierarchical clustering divided the samples (sample ID in the X-axis) into two large clusters (A and B). Each cluster was then separated into subclusters (A1, A2 and B1, B2).

DISCUSSION

In animal models and in humans, innate and adaptive immune responses have been described to be different in females and in males suggesting a strong need for studying more female cohorts [31]. For instance, women infected with HIV-1 exhibit lower plasma viral load but have a 1.6 fold higher risk to faster progression to AIDS [32]. Furthermore, vaccine responses are also different according to sex, and women have higher antibody responses and more local and systemic reactions to bacterial and viral vaccines compared to males [33, 34]. The effect of hormones on immune cell recruitment, activation and on soluble factor concentrations in peripheral blood and in cervicovaginal lavages of the FRT at each menstrual phase in women, has been partially studied. However, there are technical disparities among some of the studies, such as absence of hormone measurement and non-longitudinal analysis.

In this study on female cynomolgus macaques, we investigated the variation of vaginal microbiota, cytokine expression and neutrophil subpopulations in the vagina and in the blood in a longitudinal study covering three complete menstrual cycles. We show that cervicovaginal environmental factors such as cytokine and chemokine concentrations, neutrophil populations and microbiota vary during the menstrual cycle. We highlight that menstruation induce significant alteration of the environment by increasing local inflammation associated with cytokine production and accumulation of mature/activated neutrophils that might originate from the blood.

In peripheral blood, at steady state, we show that cytokine concentrations are weakly expressed compared to their expression in cervicovaginal fluids. Analysis of culture supernatant of cells isolated from the human female reproductive tract and PBMC, describe similar differences in terms of cytokine and chemokine expression between the systemic and the local compartment [35]. Our findings show variabilities in terms of cytokine production in both compartments according to animal and time. Nevertheless, only cervicovaginal cytokine production is influenced by hormonal phases, characterized by a significant increase of cytokine and chemokine concentration during menstruation. In women, comparison of cytokine concentration

between menstrual and peripheral blood highlighted differences, similar to our results in macaques [36]. Several human cohort-based studies describe a variation of cytokine and chemokine expression according to hormonal cycle in cervicovaginal fluids, however no consistent results were observed and none of them collected samples during menstruation [37–39].

Together with the cytokine profiles, we have characterized the neutrophil subpopulations in the blood and in cervicovaginal cytobrushes in the same female cynomolgus macaques at the same time points. In cynomolgus macaques, J. Lemaître et al. described neutrophils in the blood as CD45⁺ CD66⁺ Lin^{CD3/CD20/CD8a/CDw125/CD14-} and CD11b/CD32a expression was used to determine the maturation stage [40]. In our study, we added, to the mentioned markers, CD10 and CD101 to confirm the maturation status of the neutrophils. In female cynomolgus macaques, neutrophil percentages among CD45⁺ cells are similar between the peripheral blood and the cervicovaginal cytobrushes. Throughout this study, peripheral blood neutrophils are mainly CD11b⁺ CD101⁺ CD32a⁺ CD62L⁺ with a part expressing CD10 suggesting a combination of immature and mature neutrophils in the blood. Despite the stability of this population throughout menstrual cycles, total count of blood neutrophils decreases during menstruation. In cervicovaginal cytobrushes, several neutrophil subpopulations are identified with three main populations: CD11b^{high} CD101⁺ CD32a⁺ CD10⁺ (population 8), CD11b^{high} CD101⁺ CD32a⁺ CD10⁻ (population 6) and CD11b^{low} CD101^{low} CD32a⁻ CD10⁻ (population 7). Based on human blood characterization of neutrophils, those populations can be assigned to mature/activated for population 8 and immature/activated for population 6. The population 7 has a phenotype of pre-neutrophils, a population usually found specifically in the bone marrow [41]. The morphology of neutrophils from cervicovaginal cytobrushes observed after May-Grünwald Giemsa staining are mainly poly-nuclear and no identification of pre-neutrophils with round shape nucleus is observed [Suppl figure 3]. Neutrophil main populations in cervicovaginal cytobrushes are modified according to hormonal phases. The mature/activated subset expressing CD62L increases during menstruation. This

could be due to an infiltration of mature/activated blood neutrophils within the FRT because CD62L expression on neutrophils decreases after transmigration into the tissue [28]. Moreover, increased concentration of CCL2, G-CSF, GM-CSF and IL-6 during menstruation was previously described to be associated with neutrophil recruitment locally upon infection [42–45] .

Neutrophil populations in human blood have been extensively described and characterized as CD15⁺ CD66b⁺ CD49d^{mid} CD101⁺ CD10⁺ CD16⁺ CD62L⁺ [41]. Moreover, they increased during the secretory phase (high progesterone concentration) as observed in cynomolgus macaques [46]. So far, neutrophils have been studied in the human endometrium and were shown to increase at early menstrual phase leading the authors to hypothesize that this increase is necessary for tissue repair after menstruation [47]. To our knowledge, we performed the first study analyzing the evolution of innate immune markers including cervicovaginal neutrophils and cytokine profiles together with vaginal microbiota composition according to menstrual cycle phases.

Since the vaginal microbiota has been described to be closely involved in local immune regulation [10], we longitudinally monitored the vaginal microbiota composition in parallel of the immune marker analysis. We show a predominance of four phyla: Firmicutes, Bacteroidota, Fusobacteriota and Actinobacteriota. The predominant genus are *Sneathia*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Streptococcus*, *Bifidobacterium*, *Peptoniphilus*, *Peptostreptococcus* and *Parvimonas* in accordance with our previously published study on the characterization of the vaginal and rectal microbiota of female cynomolgus macaques [20]. We highlight that the vaginal microbiota composition is subjected to hormonal changes. In our previously published study, we did not take into consideration the menstruation. Here, we show that vaginal microbiota diversity is increased during menstruation. In women, an increased bacterial diversity during menstruation was also observed translated into a trend for increased abundances of several bacterial taxa including *Finegoldia*, *Streptococcus* and *Peptostreptococcus* [2, 48]. None of these genus was observed to significantly increase

during menstruation in cynomolgus macaques. These differences could be due to the fact that most of samples in Song et al., belong to women with a *Lactobacillus spp.* dominant microbiota whereas macaques have a diverse vaginal microbiota associated to a dysbiosis in women.

Finally, relationships between immune factors and bacterial taxa that are differentially abundant according to hormonal phases were analyzed. The analysis resulted in a clear clustering of samples collected during menstruation. This clustering suggests an increased inflammation (*ie* increase in cytokine production and in mature/activated neutrophils) during menstruation that is not observed at other hormonal phases. On the contrary, high progesterone (secretory phase) and low progesterone (proliferative phase) samples exhibit mainly a lower cytokine expression (CCL2, CCL4, IL-6, sCD40L mainly) and higher number of less mature and activated neutrophil populations compared to menstruation. Few differences are noticed between samples collected during high and low progesterone. The analysis also show animal specific clusters for four females whereas other females have more variable distribution. This observation demonstrates strong variabilities between animals but also within each animal confirming an individual specific mucosal environment. Most probably for this reason, we were not able to associate a group of bacteria or a specific genus to a profile of cytokines and/or a neutrophil phenotype. Modifications of the vaginal microbiota composition by antibiotic treatment and/or probiotic inoculations could be helpful for this purpose. The determination of such associations would be useful to develop therapeutic approaches aiming to modify the vaginal microbiota to reduce local inflammation.

In terms of cytokine profiles and neutrophil subpopulations, both peripheral blood and cervicovaginal compartments are significantly different highlighting that the blood compartment does not recapitulate what happens in the mucosal compartment. It is thus necessary to investigate mucosal compartment and not only the systemic ones. Our study was performed at steady state but infection or vaccination could exacerbate the differences between compartments. Moreover, this work suggests that within the

FRT there is a fluctuation of immune markers and vaginal microbiota at basal state. Those fluctuations are mainly due to the hormonal cycle and have to be taken into account while studying STI infection or mucosal vaccine responses in the mucosa. Indeed, an increased in cytokine expression could be affiliated to a vaccine response or immune response against a pathogen while this increase might in fact be due to a physiological change, for example, during menstruation. The impact of hormones has been described on STI acquisition. Indeed, estradiol has a positive effect on the mucosal environment by increasing the thickness of the vagina and favoring the growth of *Lactobacillus* [2, 7]. On the contrary, progesterone reduces the thickness of the vaginal epithelium in rhesus macaque and increases SIV incidence [49]. These informations highlight that there is a need for in deep characterization of the hormonal impact within the FRT mucosae by an appropriate sampling schedule including several time points. Our data point out that longitudinal studies are essential for analyzing immune markers or vaginal microbiota composition in Human or animal models. The sampling has to be repeated to take into consideration potential hormonal cycle effects otherwise the results obtained might be biased.

Conclusions

This longitudinal study gives insight into how the menstrual cycle phases affect the vaginal microbiota composition as well as immune markers such as cytokine profiles and neutrophil subpopulations. It consequently highlights the difficulties to study the local environment in the FRT and the need for more women based cohort studies with an appropriated sampling schedule. This complex hormonal regulation need to be further addressed by including other factors involved in mucosal immunity such as IgA/IgG quantification and immune cells that have been described to vary in the human endometrium according to hormonal phases (T and B lymphocytes, NK and myeloid cells) [50]. A better understanding of how hormones regulate the microbiota and immune factors within the FRT is essential to better characterize their impact on STI acquisition or control and mucosal vaccine responses.

Declarations**Consent for publication**

Not applicable

Availability of data and material

The deposit of raw sequenced paired-end reads of this macaque microbiota dataset on the SRA database through BioProject accession are ongoing.

Other datasets generated or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Funding

The program was funded by the Infectious Disease Models and Innovative Therapies (IDMIT) research infrastructure supported by the “Programme Investissements d’Avenir”, managed by the ANR under reference ANR-11-INBS-0008 as well as Sidaction “financement jeune chercheur” under reference “2019-2-FJC-1234”. The Non-Human Primate study is part of the TracVac project which received financial support from the European Union’s Horizon 2020 research and innovation programme under the grant agreement No. 733373.

Authors’ contributions

Study conception and design: CA, LR, NN, RM, JL, RLG, MTN and EM. Acquisition of data: CA, LR, CC and MTN. Analysis and interpretation of data: CA, LR, NN, LDA, EG, EG, MTN and EM. Drafting of manuscript: CA and EM. Critical revisions: CA, LR, NN, RM, JL, RLG, MTN and EM.

Acknowledgments

This work has benefited from the facilities and expertise of @BRIDGE (GABI, INRA, AgroParisTech, Paris-Saclay University). The authors would like to thank all members of the ASW (Raphaël Ho Tsong Fang, Nina Dhooge) and L2I (Nathalie Bosquet, Laetitia Bossevot, Julie Morin) teams of the IDMIT infrastructure as well as the members of the Animalliance group (Thierry Prot, Marjorie Benfissa). The authors also would like to thank the service of bacteriology-virology of Lariboisière Hospital (Paris) including Dr Hervé Jacquier for their help in setting up Nugent scoring in the vaginal samples from the cynomolgus macaques.

Bibliography

1. Wira CR, Rodriguez-Garcia M, Patel MV. The role of sex hormones in immune protection of the female reproductive tract. *Nat Rev Immunol*. 2015;15:217–30.
2. Song SD, Acharya KD, Zhu JE, Deveney CM, Walther-Antonio MRS, Tetel MJ, et al. Daily Vaginal Microbiota Fluctuations Associated with Natural Hormonal Cycle, Contraceptives, Diet, and Exercise. *mSphere*. 2020;5. doi:10.1128/mSphere.00593-20.
3. Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UME, Zhong X, et al. Temporal dynamics of the human vaginal microbiota. *Sci Transl Med*. 2012;4:132ra52.
4. Boily-Larouche G, Lajoie J, Dufault B, Omollo K, Cheruiyot J, Njoki J, et al. Characterization of the Genital Mucosa Immune Profile to Distinguish Phases of the Menstrual Cycle: Implications for HIV Susceptibility. *J Infect Dis*. 2019;219:856–66. doi:10.1093/infdis/jiy585.
5. Mihm M, Gangooly S, Muttukrishna S. The normal menstrual cycle in women. *Animal Reproduction Science*. 2011;124:229–36. doi:10.1016/j.anireprosci.2010.08.030.
6. Dei M, Di Maggio F, Di Paolo G, Bruni V. Vulvovaginitis in childhood. *Best Practice & Research Clinical Obstetrics & Gynaecology*. 2010;24:129–37. doi:10.1016/j.bpobgyn.2009.09.010.
7. Mirmonsef P, Hotton AL, Gilbert D, Burgad D, Landay A, Weber KM, et al. Free glycogen in vaginal fluids is associated with Lactobacillus colonization and low vaginal pH. *PLoS ONE*. 2014;9:e102467.
8. Gosmann C, Anahtar MN, Handley SA, Farcasanu M, Abu-Ali G, Bowman BA, et al. Lactobacillus-Deficient Cervicovaginal Bacterial Communities are Associated with Increased HIV Acquisition in Young South African Women. *Immunity*. 2017;46:29–37. doi:10.1016/j.immuni.2016.12.013.
9. Tamarelle J, Thiébaud ACM, de Barbeyrac B, Bébéar C, Ravel J, Delarocque-Astagneau E. The vaginal microbiota and its association with human papillomavirus, Chlamydia trachomatis, Neisseria gonorrhoeae and Mycoplasma genitalium infections: a systematic review and meta-analysis. *Clinical Microbiology and Infection*. 2019;25:35–47. doi:10.1016/j.cmi.2018.04.019.
10. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, et al. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity*. 2015;42:965–76. doi:10.1016/j.immuni.2015.04.019.
11. Stevens JS, Criss AK. Pathogenesis of Neisseria gonorrhoeae in the female reproductive tract: neutrophilic host response, sustained infection, and clinical sequelae. *Curr Opin Hematol*. 2018;25:13–21.
12. Lijek RS, Helble JD, Olive AJ, Seiger KW, Starnbach MN. Pathology after Chlamydia trachomatis infection is driven by nonprotective immune cells that are distinct from protective populations. *Proc Natl Acad Sci U S A*. 2018;115:2216–21. doi:10.1073/pnas.1711356115.

13. Deshmukh HS, Liu Y, Menkiti OR, Mei J, Dai N, O'Leary CE, et al. The microbiota regulates neutrophil homeostasis and host resistance to *Escherichia coli* K1 sepsis in neonatal mice. *Nat Med*. 2014;20:524–30.
14. Zhang D, Frenette PS. Cross talk between neutrophils and the microbiota. *Blood*. 2019;133:2168–77.
15. Zhang D, Chen G, Manwani D, Mortha A, Xu C, Faith JJ, et al. Neutrophil ageing is regulated by the microbiome. *Nature*. 2015;525:528–32. doi:10.1038/nature15367.
16. Hensley-McBain T, Wu MC, Manuzak JA, Cheu RK, Gustin A, Driscoll CB, et al. Increased mucosal neutrophil survival is associated with altered microbiota in HIV infection. *PLoS Pathog*. 2019;15:e1007672.
17. Van Esch E, Cline JM, Buse E, Wood CE, de Rijk EPCT, Weinbauer GF. Summary Comparison of Female Reproductive System in Human and the Cynomolgus Monkey (*Macaca fascicularis*). *Toxicol Pathol*. 2008;36 7_suppl:171S-172S. doi:10.1177/0192623308327415.
18. Weinbauer GF, Niehoff M, Niehaus M, Srivastav S, Fuchs A, Van Esch E, et al. Physiology and Endocrinology of the Ovarian Cycle in Macaques. *Toxicol Pathol*. 2008;36:7S-23S.
19. Shaikh AA, Naqvi RH, Shaikh SA. Concentrations of oestradiol-17beta and progesterone in the peripheral plasma of the cynomolgus monkey (*Macaca fascicularis*) in relation to the length of the menstrual cycle and its component phases. *J Endocrinol*. 1978;79:1–7.
20. Nugeyre M-T, Tchitchek N, Adapen C, Cannou C, Contreras V, Benjelloun F, et al. Dynamics of Vaginal and Rectal Microbiota Over Several Menstrual Cycles in Female Cynomolgus Macaques. *Front Cell Infect Microbiol*. 2019;9. doi:10.3389/fcimb.2019.00188.
21. Virtanen P, Gommers R, Oliphant TE, Haberland M, Reddy T, Cournapeau D, et al. SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat Methods*. 2020;17:261–72. doi:10.1038/s41592-019-0686-2.
22. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol*. 1991;29:297–301. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC269757/>. Accessed 15 Apr 2020.
23. Nadkarni MA, F. Elizabeth Martin, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*. 2002;148:257–66. doi:10.1099/00221287-148-1-257.
24. Escudié F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, et al. FROGS: Find, Rapidly, OTUs with Galaxy Solution. *Bioinformatics*. 2018;34:1287–94. doi:10.1093/bioinformatics/btx791.
25. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: a versatile open source tool for metagenomics. *PeerJ*. 2016;4:e2584. doi:10.7717/peerj.2584.
26. Paulson JN, Stine OC, Bravo HC, Pop M. Differential abundance analysis for microbial marker-gene surveys. *Nat Methods*. 2013;10:1200–2. doi:10.1038/nmeth.2658.
27. Condliffe AM, Chilvers ER, Haslett C, Dransfield I. Priming differentially regulates neutrophil adhesion molecule expression/function. *Immunology*. 1996;89:105–11.
28. Ivetic A. A head-to-tail view of L-selectin and its impact on neutrophil behaviour. *Cell Tissue Res*. 2018;371:437–53.
29. Lee D, Schultz JB, Knauf PA, King MR. Mechanical Shedding of L-selectin from the Neutrophil Surface during Rolling on Sialyl Lewis x under Flow*. *Journal of Biological Chemistry*. 2007;282:4812–20. doi:10.1074/jbc.M609994200.
30. Patera AC, Drewry AM, Chang K, Beiter ER, Osborne D, Hotchkiss RS. Frontline Science: Defects in immune function in patients with sepsis are associated with PD-1 or PD-L1 expression and can be restored by antibodies targeting PD-1 or PD-L1. *J Leukoc Biol*. 2016;100:1239–54. doi:10.1189/jlb.4HI0616-255R.
31. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol*. 2016;16:626–38. doi:10.1038/nri.2016.90.
32. Farzadegan H, Hoover DR, Astemborski J, Lyles CM, Margolick JB, Markham RB, et al. Sex differences in HIV-1 viral load and progression to AIDS. *The Lancet*. 1998;352:1510–4. doi:10.1016/S0140-6736(98)02372-1.

33. Cook IF. Sexual dimorphism of humoral immunity with human vaccines. *Vaccine*. 2008;26:3551–5. doi:10.1016/j.vaccine.2008.04.054.
34. Engler RJM, Nelson MR, Klote MM, VanRaden MJ, Huang C-Y, Cox NJ, et al. Half- vs Full-Dose Trivalent Inactivated Influenza Vaccine (2004–2005): Age, Dose, and Sex Effects on Immune Responses. *Archives of Internal Medicine*. 2008;168:2405–14. doi:10.1001/archinternmed.2008.513.
35. Benjelloun F, Quillay H, Cannou C, Marlin R, Madec Y, Fernandez H, et al. Activation of Toll-Like Receptors Differentially Modulates Inflammation in the Human Reproductive Tract: Preliminary Findings. *Front Immunol*. 2020;11:1655. doi:10.3389/fimmu.2020.01655.
36. Guterstam YC, Strunz B, Ivarsson MA, Zimmer C, Melin A-S, Jonasson AF, et al. The cytokine profile of menstrual blood. *Acta Obstetrica et Gynecologica Scandinavica*. 2021;100:339–46. doi:10.1111/aogs.13990.
37. Al-Harathi L, Wright DJ, Anderson D, Cohen M, Matityahu D, Cohn J, et al. The Impact of the Ovulatory Cycle on Cytokine Production: Evaluation of Systemic, Cervicovaginal, and Salivary Compartments. *Journal of Interferon & Cytokine Research*. 2000;20:719–24. doi:10.1089/10799900050116426.
38. Kyongo JK, Jespers V, Goovaerts O, Michiels J, Menten J, Fichorova RN, et al. Searching for Lower Female Genital Tract Soluble and Cellular Biomarkers: Defining Levels and Predictors in a Cohort of Healthy Caucasian Women. *PLoS One*. 2012;7:e43951. doi:10.1371/journal.pone.0043951.
39. Francis SC, Hou Y, Baisley K, Wijgert J van de, Watson-Jones D, Ao TT, et al. Immune Activation in the Female Genital Tract: Expression Profiles of Soluble Proteins in Women at High Risk for HIV Infection. *PLOS ONE*. 2016;11:e0143109. doi:10.1371/journal.pone.0143109.
40. Lemaitre J, Cosma A, Desjardins D, Lambotte O, Le Grand R. Mass Cytometry Reveals the Immaturity of Circulating Neutrophils during SIV Infection. *J Innate Immun*. 2020;12:170–81.
41. Evrard M, Kwok IWH, Chong SZ, Teng KWW, Becht E, Chen J, et al. Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions. *Immunity*. 2018;48:364–379.e8. doi:10.1016/j.immuni.2018.02.002.
42. Symons LK, Miller JE, Tyryshkin K, Monsanto SP, Marks RM, Lingegowda H, et al. Neutrophil recruitment and function in endometriosis patients and a syngeneic murine model. *The FASEB Journal*. 2020;34:1558–75. doi:10.1096/fj.201902272R.
43. McLoughlin RM, Witowski J, Robson RL, Wilkinson TS, Hurst SM, Williams AS, et al. Interplay between IFN- γ and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation. *J Clin Invest*. 2003;112:598–607. doi:10.1172/JCI200317129.
44. Balamayooran G, Batra S, Balamayooran T, Cai S, Pacher P, Jeyaseelan S. Intrapulmonary G-CSF Rescues Neutrophil Recruitment to the Lung and Neutrophil Release to Blood in Gram-negative Bacterial Infection in MCP-1 $^{-/-}$ Mice. *J Immunol*. 2012;189:5849–59. doi:10.4049/jimmunol.1200585.
45. Laan M, Prause O, Miyamoto M, Sjöstrand M, Hytönen AM, Kaneko T, et al. A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF- α . *Eur Respir J*. 2003;21:387–93.
46. Nowak J, Borkowska B, Pawlowski B. Leukocyte changes across menstruation, ovulation, and mid-luteal phase and association with sex hormone variation. *American Journal of Human Biology*. 2016;28:721–8. doi:10.1002/ajhb.22856.
47. Armstrong GM, Maybin JA, Murray AA, Nicol M, Walker C, Saunders PTK, et al. Endometrial apoptosis and neutrophil infiltration during menstruation exhibits spatial and temporal dynamics that are recapitulated in a mouse model. *Sci Rep*. 2017;7:17416. doi:10.1038/s41598-017-17565-x.
48. Kaur H, Merchant M, Haque MM, Mande SS. Crosstalk Between Female Gonadal Hormones and Vaginal Microbiota Across Various Phases of Women's Gynecological Lifecycle. *Front Microbiol*. 2020;11. doi:10.3389/fmicb.2020.00551.
49. Marx PA, Spira AI, Gettie A, Dailey PJ, Veazey RS, Lackner AA, et al. Progesterone implants enhance SIV vaginal transmission and early virus load. *Nat Med*. 1996;2:1084–9. doi:10.1038/nm1096-1084.
50. Hickey DK, Patel MV, Fahey JV, Wira CR. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *J Reprod Immunol*. 2011;88:185–94.

Strategies tested in vivo for the *Lactobacillus* spp. enrichment of the cynomolgus macaque vaginal microbiota

I. Introduction and objectives

Human vaginal microbiota has been described to modulate local host inflammation. Indeed, women suffering from bacterial vaginosis (*i.e.* diverse vaginal microbiota rich in anaerobic bacteria) exhibited higher local inflammation, such as an increased expression of IL-1 α , IL-1 β , G-CSF, GM-CSF, TNF α , IFN γ , IL-8, IL12-p70, IL-4, IL-10 for instance [195, 248]. This higher inflammation has been associated to several anaerobic bacteria: *Fusobacterium*, *Aerococcus*, *Sneathia*, *Mobiluncus*, *Prevotella*, *Parvimonas*, etc [195, 246]. Those bacteria are also present in the natural vaginal microbiota of female cynomolgus macaques [317]. Indeed, we have shown that the vaginal microbiota of cynomolgus macaques displays similar composition and abundance than the one of women belonging to CST IV (*i.e.* diverse vaginal microbiota rich in anaerobic bacteria poor in *Lactobacillus* spp.) [317].

To better understand the impact of the vaginal microbiota composition on immune response, we wanted to compare two groups of animals: one group with a higher abundance of *Lactobacillus* spp., and the other one with an anaerobic dominant microbiota. The aims were to study, first the susceptibility of both groups to a single STI (CT infection) or co-infection (CT and SIV); Second, the impact of the vaginal microbiota composition on the innate and adaptive immune responses induced by the infection [Fig25]. We wanted to determine if the vaginal microbiota composition will have an impact on the susceptibility to infection and if the inflammation induced by the infection will be modified according to vaginal microbiota composition.

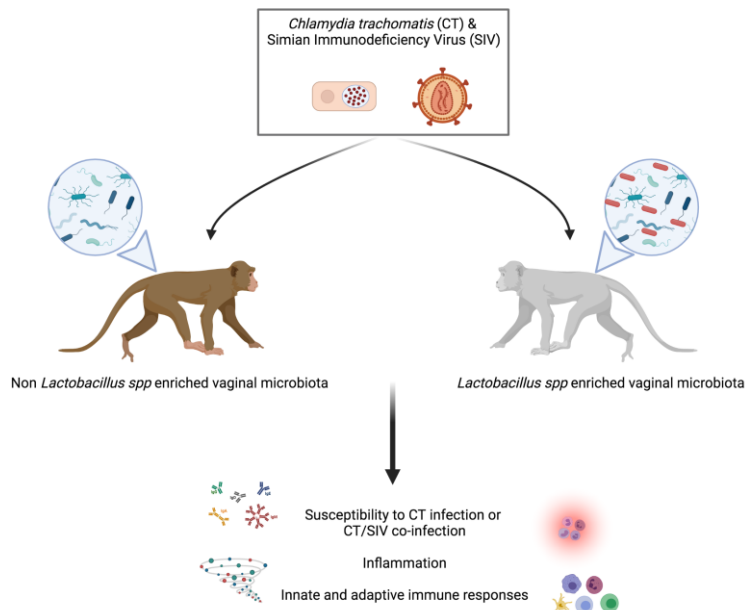


Figure 25: Futur aims.

Female cynomolgus macaques with an enriched *Lactobacillus spp.* vaginal microbiota or not will be infected with *Chlamydia trachomatis* (CT) or co-infected with CT and Simian Immunodeficiency virus (SIV). Then, different parameters will be studied: the susceptibility to infection/co-infection, antibodies production, local and systemic inflammation including the analysis of local and systemic innate and adaptive immune responses. Created with BioRender.com.

Several research teams have developed macaque's model vaginally enriched with *Lactobacillus*. Chinese rhesus macaques vaginally inoculated with a human vaginal isolate of *L. jensenii* during seven consecutive days showed a colonization for up to 5 days after the last inoculation [319]. Moreover, Lagenaur et al. have observed *Lactobacillus spp.* colonization of two rhesus macaques after five successive days of inoculation of a modified strain of *L. jensenii* expressing HIV-1 entry inhibitor cyanovirin-N. This colonization was sustained for three to six weeks. One animal did not sustain the colonization after week 3 due to the apparition of menstruation [320]. Finally, a comparative study between rhesus and cynomolgus macaque was made to evaluate the ability for vaginal *Lactobacillus spp.* colonization in both species. These ones were observed to be able to be colonized by *Lactobacillus spp.* after antibiotic pretreatment as well as lactic acid flushing of the vaginal cavity prior to inoculation of

10^{10} *L. rhamnosus*. Rhesus macaque seems to sustain the colonization longer compared to cynomolgus (7 weeks vs 3 weeks). However, a strong variability between animals was observed in rhesus macaques [321]. Another study, aiming at promoting the growth of natural *Lactobacillus spp.* in rhesus macaques was performed by inoculating a sucrose gel intravaginally. The authors observed a significant increase of *Lactobacillus spp.* after 5 days of inoculations [322].

II. Results

a. Single inoculation of *L. crispatus* capsule

L. crispatus has been described to induce an anti-inflammatory environment and a protection against STI acquisition mainly by its ability to produce D and L-lactic acid [195, 207, 255]. Only one probiotic, composed of only *L. crispatus* (10^9) and not a combination of different species, is available for sale: Physioflor LP[®] developed by the IPRAD laboratory. This capsule is mucoadhesive and at extended release during 4 days. Three female cynomolgus macaques were included in this study and Physioflor LP[®] was used to enrich the vaginal microbiota of those females.

At baseline, all the females had a Nugent score of 8, thus a highly diverse microbiota with no *Lactobacillus spp* [Fig 26A]. We did not observe *Mobiluncus* on the gram staining, so the Nugent score did not reach 10. Vaginal pH was quite stable around 7 (Mean: 6.68; SD: 0.24) for all individuals at baseline. Out of three females, only one female (CCB119) exhibited a decrease Nugent score (between 3 and 0) and pH (between 6 and 6.5) after one capsule, with a large amount of *Lactobacillus* and a decrease in anaerobic bacteria. However, the presence of *Lactobacillus* gradually decreased after day 7, associated with an increased in anaerobic bacteria and vaginal pH [Fig 26B]. The loss of *Lactobacillus* is more drastic during menstruation. Indeed, the menstruation interferes with the enrichment of *Lactobacillus* within the vagina. The two other females exhibited neither an increase of *Lactobacillus* nor a decrease of the pH after one treatment a week during 4 weeks. Given those results, antibiotic treatment

before inoculation of the *Lactobacillus crispatus* capsule was decided to increase the reproductibility.

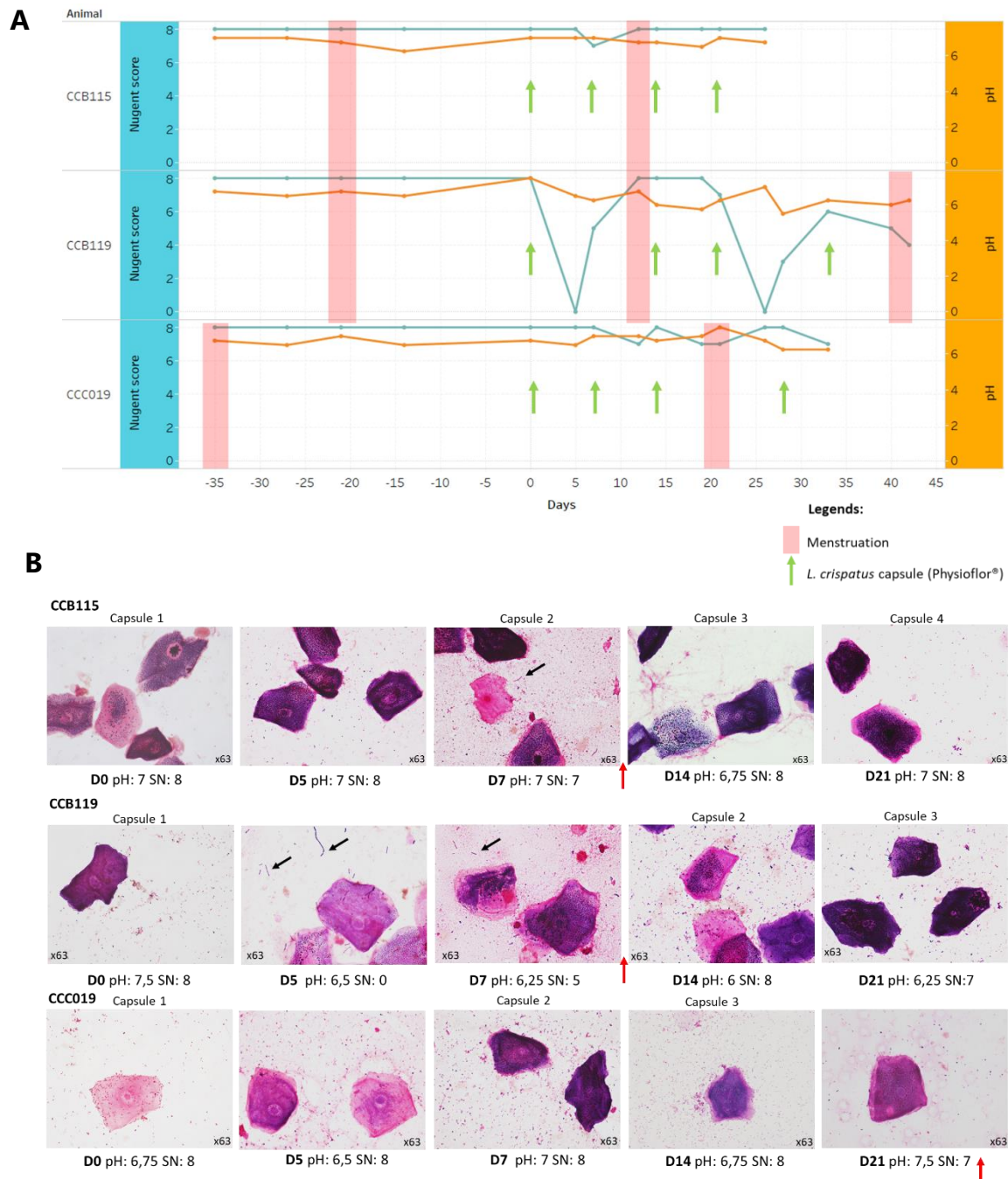


Figure 26: Nugent score and vaginal pH of three female cynomolgus macaques.

(A) Nugent score is represented in blue and pH in orange. Green arrows represent intravaginal inoculation of *L. crispatus* capsule (Physioflor® LP). Menstruation are shown by a red zone. (B) Gram staining of vaginal fluids from the first capsule of *L. crispatus* inoculation (D0; Physioflor® LP) to D21 in the three female cynomolgus macaques. Black arrows represent *Lactobacillus spp.* and red arrow the menstruation.

b. Metronidazole pretreatment followed by *L. crispatus* caspule inoculation

Metronidazole is an antibiotic, often prescribed for women suffering from bacterial vaginosis (BV). BV+ women that use metronidazole gel for 5 days displayed a decrease in BV associated bacteria: *BVAB1/2/3*, *Megashera*, *Atopium*, *Sneathia* and *G. vaginalis* [200]. Furthermore, monthly oral treatment with metronidazole, in association with fluconazole, induces an increased abundance of *Lactobacillus* spp. [201]. We decided to use this antibiotic locally to remove anaerobic bacteria, since it was described to be efficient against a wide range of anaerobic bacteria that are highly abundant in the vaginal microbiota of cynomolgus macaques. Intravaginal treatment with a metronidazole suppository (Flagyl®; SANOFI aventis) was performed in five female cynomolgus macaques, two days before each inoculation of the capsule of *L. crispatus* (Physioflor® LP; IPRAD).

Interestingly, after antibiotic treatment, we observed on gram staining of vaginal fluids that a female had *Lactobacillus* spp. [Fig27]. This observation was confirmed by spreading the vaginal fluids on a MRS agar, which allows the growth of *Lactobacillus*. Based on these results, we decided to culture the vaginal fluids collected before antibiotic treatment on MRS agar to confirm the presence of natural *Lactobacillus* spp.. Isolated colonies were observed by gram staining to confirm bacterial morphologies (gram + bacilli shape bacteria), then each isolated colony was amplified and frizzed [Fig28]. The regions V3 and V4 of 16S rRNA of the isolated colonies were sequenced to confirm their affiliations to the *Lactobacillus* genus, and Basic Local Alignment Search Tool (BLAST) analysis gave us the species name [Table 7]. Cynomolgus macaques have very few *Lactobacillus* at baseline but metronidazole treatment, by reducing the number of anaerobic bacteria, facilitates the growth of natural *Lactobacillus* strains: *L. crispatus*, *L. casei*, *L. animalis*, *L. murinus* and *L. johnsonii*.

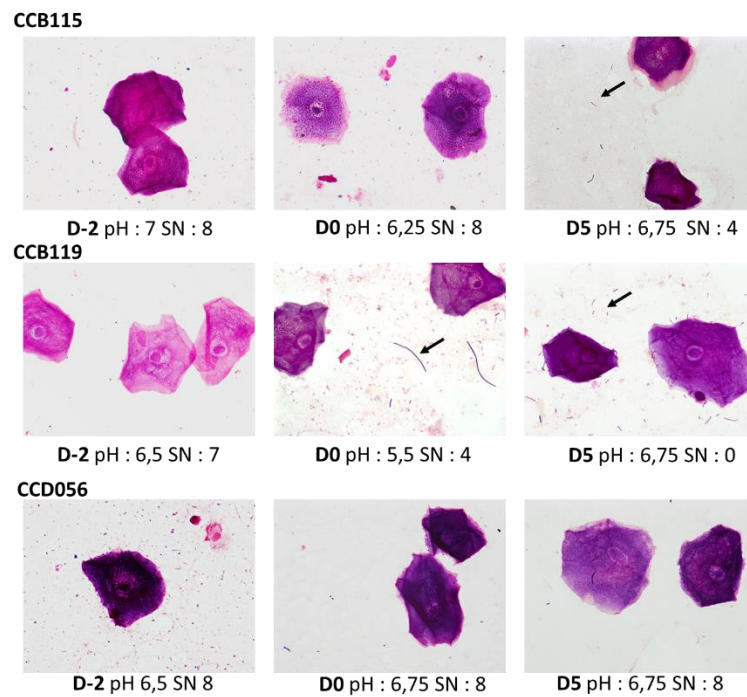


Figure 27: Nugent scores and vaginal pH of cynomolgus macaques.

Gram staining of vaginal fluids at D-2 (baseline), D0 (2 days after metronidazole treatment) and D5 (5 days after *L. crispatus* inoculation) in the three female cynomolgus macaques. Black arrow represents *Lactobacillus spp.*

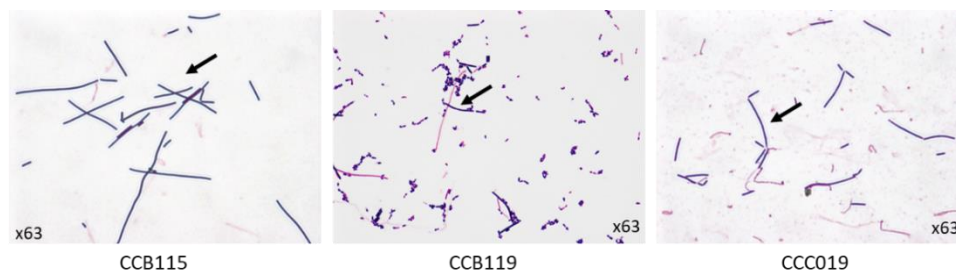


Figure 28: Isolated colonies from the vaginal microbiota of three cynomolgus macaques.

Black arrow show *Lactobacillus spp.*

Table 7: *Lactobacillus* spp. species isolated in the vaginal microbiota of five cynomolgus macaques.

Animals	Colony 1	Colony 2
CCB115	<i>L. crispatus</i> strain CO3MRS11 or <i>L. casei</i> strain CAU6147	/
CCB119	<i>L. animalis</i> strain FR12	/
CCC019	<i>L. animalis</i> strain FR12	/
CCD056	<i>L. murinus</i> strain LbP2	<i>L. johnsonii</i> strain Egg-4
CDC034	<i>L. animalis</i> FR12	<i>L. johnsonii</i> strain KLDS

Metronidazole treatment allows anaerobic bacteria number to decrease in the vaginal microbiota, as observed on the gram staining [Fig27]. Three females (CCB119, CCB115, CCC019) out of five exhibit a decrease in their Nugent score (between 0 and 3) for around 20 days after one antibiotic treatment and one inoculation of capsule. Indeed, the enrichment of *Lactobacillus* was observed until the menstruation [Fig29]. However, very few impact of the pH was observed. This observation suggests a low production of lactic acid, which can be due to an insufficient availability of glycogen breakdown, essential for lactic acid production and *Lactobacillus* growth. It can be also due to an insufficient presence of *Lactobacillus*. Menstruations removed *Lactobacillus* presence by disturbing the local environment, as observed already in the first phase. In order to sustain the enrichment with *Lactobacillus*, we decided to treat the three females, where we observed an enrichment of *Lactobacillus*, with high dose of Depo-provera®. Depo-provera® is a contraceptive composed of medroxyprogesterone acetate (MPA) which, at high dose, will affect the menstrual cycle by maintaining high level of progesterone, thus suppressing the menstruation. One month after the injection of Depo-provera by intramuscular route, the animals received the same protocol as before (antibiotic treatment two days before the capsule of *Lactobacillus*). Surprisingly, only one female exhibits a partial enrichment with *L. crispatus* during 10 days, but we could not see this enrichment after a second round of treatment [Fig30]. Depo-provera treatment has been described to reduce the thickness of the epithelium [177, 323]. Indeed, Depo-

provera is known to reduce the number of glycogen bearing cells in the epithelium, which in return reduce the growth of *Lactobacillus* [324].

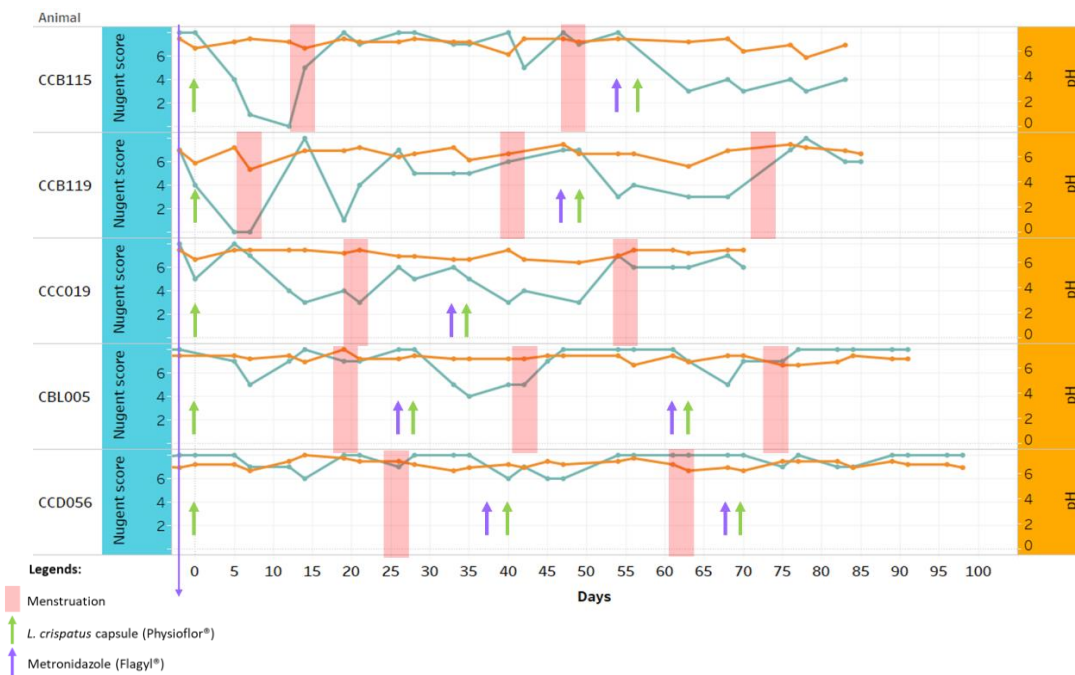


Figure 29: Nugent scores and vaginal pH of cynomolgus macaques after metronidazole treatment.

Nugent score is represented in blue and pH in orange. Green arrows represent intravaginal inoculation of *L. crispatus* capsule (Physioflor® LP) and purple arrows the intravaginal metronidazole treatment. Menstruations are shown by a red zone.

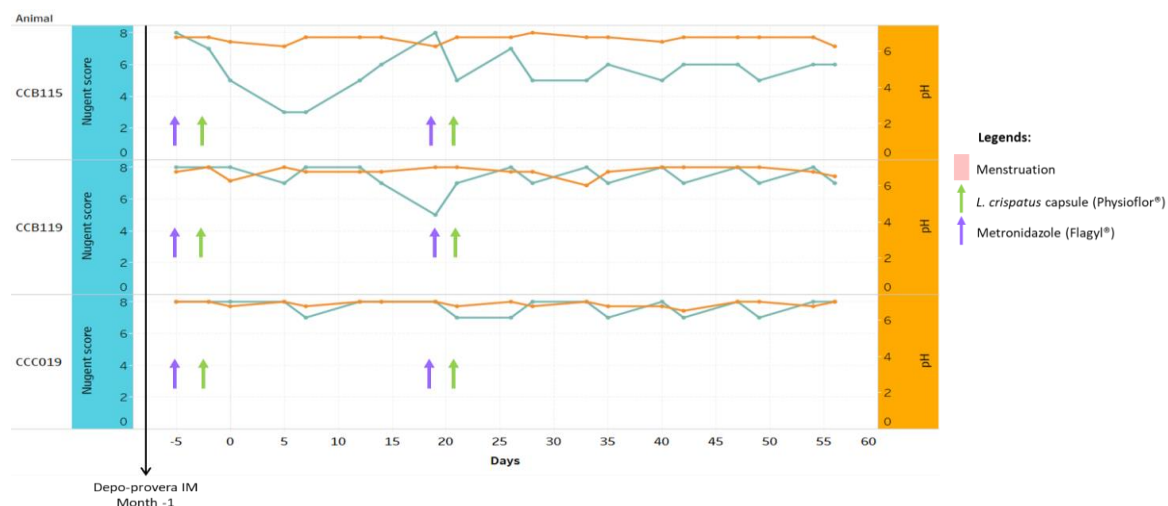


Figure 30: Nugent scores and vaginal pH in three cynomolgus macaques after Depo-provera treatment.

Depo-provera was injected by intramuscular route one month before the first metronidazole inoculation. Nugent score is in blue and pH value in orange. Green arrows represent intravaginal inoculation of *L. crispatus* capsule (Physioflor® LP) and purple arrows the intravaginal metronidazole treatment.

c. Sucrose intravaginal treatment

We have shown that menstruation decreases the enrichment of *Lactobacillus*. Depo-provera treatment allows the absence of menstruation and, in theory, facilitates the *Lactobacillus* enrichment. As observed in the previous phase, Depo-provera treatment abrogates the ability of *L. crispatus* to colonize the vagina of cynomolgus macaques. Since the effect might be linked to a decreased availability of glycogen breakdown, which is essential for *Lactobacillus* growth, we decided to treat the female with sucrose as reported by Hu et al. They have shown that repeated vaginal inoculation of a 9% sucrose gel (each day during 5 days) in rhesus macaques induces an increase of *Lactobacillus* sequences, between 50.84% to 96.98% in the vaginal microbiota of those macaques [322]. Two groups of three females were studied, one group was treated with Depo-provera, and the other not. Each female received an intravaginal inoculation of a 9% sucrose gel, twice a week during 3 weeks. Unfortunately, none of the females exhibited an increase in *Lactobacillus* after all the inoculations [Fig31].

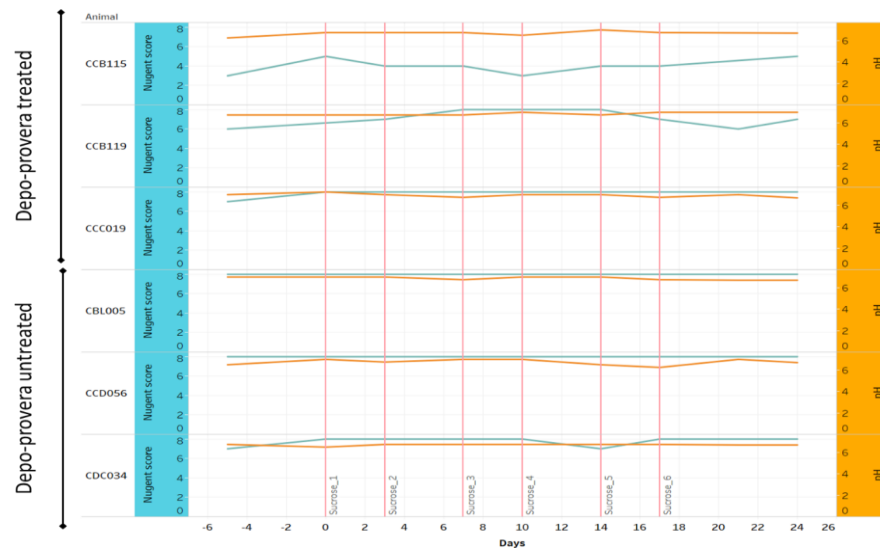


Figure 31: Nugent scores and vaginal pH of cynomolgus macaques after sucrose treatment.

CCB115, CCB119, CCC019 received Depo-provera treatment one month before the first intravaginal inoculation of sucrose, whereas CBL005, CCD056, CDC034 did not received Depo-provera. Nugent score is in blue and pH value in orange. Pink lines indicate sucrose treatments.

d. Repeated metronidazole treatments before *L. crispatus* inoculations

Based on all the results we obtained, we set up a different experimental schedule based on repeated metronidazole treatments and *Lactobacillus* inoculations without Depo-provera treatment. Five females were treated locally with metronidazole three times during week 1. Animals were then inoculated twice a week during 3 weeks with *L. crispatus* capsule. Then, animals were inoculated once a week during 3 weeks with *L. crispatus* capsule, in order to see if we can sustain the enrichment with only one treatment a week. Before each *L. crispatus* capsule inoculations, the vaginal cavity was washed with 2mL of 0.02% of lactic acid in order to decrease the pH of the vagina and improve the implantation of *Lactobacillus*.

Three females out of five had an increase in *Lactobacillus* spp. in their vaginal microbiota, with a Nugent score below 3 during the first three weeks. Moreover, pH values were lower, between 5 to 6 [Fig32]. However, switching to one treatment a week induces the loss of the enrichment of *Lactobacillus* in two females out of the three that were previously enriched. Indeed, only one female was able to have a sustained enrichment of *Lactobacillus* (CCB119) during all the protocol. CDC034 displays a decrease in Nugent score after antibiotic and *L. crispatus* inoculation. This decrease was not due to an increased presence of *Lactobacillus* spp. but to a lower abundance of anaerobic bacteria. Indeed, the treatment was able to decrease drastically the anaerobes, but no increase enrichment of *Lactobacillus* spp. was observed. V3 and V4 regions of 16S rRNA were sequenced at baseline, after antibiotic treatment, after the first three weeks and at the end of the protocol on cervicovaginal swabs [Fig33]. Significant alteration of the vaginal microbiota was observed after metronidazole treatment translated into a lower vaginal microbiota diversity in all animals. We observed an increase of *Firmicutes* and decrease of *Fusobacteria* and *Bacteroidetes* after metronidazole treatments. Three females (CBL015, CDC034 and CCD056) also displayed an increase in *Actinobacteria* after metronidazole treatments [Fig33A]. The

Firmicutes increase is mainly due to the presence of *Lactobacillus spp.* in 4 females out of 5, only CCD056 does not have *Lactobacillus spp.* after metronidazole treatments but an increase of *Streptococcus spp.* instead. The high abundance of *Lactobacillus spp.* observed in CDC034 after antibiotic treatments is not sustained with the *L. crispatus* inoculations, but instead a more diverse microbiota is observed. However, the abundance of *Lactobacillus spp.* in the other three animals (CCB115, CCB119, CBL015) is sustained during the first three weeks, and lost after switching to one *L. crispatus* inoculation a week. CCB119 had its menstruation at the end of the protocol, when we sampled cervicovaginal swab for sequencing analysis, thus we were not able to confirm the result observed on gram staining, suggesting a high abundance of *Lactobacillus spp.* even after switching to one *L. crispatus* inoculation a week. CCB115 and CBL015 exhibit, at the end of the protocol, an increase of *Peptoniphilus*, *Streptococcus* and *Escherichia* mainly. CDC034 and CCD056 did not display an increased enrichment of *Lactobacillus spp.* in their vaginal microbiota, neither on the Nugent score and sequencing. Interestingly, *G. vaginalis* abundance was higher in CCD056 and CDC034 compared to the other females [Fig33B]. To study the local inflammation, vaginal fluids were collected each week to monitor cytokine and chemokine expression. Moreover, the accumulation of neutrophils in the cervix were monitored at the end of the protocol by collecting cervicovaginal cytobrushes. At basal state, several cytokines are expressed (G-CSF, IL-1 β , IL1-RA, IL-8, IL-18, CCL2, VEGF etc) in the vagina. There is high expression of IL1-RA, IL-8, G-CSF and CCL2 at baseline. Differences in terms of expression are observed between females. After antibiotic treatment, there is a decrease in pro-inflammatory cytokines, such as G-CSF, IL-8, IL-6, IL-18, CCL2 but also IL1-RA [Fig34A]. After 2 and 4 capsules of *L. crispatus*, there is in the females enriched with *Lactobacillus*, compared to the other females, a lower expression of IL-18, TGF α , TNF α , VEGF and CCL2. CDC034, despite of a highly diverse microbiota, does not harbor a high inflammatory environment compared to CCD056. CDC034 has the same profile of cytokine/chemokine expression as CCB119, CCB115 and CBL015, except an increased expression of TGF α , TNF α and VEGF after the 6th capsules of *L. crispatus* (as soon as we

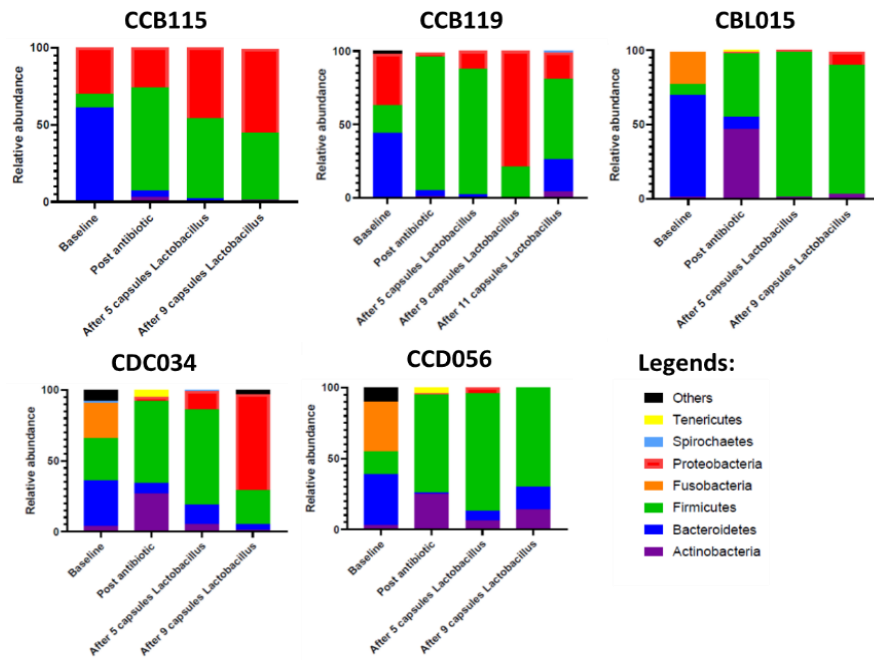
switched to one *L. crispatus* capsule a week). CCD056 exhibits a strong local inflammation compared to all the females, which might be due to the increase of *Streptococcus* in the vagina [Fig33 and 34]. We can observe that at the menstruation, there is a strong increase of cytokine and chemokine expression (G-CSF, IL-1 β , IL-6, CCL2 etc) in all animals. It seems that the effect of menstruation, in addition to switching to one treatment a week, induces an increased inflammation in all the females. Those modifications might be linked to the increased presence of anaerobic bacteria after switching to one *L. crispatus* inoculation a week. Cervicovaginal cytobrushes were collected at the end of the protocol to observe neutrophil presence by flux cytometry [Fig35A]. Cheu et al., at a keystone meeting in 2018, have shown that BV+ women have an accumulation of neutrophils within the FRT compared to BV-negative women. Moreover, they demonstrated that the neutrophils in the FRT of BV+ women are more activated and have an increased survival [R. Cheu et al., Keystone 2018]. We hypothesis that female cynomolgus macaques with a lower bacterial diversity in the vagina will have less neutrophils compared to macaques with highly diverse microbiota. Flow cytometry analysis of cells collected in the cervix at the end of the protocol, showed a strong presence of neutrophils in all animals, where between 54% to 90% of CD45⁺ cells were neutrophils. However, the female CCB119, which was the only female enriched in *Lactobacillus spp.* at the end of the protocol, exhibits the lowest percentage of neutrophils (54% of neutrophils among the CD45⁺ cells) [Fig35B]. Since this female has her menstruation at the end of the protocol, the cytobrushes were obtained the week after menstruation. The low percentage of neutrophils observed in this female could be due to a lower bacterial diversity associated with an increased *Lactobacillus* presence confirmed by Gram staining. No differences between females were observed in terms of neutrophils activation (expression of CD11b, HLA-DR, CD32a...). To have a control of the cervico-vaginal accumulation of neutrophils in untreated females, 13 female cynomolgus macaques, included in a different study, were sampled. Variable percentages of neutrophils were observed in the females: between 2.4% to 94% of neutrophils among the CD45⁺ cells [Fig35C]. Out of the 13

females, three females had a very low percentage (between 2.4% to 17%) of neutrophils. Interestingly, two of those females had a vaginal microbiota lacking in anaerobic bacteria and the third one had a vaginal microbiota dominated by gram-negative bacteria (Gram staining observation), which is not observed in females exhibiting a high percentage of neutrophils. Altogether these data indicate that, a highly diverse vaginal microbiota with anaerobes, such as *Streptococcus spp.*, displays an increased inflammation (neutrophil accumulation, cytokine/chemokine expression) in the FRT compared to a less diverse microbiota, enriched or not by *Lactobacillus spp.*



Figure 32: Nugent scores and vaginal pH of cynomolgus macaques.

(A) Nugent score is represented in blue and pH in orange. Green arrows represent intravaginal inoculation of *L. crispatus* capsule (Physioflor® LP) and purple arrows the intravaginal metronidazole treatment. Menstruations are indicated by a red zone.

A**B**

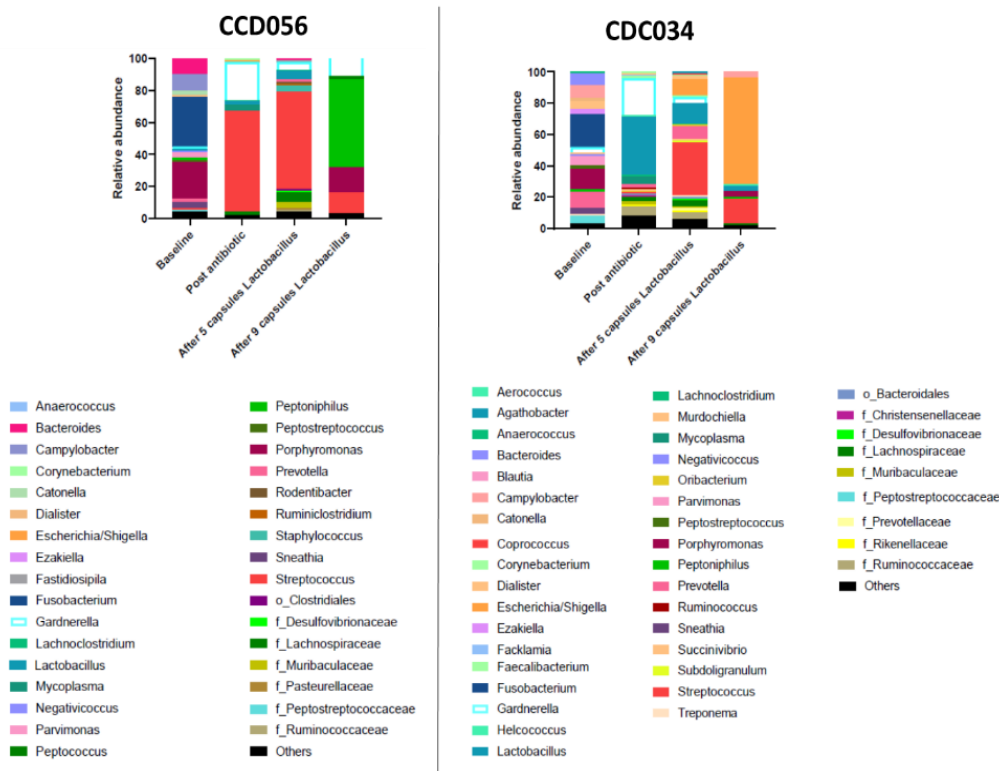
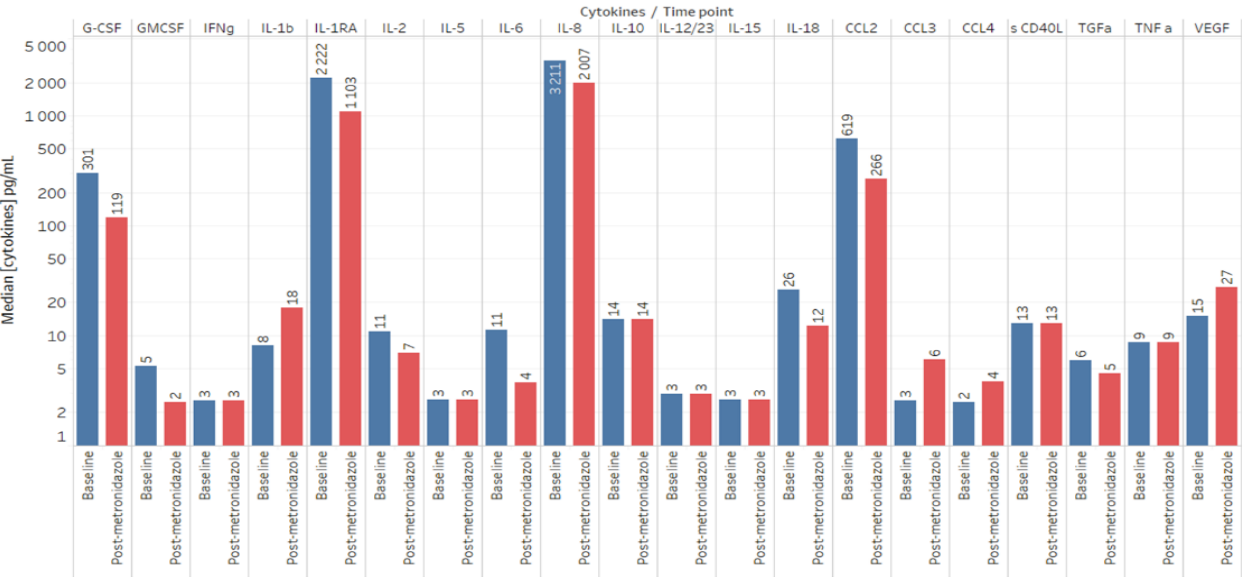


Figure 33: Vaginal microbiota composition at baseline, after metronidazole treatment and *L. crispatus* inoculations in five cynomolgus macaques.

(A) Vaginal microbiota composition at the phylum level or at the genus level (B) after two *L. crispatus* inoculations a week during two weeks (After 5 capsules) and after switching to one inoculation of *L.crispatus* a week (After 9 capsules).

A



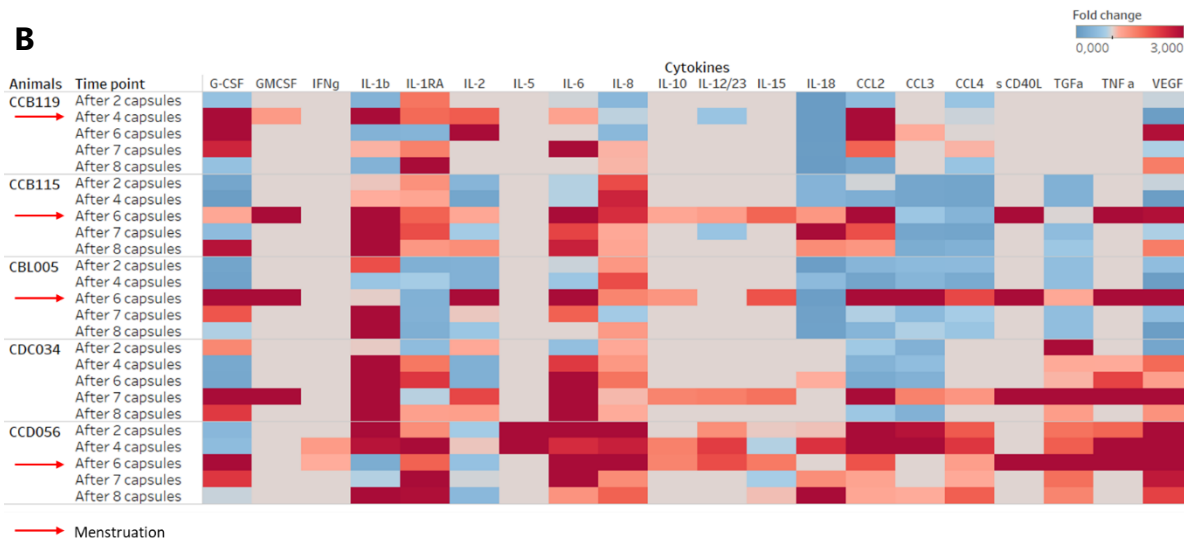


Figure 34: Cytokine and chemokine concentrations in cervicovaginal fluids before and after metronidazole and *L. crispatus* inoculations.

(A) Median cytokine and chemokine concentration (pg/mL) at baseline and after metronidazole treatment. Baseline are in blue and after antibiotic treatment in red. (B) Heatmap representing the fold change expression of cytokines/chemokines after *L. crispatus* capsule inoculations compared to the value after metronidazole treatment. Red represents increased expression and blue decreased expression.

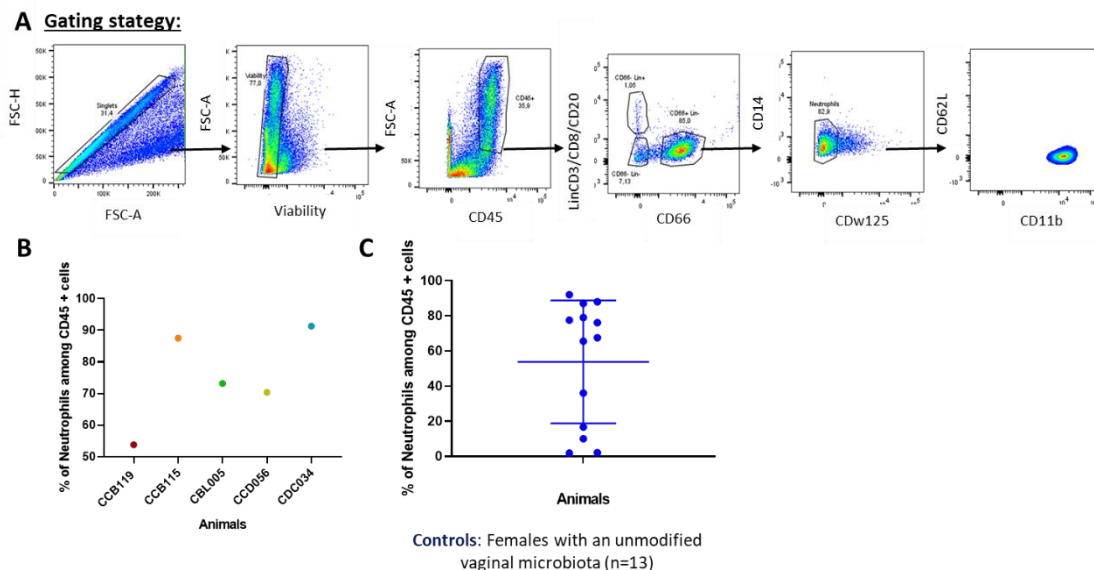


Figure 35: Neutrophil accumulation within the vagina of cynomolgus macaques.

(A) Gating strategy used on cells collected from cervicovaginal cytobrushes to determine neutrophil accumulation (B) Percentage of neutrophils among CD45⁺ cells collected by cervicovaginal cytobrushes in the five female cynomolgus macaques at the end of the protocol (three metronidazole treatments and nine *L. crispatus* inoculations). (C) Percentage of neutrophils among CD45⁺ cells collected by cervicovaginal cytobrushes in 13 healthy female cynomolgus macaques.

e. Repeated treatments with azithromycin and *L. crispatus* gel

To facilitate *L. crispatus* enrichment in the vaginal microbiota of female cynomolgus macaques, two changes were made. First of all, to induce a more drastic elimination of vaginal microbiota, we decided to replace metronidazole with azithromycin. Azithromycin is an antibiotic with a broad spectrum against bacteria, including *Streptococcus spp.* and *Chlamydia trachomatis*. Secondly, we wanted to increase *L. crispatus* inoculation from 10^9 to at least 10^{10} . For that, *L. crispatus* capsule (Physioflor® LP) was cultured and an isolated colony of *L. crispatus* was amplified and diluted in a gel composed of 9% sucrose, 0.02% of lactic acid and 1.5% of hydroxyethylcellulose (HEC). Five female cynomolgus macaques received three times during one week azithromycin (30mg/kg) through oral route. Then, twice a week during 3 weeks followed by once a week during three weeks, a gel of 10^{10} *L. crispatus* by vaginal route. Before each inoculation of the gel, the vaginal cavity was washed with 2mL of 0.02% of lactic acid. After antibiotic treatment and two inoculations of *L. crispatus* a week, there was a decrease of Nugent score in all the females [Fig36]. CCB119 had a stable Nugent score below 4 and a low pH around 5. CCB115 had a Nugent score between 4 to 6, which increases as soon as we switch to one *L. crispatus* gel inoculation a week. CBL005 had a variable Nugent score, between 3 to 6, but as observed for CCB115, the Nugent score increased as soon as we switched to one *L. crispatus* gel inoculation a week. As for CCD056 and CCD034, both females had a decrease in Nugent score, between 4 to 7, but this decrease was not due to *Lactobacillus spp.* enrichment but to decreased abundances of the anaerobe population within the vaginal microbiota. If we compare the results obtained during this protocol to the one using metronidazole instead of azithromycin, very few differences are observed. In a cohort based study, azithromycin treatment was observed to be highly effective at eradicating *M. genitalium* and *C. trachomatis*, but it was not sufficient to induce a shift in CST classification. In contrary, metronidazole treatment has a significant impact on the vaginal microbiota diversity

[218]. Moreover, azithromycin treatment for CT infection in women has been shown to induce a shift in their vaginal microbiota by inducing the selection of antibiotic resistant strains of *G. vaginalis* and *L. iners* [259]. Those strains were associated to increase risk of CT reinfection [258, 260]. Those data suggest a controversial impact of azithromycin uses for microbiota modification.

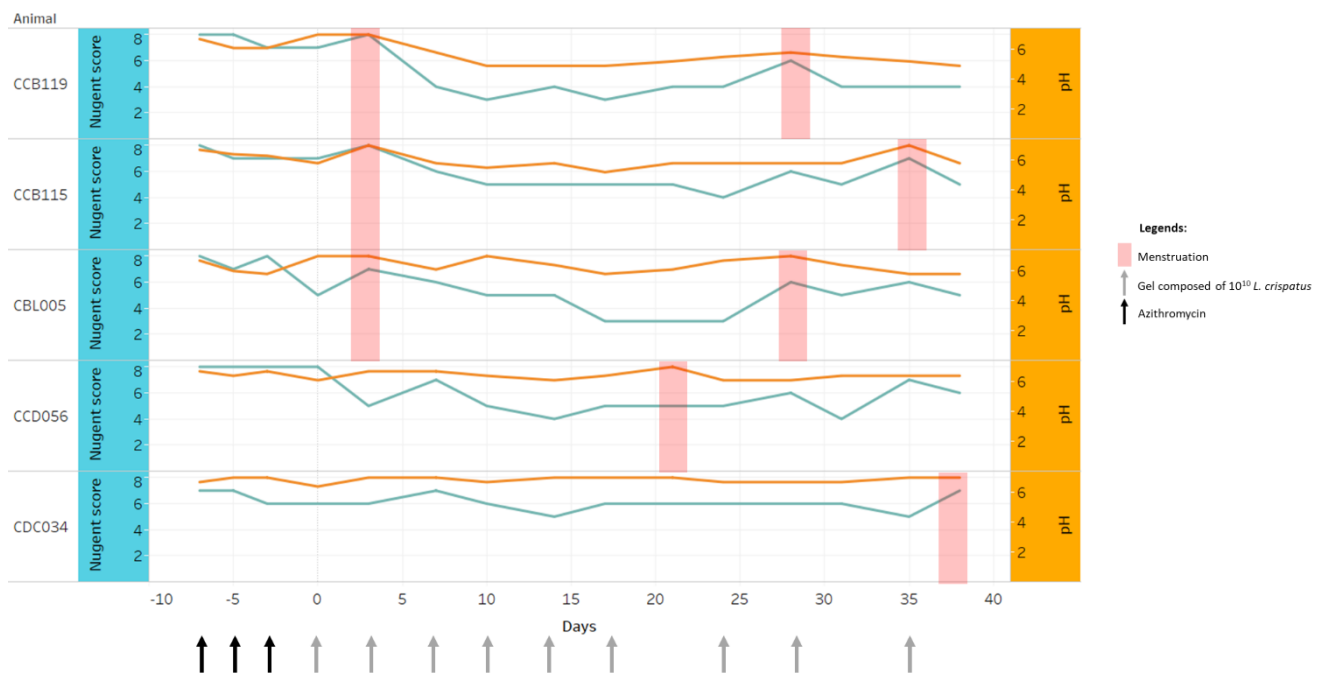


Figure 36: Nugent score and vaginal pH value after azithromycin treatment and *L. crispatus* gel inoculations.

Nugent score is represented in blue and pH in orange. Grey arrows represent intravaginal inoculation of *L. crispatus* gel and black arrows azithromycin treatment. Menstruations are shown by a red zone.

III. Data summary

Altogether, we have tried different methodologies to facilitate the colonisation of *L. crispatus* within the vaginal microbiota of cynomolgus macaques [Fig37]. The enrichment of macaque vaginal microbiota with a human strain (*L. crispatus*) was tricky due to several issues:

- Menstruation induces important environmental changes including inflammation that do not permit a sustained presence of *L. crispatus*.
- Elimination of menstruation by sustaining progesterone level (Depo-provera treatment) reduces *L. crispatus* enrichment. This phenomenon was not reversed by increasing small sugar availability described to be needed for *Lactobacillus spp* growth.

Antibiotic treatment by metronidazole induces significant microbiota modification (decrease bacterial diversity) and allow a better *L. crispatus* enrichment. However, inter-individual differences remain even after the use of a more drastic antibiotic treatment (Azithromycin). To sustain *L. crispatus* enrichment and decrease inter-individual variabilities repeated inoculations of antibiotic and *L. crispatus* are necessary. In most of the *L. crispatus* enrichment strategies that we performed, Nugent scoring and pH was used to determine the presence or absence of *Lactobacillus spp*. However these technics are not accurate enough to determine the abundance and the *Lactobacillus* species for example. To enrich the vaginal microbiota of female cynomolgus macaques with *Lactobacillus*, it was decided for further experiments to use local metronidazole treatments followed by repeated inoculations of *L. crispatus* gel (1010) after lactic acid washing of the vaginal cavity. This protocol was chosen because it was the most successful to enrich in *Lactobacillus* according to our analysis. It was such used to study the impact of the microbiota on CT infection (next chapter/manuscript).

However for these experiments we have sequenced the microbiota to quantify the abundance of *Lactobacillus spp* throughout the study which allowed to determine any variabilities according to animal. In addition, full 16S rRNA sequencing was performed

to allow the distinction between the Human strain that was inoculated and natural strains that may increase upon antibiotic treatment. Thanks to the sequencing, we were such able to precisely determine the presence of *Lactobacillus* and detect any animals that would not have been enriched with the inoculated strain of *L. crispatus*.

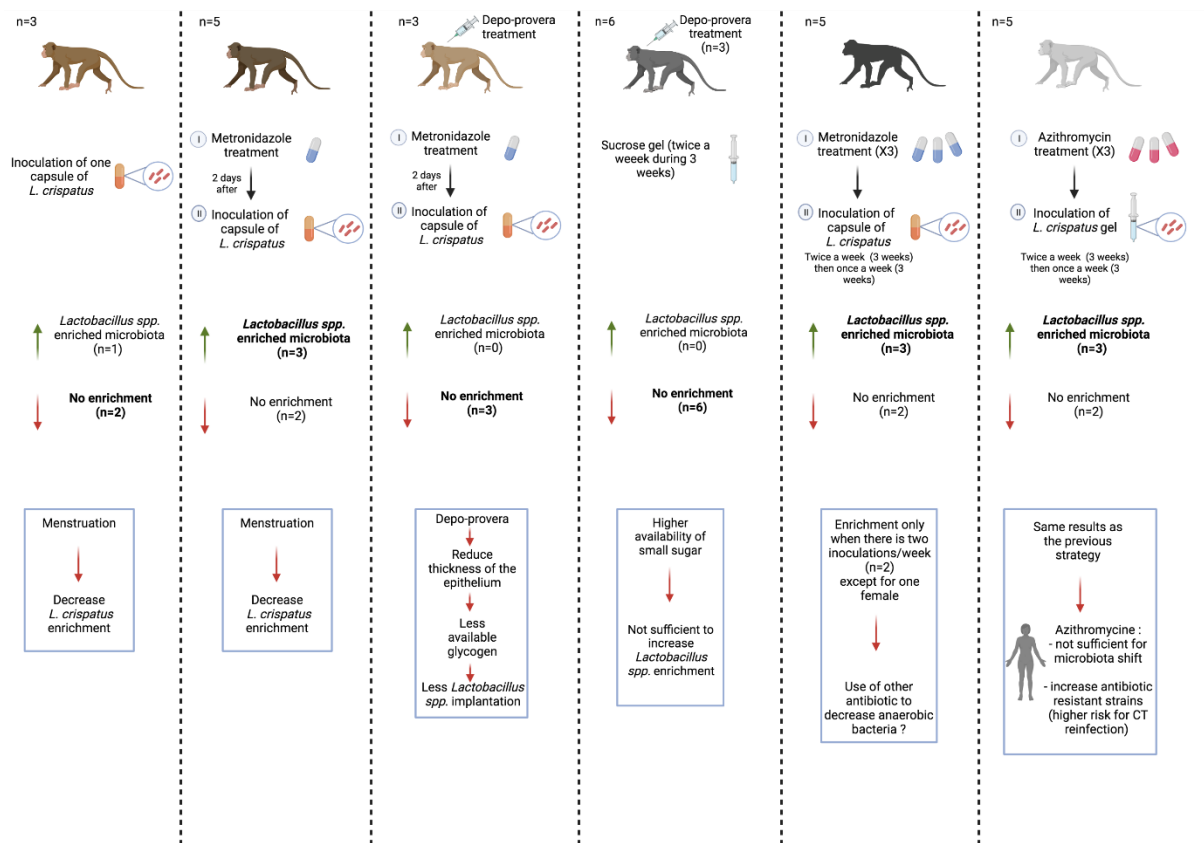


Figure 37: Summary of the strategies tested during this thesis in cynomolgus macaques to induce *L. crispatus* implantation and growth in the vaginal microbiota.

Created with BioRender.com

Third article: Influence of vaginal microbiota composition on cervicovaginal and systemic inflammation induced by *Chlamydia trachomatis* infection

Cindy Adapen, Louis Réot, Natalia Nunez, Claude Cannou, Romain Marlin, Julien Lemaître, Ségolène Diry, Léo d'Agata, Marco Leonec, Nathalie Bosquet, Roger Le Grand, Marie-Thérèse Nugeyre, Elisabeth Menu

Manuscript in preparation.

I. Introduction and objectives

Chlamydia trachomatis (CT) is an obligate intracellular bacteria responsible for genital Chlamydia infection. This sexually transmitted infection (STI) is mostly asymptomatic but prolonged infection can lead to upper tract dissemination of the pathogen [15]. CT increases local inflammation by increasing the production of cytokines and the recruitment of immune cells such as neutrophils [34, 35, 38–40]. Neutrophil recruitment has been linked to immunopathology in mice [46, 47]. However, very few is known on the impact of CT infection on cervicovaginal neutrophil phenotype. We have previously described the phenotype of systemic and vaginal neutrophils at basal state throughout the menstrual cycle in cynomolgus macaques but we do not know how resident neutrophils will react after CT infection [C. Adapen et al., Local innate markers and vaginal microbiota composition are influenced by hormonal cycle phases]. Vaginal microbiota is also a key factor within the female reproductive tract (FRT) in the regulation of local immune responses. In women, susceptibility to CT has been described to be subject to vaginal microbiota composition, indeed a *L. crispatus* dominated vaginal microbiota is associated with a decrease risk for CT infection [192]. On the contrary, a highly diverse vaginal microbiota rich in anaerobic bacteria is associated with higher local inflammation therefore higher susceptibility to

CT infection. Local inflammation is an essential parameter involved in the modulation of STI acquisition. Therefore, by modulating the local inflammation through the vaginal microbiota composition we wondered whether or not we could modify the inflammation and the susceptibility to CT infection. Cynomolgus macaques have a naturally diverse vaginal microbiota poor in *Lactobacillus spp* and rich in anaerobic bacterium that are also detected in women that suffers from dysbiosis [317]. We hypothesised that female cynomolgus macaques with a vaginal microbiota enriched with *L. crispatus* will be able to decrease the inflammation induced by CT and/or the susceptibility to CT infection.

We set up an *in vivo* study on twelve female cynomolgus macaques divided in two groups of six females. All animals received intravaginal metronidazole antibiotic treatment to eradicate anaerobic bacteria from the vaginal microbiota. Then one group received intravaginal inoculations of a Human *L. crispatus* strain to induce an enrichment of *L. crispatus* in those animals. The second group was not given any *Lactobacillus spp*, we only monitored the evolution of vaginal microbiota composition after metronidazole treatment. Subsequently, all animals received repeated intravaginal inoculations of CT. During this study, several samples were collected. To monitor local and systemic inflammation, cytokine/chemokine concentration as well as neutrophil phenotype/activation were studied in blood and cervicovaginal samples. To follow CT infection, CT load as well as anti-CT IgG were quantified in cervicovaginal swabs and peripheral blood serum respectively. Finally, vaginal microbiota composition was followed by full 16 rRNA sequencing.

II. Data summary

We have demonstrated a non-persisting CT infection in 10 animals out of 12 (1 animal in each group remained uninfected) and no association between *L. crispatus* enrichment and lower susceptibility to CT infection. However, the production of specific anti-CT IgG was higher in *L. crispatus* treated compared to untreated animal. Metronidazole treatment induced drastic modifications of the vaginal microbiota by reducing the abundance of several anaerobes and increasing the number of natural *Lactobacillus spp* (*L. johnsonii* and *L. prophage* mainly) as well as opportunistic bacteria such as *Streptococcus spp* and *Staphylococcus spp*. *L. crispatus* was detected only in *L. crispatus* treated animal but the enrichment was highly variable according to time points and animals. Surprisingly, *L. prophage* and *L. johnsonii* relative abundance was positively associated with CT load demonstrating an increased bacterial load in animals with high abundance of natural *Lactobacillus spp*. CT infection was described to induce significant alteration of vaginal microbiota during infection but also after CT infection. Indeed, we demonstrated a significant alteration of vaginal microbiota at the last time points of the protocol compared to baselines suggesting a persisting effect on the microbiota of antibiotic treatment as well as CT infection/inoculation. In terms of inflammation, peripheral blood and cervicovaginal cytokine concentrations were not significantly altered by CT infection in both groups. However, untreated animals exhibited an increased production of peripheral blood cytokines after CT infection whereas it was the opposite in the cervicovaginal compartment where *L. crispatus* treated animals exhibited an increased production of cytokines compared to untreated animals after CT infection. These results suggest a better local immune response as well as a better control on systemic inflammation upon CT infection in *L. crispatus* treated compared to untreated animals. Peripheral blood neutrophils were more mature and activated after CT infection/inoculation in both groups. As for cervicovaginal neutrophils, very few alterations of their phenotype were noticed after CT infection. We were not able so far to determine an association between a specific group of bacteria,

a higher inflammation (neutrophil activation and cytokine production) and an increased susceptibility to CT infection.

Those data highlight an impact of vaginal microbiota composition on the local and systemic immune responses induced by CT infection. Interestingly, we demonstrated that increase abundance of *L. johnsonii* was associated with higher CT load. Moreover, a significant and persisting alteration of the vaginal microbiota upon metronidazole and CT infection/inoculation was described. This study confirmed that vaginal microbiota composition can be a powerful tool to modulate local inflammation and STI susceptibility.

III. Article

Influence of vaginal microbiota composition on cervicovaginal and systemic inflammation induced by *Chlamydia trachomatis* infection.

Cindy Adapen¹, Louis Réot¹, Natalia Nunez², Claude Cannou^{1,3}, Romain Marlin¹, Julien Lemaitre¹, Ségolène Diry², Léo d'Agata³, Marco Leonec¹, Nathalie Bosquet¹, Roger Le Grand¹, Marie-Thérèse Nugeyre^{1,3}, Elisabeth Menu^{1,3}

¹Université Paris-Saclay, Inserm, CEA, Center for Immunology of Viral, Auto-immune, Hematological and Bacterial diseases (IMVA-HB/IDMIT), Fontenay-aux-Roses, France

²Life&Soft, 92260 Fontenay-aux-Roses, France

³MISTIC group, department of Virology, Institut Pasteur, Paris, France

INTRODUCTION

The World Health Organization (WHO) estimates that every year, 357 million people are infected with one of the following curable sexually transmitted diseases (STI): Chlamydia, Gonorrhea, Syphilis or Trichomonas [1]. Those STI continue to represent a major public health issue. In many countries, women between 15-24 years old that are sexually active have the highest risk of Chlamydia acquisition. *Chlamydia trachomatis* (CT) is an obligate intracellular gram-negative bacterium and serovar D to K are responsible for genital *Chlamydia* infection. Around 70% to 90% of CT infection in women are asymptomatic and without active screening, individuals stay infected and continue to spread the pathogen [2]. About 20-54% of CT infected patients self-resolve within a year whereas 23-30% become repeatedly infected [3–5]. Asymptomatic or symptomatic CT infection can induce chronic inflammation resulting in several diseases including cervicitis, cystalgias, urethral syndrome but the infection can also progress to the upper genital tract and induce salpingitis, inflammatory chronic pelvic disease and sterility.

CT preferentially infects the endocervical columnar epithelial cells of the female reproductive tract (FRT). In the FRT, pro-inflammatory cytokines such as IL-1 α , IL-1 β , IL-6, IL-8, TNF α , IL-12, IFN γ , IL-18 but also IL-10, VEGF, G-CSF and GM-CSF increase upon infection, resulting in a strong local inflammation [6–10]. Polymorphonuclear leukocytes (PMN) as well as other innate immune cells are recruited to the FRT during CT infection [7]. Neutrophils have been linked, in several studies, to immunopathology and are not directly necessary for the decrease of the bacterial burden upon CT infection [11, 12]. However, a study, in which serum from mice that resolve CT infection was passively transferred to CT reinfected mice, demonstrates that PMN are involved in bacterial burden reduction through antibody-mediated anti-CT immunity [13]. Those studies clearly demonstrated a dual effect of PMN during infection, on one hand they are essential for bacterial clearance and on another hand, when over activated and recruited, they increase the pathology. Those opposite roles could be due to differences in PMN subpopulation or activation status in the blood or in the FRT, which to our knowledge has not been described yet.

To clear CT infection, a Th1 response is essential; indeed production of IFN γ has a central role in the immune response against CT infection. IFN γ induces the depletion of the intracellular pool of tryptophan through induction of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO). Tryptophan is converted into kynurenine, resulting in the inhibition of *Chlamydia* growth. Interestingly, the microbiota composition has also an impact on CT infection. Indeed, indole which is produced by several anaerobic bacteria present within the vaginal microbiota, promotes CT infection in presence of IFN γ [3]. In fact, the kynurenine/tryptophan ratio is increased in CT infected women, and correlates with the presence of *Streptococcus spp.* and *Peptoniphilus spp.* [14]. Indole producing bacteria such as *Porphyromonas*, *Fusobacterium* or *Peptoniphilus* as well as *G. vaginalis* are highly represented in women suffering from bacterial vaginosis. Therefore a highly diverse microbiota with a predominance of anaerobic bacteria and absence or few *Lactobacillus spp.* increases the risk of CT infection [14–16]. On the contrary, a vaginal microbiota dominated by

Lactobacillus spp. is associated with a decreased risk for CT infection [17]. An *in vitro* study demonstrated that *L. crispatus* strains, by producing both L and D-lactic acid, have the best anti-CT effect [18].

Many studies were performed in mice model with *C. muridarum* to understand CT infection. However, mice reproductive tract exhibits structural and functional differences compared to women FRT [19]. Moreover, pretreatment with progestins is necessary to induce CT and consistent *C. muridarum* infection in mice [2]. Cynomolgus macaques, in contrast to mice, have a FRT that is closely similar to the one of women in terms of morphology, endocrine system, menstrual cycle but also immune cells distribution within the FRT [20, 21]. In addition, macaques are susceptible to STI such as CT or Simian Immunodeficiency Virus (SIV) [22, 23]. Female cynomolgus macaques have a highly diverse vaginal microbiota with few *Lactobacillus spp.* and many anaerobic bacteria such as *Sneathia*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Peptoniphilus*, *Bacteroides* and *Dialister*. Vaginal microbiota composition of cynomolgus macaques is thus similar to the one of women suffering from bacterial vaginosis [24].

To better study the impact of the vaginal microbiota composition on CT susceptibility and on the inflammation induced by the infection, the vaginal microbiota of female cynomolgus macaques was modified to enrich the microbiota in *Lactobacillus* species. We characterized the impact of the microbiota composition on CT infection susceptibility, specific immune responses and inflammation (ie PMN and soluble factors).

MATERIALS AND METHODS

Ethics statement

Eighteen sexually mature adult female cynomolgus macaques (*Macaca fascicularis*), aged of 5 to 7 years old and originating from Mauritian AAALAC certified breeding centers were included in this study. All animals were housed in IDMIT facilities (CEA, Fontenay-aux-roses), under BSL-2 containment (Animal facility authorization #D92-032-02, Préfecture des Hauts de Seine, France) and in compliance with European Directive 2010/63/EU, the French regulations and the Standards for Human Care and Use of Laboratory Animals, of the Office for Laboratory Animal Welfare (OLAW, assurance number #A5826-01, US). This study was approved and accredited by the institutional ethical committee "Comité d'Ethique en Expérimentation Animale du Commissariat à l'Energie Atomique et aux Energies Alternatives" (CEtEA #44) under statement numbers A18-083, A18_107, A17_036 and A16_048. The study was authorized by the "Research, Innovation and Education Ministry" under registration number APAFIS#20692-2019051709424034v1. The twelve animals were housed in the same room in social groups of six animals under controlled conditions of humidity, temperature and light (12h light/dark cycles). The animals were fed once or twice a day with commercial monkey chow and fruits. Water was available *ad libitum*. They were provided with environmental enrichment including toys and novel food under the supervision of the CEA Animal Welfare Officer.

Experimental design

Several protocols were tested to induce the enrichment of exogenous *Lactobacillus spp.* in five female cynomolgus macaques [Figure 1]: (1) A single inoculation of a capsule of *L. crispatus* (Physioflor LP®; IPRAD PHARMA, Paris) ; (2) A single pretreatment with metronidazole two days before the inoculation of *L. crispatus* capsule; (3) Depo-provera® intra-muscular injection (30mg) one month before metronidazole pretreatment two days before the inoculation of *L. crispatus* capsule (Physioflor LP®; IPRAD PHARMA, Paris); (4) Depo-provera® intra-muscular injection (30mg) in 3

animals out of 6, one month before repeated sucrose gel inoculation; (5) Repeated treatment of metronidazole before repeated inoculation of *L. crispatus* capsule (Physioflor LP®; IPRAD PHARMA, Paris); (6) Repeated treatment of azithromycine before repeated inoculation of *L. crispatus* gel. Menstruation and Depo-provera injection reduced the enrichment of *L. crispatus*. However, repeated pretreatment with metronidazole or azithromycin followed by repeated inoculation of *L. crispatus* induced a higher rate of *L. crispatus* enrichment.

Twelve female cynomolgus macaques divided in two groups (Untreated and *L. crispatus* treated) were then studied during four months and a half. Age, weight, haplotype and group distribution are summarized in Table 1. According to the data previously obtained by our team (summary presented in Figure 1), the following protocol was selected to modify the microbiota composition. The experimental design is represented in the Figure 2A. On days -23, -21 and -19, all females received a vaginal ovule of 187.7 mg \pm 17.8 (mean \pm SD) of metronidazole (FLAGYL 500mg ; SANOFI-AVENTIS). Then, a gel composed of 10^{10} *L. crispatus* was inoculated intravaginally on days -16, -13, -9 and -6 to the females of the treated group. At day 0, all the animals received intravaginally 10^6 IFU of *C. trachomatis* serovar D and two days before (D-2) the animals from the treated group also received intravaginally the gel of *L. crispatus*. The experimental design of D-2 and D0 was repeated during six weeks so each animal received six inoculation of CT. After the six inoculations, the animals were followed up for four consecutive weeks [Fig2A].

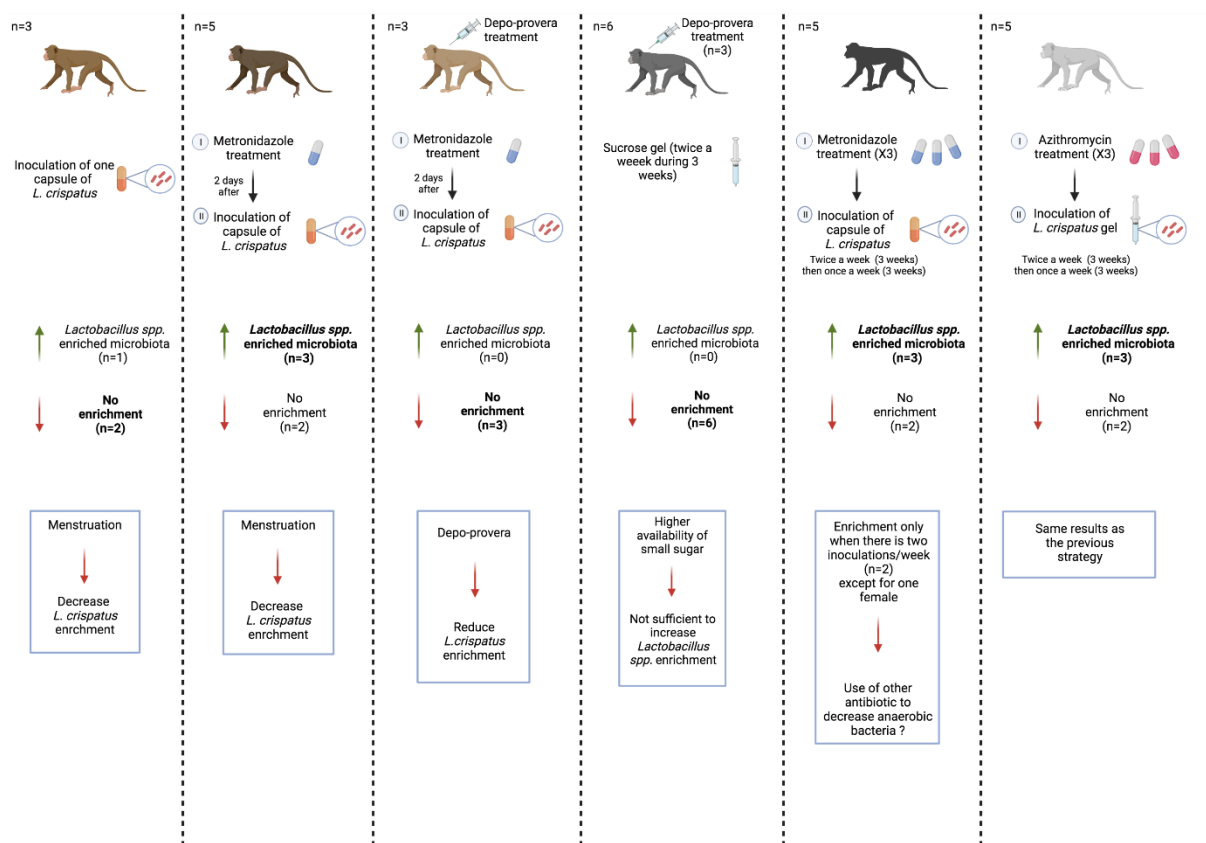
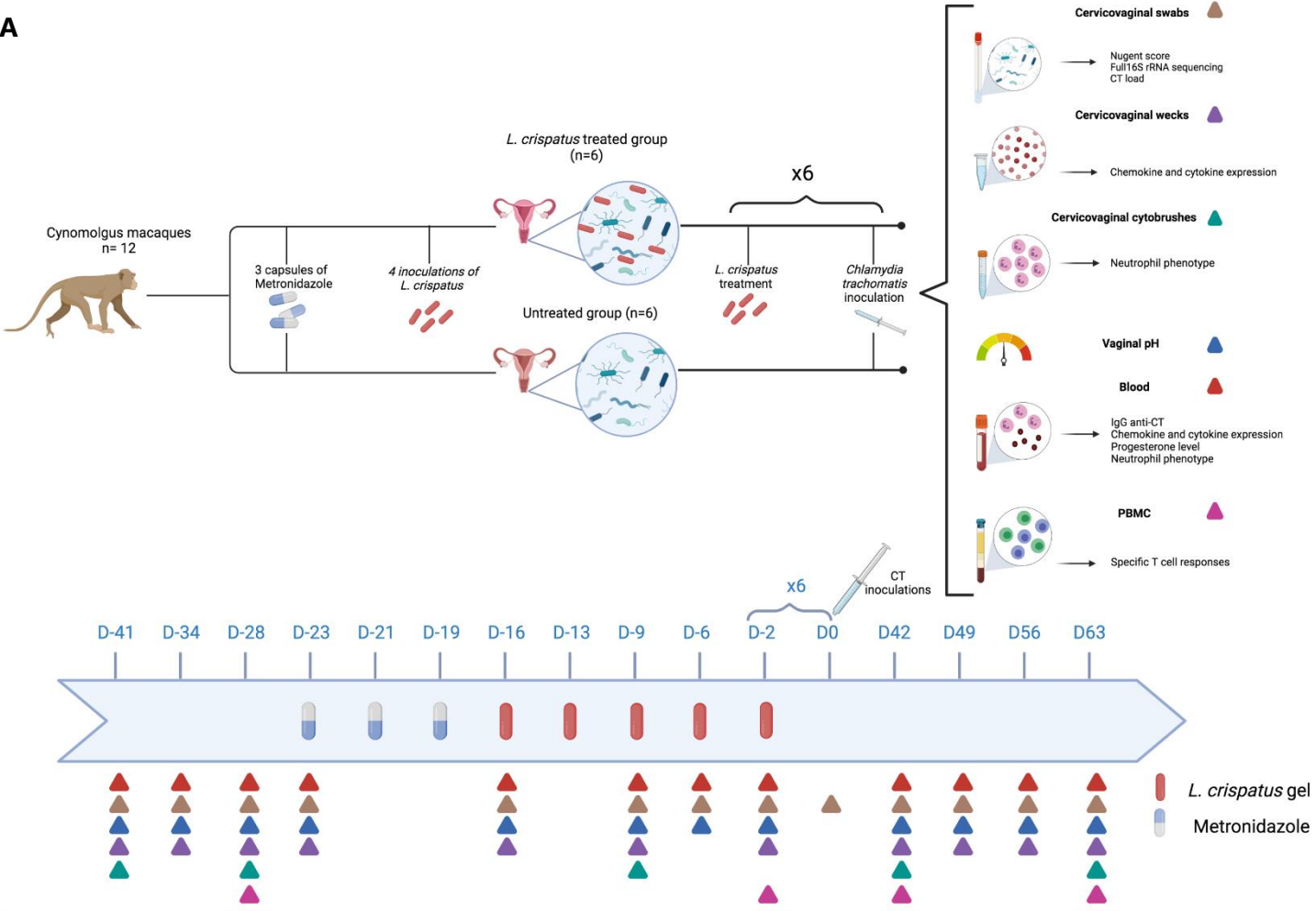


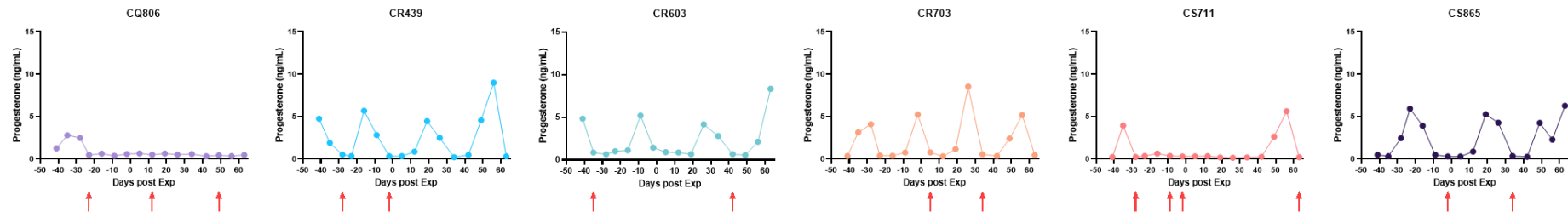
Figure 1: Summary of the results obtained to enrich the vaginal microbiota of female cynomolgus macaques with a human strain of *L. crispatus*. Several protocols were tested: (1) A single intravaginal inoculation of *L. crispatus* capsule (Physioflor LP®; IPRAD PHARMA, Paris) induced an increased presence of *L. crispatus* in only 1 animal out of 3. Moreover, menstruation disturbed *L. crispatus* enrichment. (2) Intravaginal pretreatment with metronidazole two days before *L. crispatus* capsule inoculation allowed a better *L. crispatus* enrichment (3 animals out of 5). (3) Depo-provera injection, to avoid menstruation, before antibiotic and *L. crispatus* inoculation reduced *L. crispatus* enrichment. (4) Sucrose gel inoculation in depo-provera treated or untreated animals did not allow the growth of natural *Lactobacillus* spp present within the vaginal microbiota of female macaques. (5) Repeated intravaginal treatment with metronidazole followed by repeated inoculation of *L. crispatus* capsule (twice a week) induced a *L. crispatus* enrichment in 3 females out of 5. (6) Identical strategy as the previous protocol but with oral administration of azithromycin and intravaginal inoculation of a more concentrated gel of *L. crispatus* gave similar results as previously obtained. Created with BioRender.com.

Table 1: Information (identification name, date of birth, haplotype and groups) concerning the 12 animals included in the study.

ID	Date of birth	Weight	Haplotype	Groups
CQ806	2015-02-05	3.59 ± 0.09	Rec H4 H7 Rec H4 H7	<i>L. crispatus</i> treated group
CR439	2015-03-24	5.47 ± 0.27	H3 H2	<i>L. crispatus</i> treated group
CR603	2015-04-12	4.97 ± 0.27	Rec H3 H4 H7 Rec H7 H1 H2	<i>L. crispatus</i> treated group
CR703	2015-04-19	4.21 ± 0.09	H1 Rec H1 H2	<i>L. crispatus</i> treated group
CS711	2015-08-08	4.22 ± 0.25	H1 H2	<i>L. crispatus</i> treated group
CS865	2015-08-27	4.78 ± 0.33	H1 H6	<i>L. crispatus</i> treated group
CQ819	2015-02-06	2.84 ± 0,06	Rec H4 H7 Rec H3 H1 H2	Untreated group
CR403	2015-03-22	5.31 ± 0.27	H1 Rec H4 H5	Untreated group
CR574	2015-04-06	4.64 ± 0.33	H1 Rec H2 H4 H7	Untreated group
CS693	2015-08-06	4.70 ± 0.17	Rec H1 H4 H7 H3	Untreated group
CS742	2015-08-11	6.75 ± 0.31	H3 Rec H1 H5	Untreated group
CS794	2015-08-18	3.35 ± 0.08	Rec H3 H4 Rec H7 H1 H3 H6	Untreated group



B

L. crispatus treated group

Untreated group

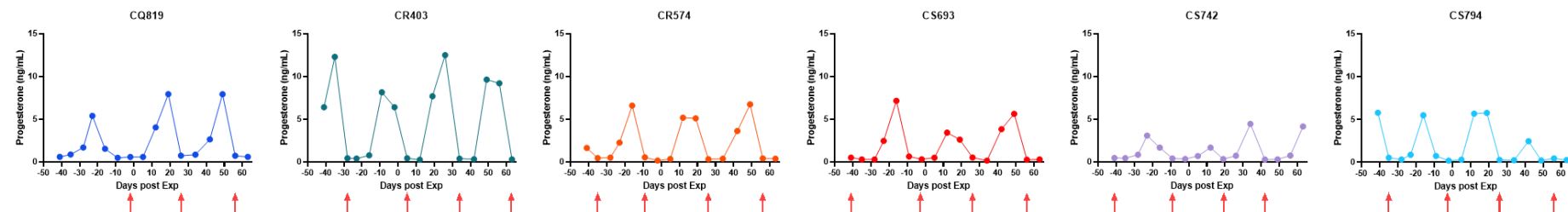


Figure 2: Experimental design and progesterone level. (A) Twelve female cynomolgus macaques were included in this study and divided in two groups of six animals. After four weeks of baseline, all animals received ovules of metronidazole (three during one week) intravaginally (IVAG). Then animals from the *L. crispatus* treated group ($n=6$) received four IVAG inoculations of *L. crispatus* gel at 10^{10} CFU (twice during two weeks) after washing the vaginal cavity with 0.02% of lactic acid. At D0, all animals were inoculated with a low dose of CT (10^6 IFU). At D-2 all animals within the *L. crispatus* treated group received the gel of *L. crispatus*. This design D-2/D0 was repeated six times, therefore all animals received six doses of CT. Then animals were followed during four weeks. Sample collection included: cervicovaginal swabs, cervicovaginal wecks, cervicovaginal cytobrushes, whole blood, PBMC and pH measurement. Created with BioRender.com. (B) Progesterone quantification (ng/mL) in each animal throughout the study (once a week). Red arrows represent menstruation.

Sample collection

Whole blood, cervicovaginal fluids and samples were collected [Fig2A] at baseline (days -42, -35, -28 and -23), after metronidazole treatments (day -16), after one and two weeks of *L. crispatus* inoculations or not (days -16 and -9), two days before each CT inoculation (days -2, 5, 12, 19, 26 and 34) and once a week during the follow up (days 42, 49, 56 and 63). Cervicovaginal samples for microbiota analysis were also collected before each CT inoculation (days 7, 14, 21, 28 and 36). Cervicovaginal cytobrushes were collected at five time points: at baselines (days -42 and -28), after antibiotic treatments and *L. crispatus* inoculations or not (day -9) and during follow up (days 42 and 63). Peripheral blood mononuclear cells (PBMC) were gathered at baseline (day -28), two days before the first CT inoculation (day -2) and during the follow up (days 42 and 63). Sample collection order was as followed: Weck-Cel Spear during blood withdrawal, vaginal pH measurement, cervicovaginal swab and cervicovaginal cytobrushes. Cervicovaginal fluids were collected with a Weck-Cel Spear (Medtronic) placed in the vaginal vault for 2 min. Secretions were recovered from the spears by adding 600uL of elution buffer (PBS, NaCl 0.25M and protease inhibitor mixture; Merck Millipore, Fontenay-sous-Bois, France) and centrifuged at 13000xg for 20min. Secretions were then aliquoted and stored at -80°C before cytokine/chemokine quantification. Blood was collected and used for Complete Blood Count (CBC), then plasma and serum were collected, aliquoted and stored at -80°C for cytokine/chemokine and progesterone quantification. Cell Preparation tube (CPT) with Sodium Heparin were used to isolated mononuclear cells from peripheral blood (BD Biosciences, Le Pont-de-Claix, France) after centrifugation for 30 min at 3000 rpm. PBMC were collected at the top of the CPT gel surface and washed twice. Vaginal pH measurements were done by inserting a neonate nasal swab (Copan Diagnostics Inc., Murrieta, CA, USA) into the vaginal cavity for few second then vaginal fluids were spread on a pH paper ranging from 4 to 7. Cervicovaginal samples for microbiota analysis were collected with nylon flocked swabs inserted in the vaginal vault and turned four to five times before storing in amies liquid (ELITech Microbio, France). Swabs were then aliquoted and either used for Nugent

score or stored frozen at -80°C until DNA extraction. Cervicovaginal cells were collected using two successive cytobrushes (VWR;Belgium) inserted in the vaginal cavity and turned 4 to 5 times. After collection, the cytobrushes were put in a 15 mL tube containing 5 mL of RPMI with 10% Fetal Calf Serum (FCS) and 5% Penicillin streptomycin neomycin (PSN). The samples were conserved on ice block before processing.

Metronidazole ovules

Metronidazole ovules (Flagyl[®], Sanofi Aventis; Paris) of 500mg were put in a 5 mL tube and weight before putting in a water bath set at 37°C for few hours until complete melting. Melted ovules were then used to fill pediatric ovule (Herboristerie du Valmont; Belgium). Ovules were then weighted to determine the concentration of each antibiotic ovule and stored at 4°C before inoculation.

***L. crispatus* gel**

A capsule of 10⁹ *L. crispatus* (Physioflor LP[®]; IPRAD PHARMA, Paris) was cultured in Mann Rogosa and Sharpe (MRS) liquid culture media (BD Bioscience) supplemented with L-cysteine (Sigma Aldrich) for 12h at 37°C with 10% CO₂. Then, 50µL were spread on a MRS agar (BD Bioscience) for 72h at 37°C with 10% CO₂. An isolated colony (*Lactobacillus crispatus* strain IP174178) was amplified in liquid MRS culture medium (BD Bioscience) supplemented with L-cysteine (Sigma Aldrich) for 12h at 37°C with 10% CO₂. The culture was diluted 1/100 and cultured until the exponential phase was reached (DO: 0,6). Three consecutive washing steps were performed with 1X PBS and centrifugation at 4000xg 20min to obtain the bacteria. Resuspension was performed with a gel composed of 1,5% Hydroxyethylcellulose gel (HEC; Sigma Aldrich), 9% sucrose (Sigma Aldrich), 0,05% lactic acid (Sigma Aldrich) and citrate buffer (Citric acid monohydrate; trisodium citrate dihydrate; H₂O milliQ). Syringes were filled with 1 mL of *L. crispatus* gel and freezed until use. Multiplicity of infection were determined after serial dilutions of the gel and MRS plating.

C. trachomatis infection

Chlamydia trachomatis serovar D (D/UW3/Cx) were obtained from Dr Frank Follmann laboratory (SSI, Denmark) [25]. Briefly, batches of CT was produced by infecting HeLa cells then 2-3 days after infection, cells were harvested, and CT was purified from the cells. For animal inoculation, CT was diluted in Sucrose Phosphate Glutamate (SPG) buffer to achieve the expected concentration. Each animal received intravaginally 800µL of CT inoculum (10^6 IFU/800 µL).

Progesterone quantification

Progesterone level was determined each week in peripheral blood plasma sample by ELISA (IBL international; Germany) according to manufacturer's instructions [Fig2B].

***Chlamydia trachomatis* load**

Detection of CT in cervicovaginal samples was performed by qPCR. DNA was extracted using the kit QIAamp DNA Mini Kit (Qiagen; Germany) according to manufacturer instructions. As for qPCR, the kit Presto combined quantitative real time *Chlamydia trachomatis/Neisseria gonorrhea* (Goffin Molecular Technologies; Netherlands) as well as a thermocycler CFX96™ real-time system (Biorad; USA) was used according to manufacturer instructions.

Specific antibodies against CT

Specific IgG against CT were quantified by ELISA (Demeditec; Germany) in serum according to manufacturer's instructions. Goat anti-monkey IgG coupled with HRP (Biorad, USA) was used for revealing. Optical density values at 450nm were obtained using a multimode microplate reader, the Spark 10M (TECAN; Switzerland). Units of IgG anti-CT was calculated using the following formula: $(OD_{\text{sample}} \times 10) / OD_{\text{cutoff}}$ and multiplied by the dilution factor.

Nugent score

Vaginal fluids obtained by swabs were spread on a slide, fixed with heat (50°C) and 100% ethanol before Gram staining (RAL diagnostics; France). Slides were then observed with a 100X oil immersion lens using a Axioplan 2 microscope (ZEISS). Gram-positive rod shaped bacteria as well as non-rod shaped bacteria were counted in 10 random fields for each slide. The Nugent score was determined by adding the score of non-rod and rod shaped bacteria [Table 2]. A score between 7-10 indicates bacterial vaginosis, score 4 to 6 indicates an intermediate flora and score 0 to 3 indicates a normal flora [26].

Table 2: Nugent scoring

Score	<i>Lactobacillus</i> (number of bacteria/field)	Anaerobic bacteria (number of bacteria/field)	<i>Mobiluncus</i> (number of bacteria/field)
0	>30	0	0
1	5 to 30	<1	<1 to 4
2	1 to 4	1 to 4	5 to >30
3	<1	5 to 30	
4	0	>30	

DNA extraction and full 16S rRNA gene sequencing

The PowerFecal DNA Pro isolation kit (Qiagen;Germany) was used following the manufacturer's instructions. 16S rRNA gene was amplified by PCR using specific 16S primers (27F and 1492R) of the 16S Barcoding Kit 1-24 (SQK-16S024, Oxford Nanopore Technologies) and LongAmp™ Taq 2× Master Mix (New England Biolabs. PCR condition used were 1 min of denaturation at 95 °C, 30 cycles of denaturation (20 secs at 95 °C), annealing (30 secs at 55 °C) and extension (2 min at 65 °C). A final extension step was added for 5 min at 65 °C. PCR products were washed using AMPure XP beads (Beckman Coulter, USA) and quantified using QuBit fluorometer abd HS DNA kit (Invitrogen). Twenty-four barcoded samples were pooled and 100 fmol (~100 ng) were

used for library preparation on one R9.4 flow cell. GridION™ was used for sequencing (Oxford Nanopore Technologies) according to the manufacturer's instructions.

Cytokine and chemokine quantification

Pro- and anti-inflammatory cytokines as well as chemokines were quantified in cervicovaginal fluids and plasma by 23plex assay for the detection of: G-CSF, GM-CSF, IFN γ , IL-1 β , IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23(p40), IL-13, IL-15, IL-17A, CCL2, CCL3, CCL4, sCD40L, TGF α , TNF α , VEGF, IL-18 (NHP cytokine magnetic bead panel kit; Merck Millipore; Germany), according to manufacturer's instructions.

Cytospin and blood smear

Cytospin was performed at each time point on cervicovaginal cell suspension. Briefly, between 10⁴ and 10⁵ cells were seeded and spun at 800rpm during one minute. Whole blood smear was also done at each time point. Dry cytopins and whole blood smears were then fixed with Labofix Q path (VWR Avantor; Belgium) and a May-Grunwald Giemsa staining were performed (RAL diagnostics; France). Slides were observed with a Eclipse 80i microscope (NIKON). Images were acquired with a 60x lens using a high-definition cooled color digital camera (DXM1200C; NIKON).

Neutrophil phenotyping

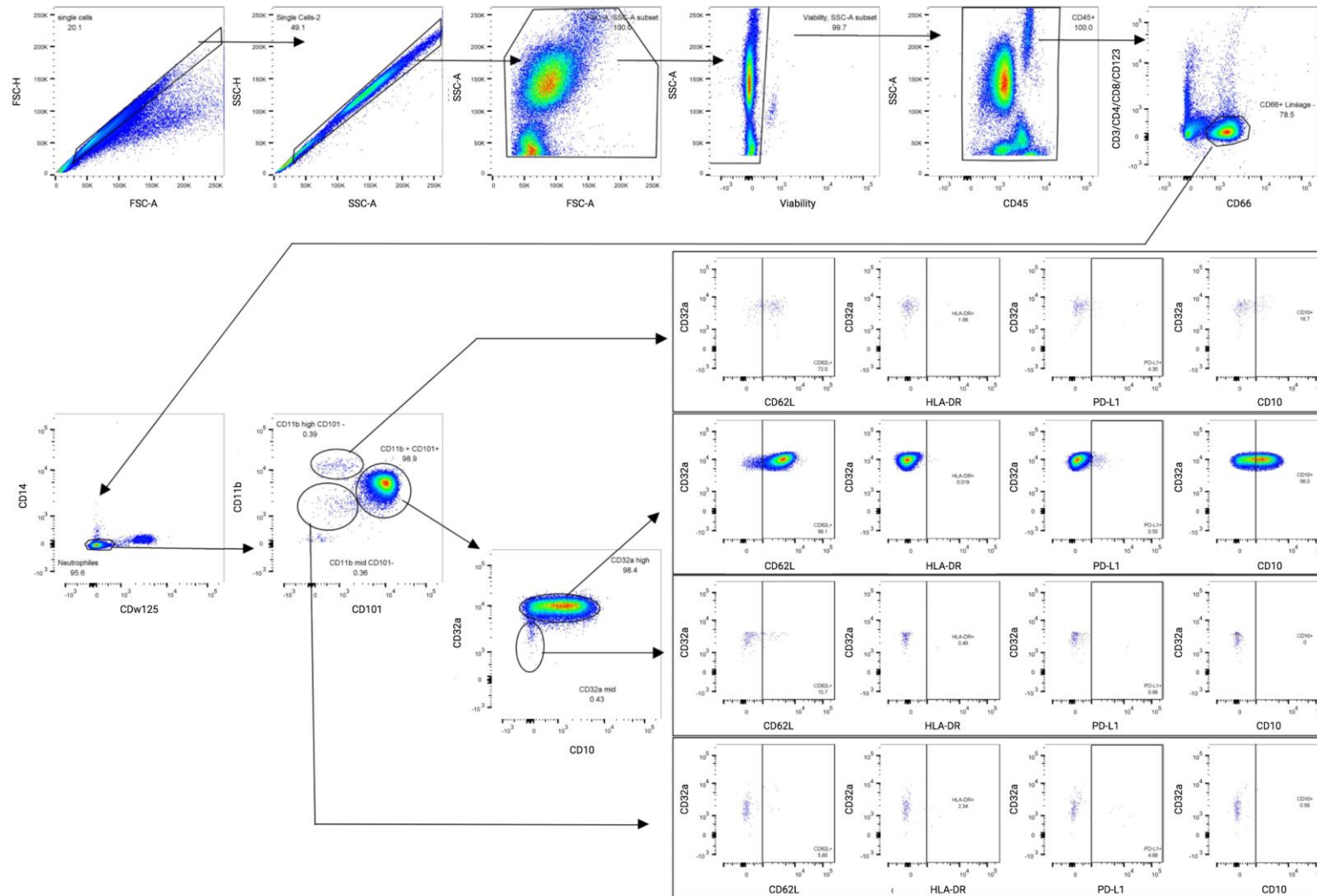
Neutrophil populations were analyzed in whole blood and cervicovaginal cells. Cervicovaginal cells were filtered with 35 μ m filter (Corning Falcon ; USA). Then, cervicovaginal cells and whole blood were incubated with the antibodies listed in Table 3, washed and fixed with FACS lysing buffer (BD, Biosciences) or BD cell Fix solution (BD, Biosciences). A fourteen color panel, containing neutrophil surface makers of maturation and activation, were used. Neutrophils were identified as CD45 positive Lineage negative (CD3, CD8, CD20, CD123, CD14, CDw125) and CD66abce positive. CD10 and CD101 were used to determine maturity. CD62L shedding and CD11b increased expression were assessed to attest the priming status of blood neutrophils

[27]. CD32a, CD64, PD-L1 and HLA-DR complete the panel to study the activation of neutrophils in both compartments. Phenotyping was performed on a Fortessa (BD, Biosciences) with DIVA (BD) and FlowJo (Tristar, USA) software. The gating strategy in cervicovaginal cytobrushes is described in the supplementary figure 1.

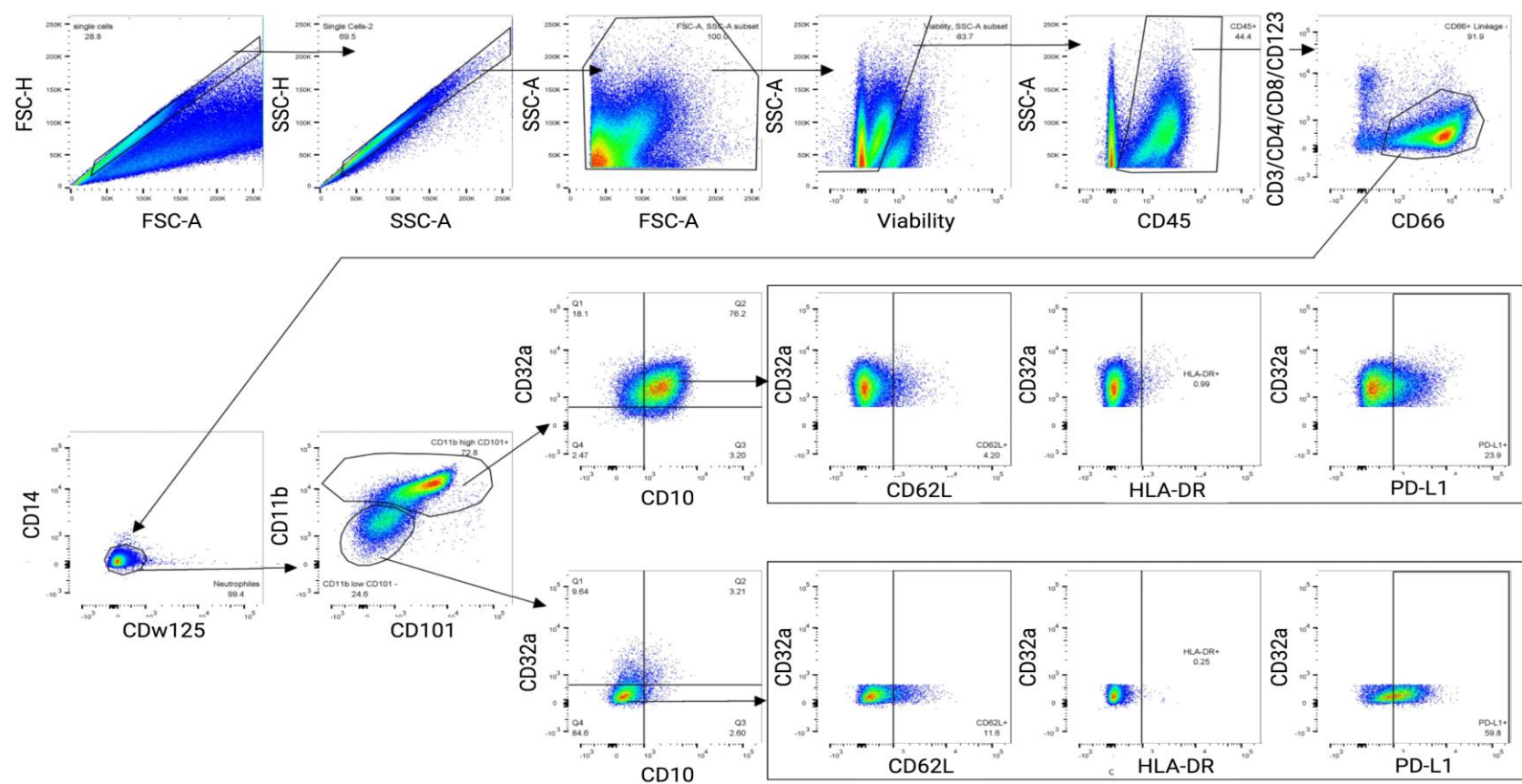
Table 3: Antibody panel for neutrophils determination in blood and cervicovaginal cytobrushes

Antibody	Label	Reference	Manufacturer
Bluevid	BUV736	L23105	Lifetechnologies
CD64	BUV737	612776	BD
CD11b	FITC	130-110-552	Milteny
CD45	Viogreen	130-177-193	Milteny
CD3	BV650	563916	BD
CD8	BV650	563821	BD
CD20	BV650	563780	BD
CD123	BV650	563405	BD
CD62L	BV711	565040	BD
CD14	Vioblue	130-110-524	Milteny
CD10	PercP-Cy5,5	312216	Biolegend
CDw125	PE	130-110-544	Milteny
PD-L1	PE-Dazzle594	329732	Biolegend
CD101	PE-Vio770	130-115-832	Milteny
CD32a	AF647	60012	Stemcell
HLA-DR	AF700	307626	Biolegend
CD66	APC-Vio770	130-119-847	Milteny

A



B



Supplementary figure 1: FACS gating strategy for neutrophil phenotyping in blood samples (A) and cervicovaginal cytobrushes (B).

Peripheral T cell responses

Specific Th1, Th2 and Th17 responses were assessed after *in vitro* stimulation of PBMC. Cells were plated with co-stimulation markers (CD28 and CD49d) and Brefeldin A (10µg/mL; Sigma-Aldrich) in addition to different stimuli. Cells were either unstimulated, stimulated with PMA (6,2µg/mL; Sigma-Aldrich) and ionomycin, inactivated *Chlamydia trachomatis* (iCT ; 5µg/mL), CT043 or CT681 CT peptides (2µg/mL) (ProteoGenix, France) overnight at 37°C. Cells were then permeabilized using Cytofix/Cytoperm reagent (BD Biosciences) and Perm/Wash buffer (BD Biosciences) and frozen. The antibody list in Table 3 were used for intracellular staining and a LSRII with DIVA (BD Biosciences) and FlowJo software (Tristar, USA) were used for the analysis. Cytokines measured are summarized in Table 4.

Table 4: Antibody panel for T cells intracellular staining.

Antibody	Label	Reference	Manufacturer
Bluevid	BUV736	L23105	Life Technologies
IFN γ	V450	560371	BD
CD4	BV510	563094	BD
TNF α	BV605	502936	Biolegend
IL-13	BV711	564288	BD
CD154	FITC	555699	BD
IL-5	PE	559332	BD
IL-2	PerCP-Cy5,5	560708	BD
CD8	PE-Vio770	130-113-159	Miltenyi
CD137	APC	550890	BD
IL-17A	AF700	560613	BD
CD3	APC-Cy7	557757	BD

Statistical analysis

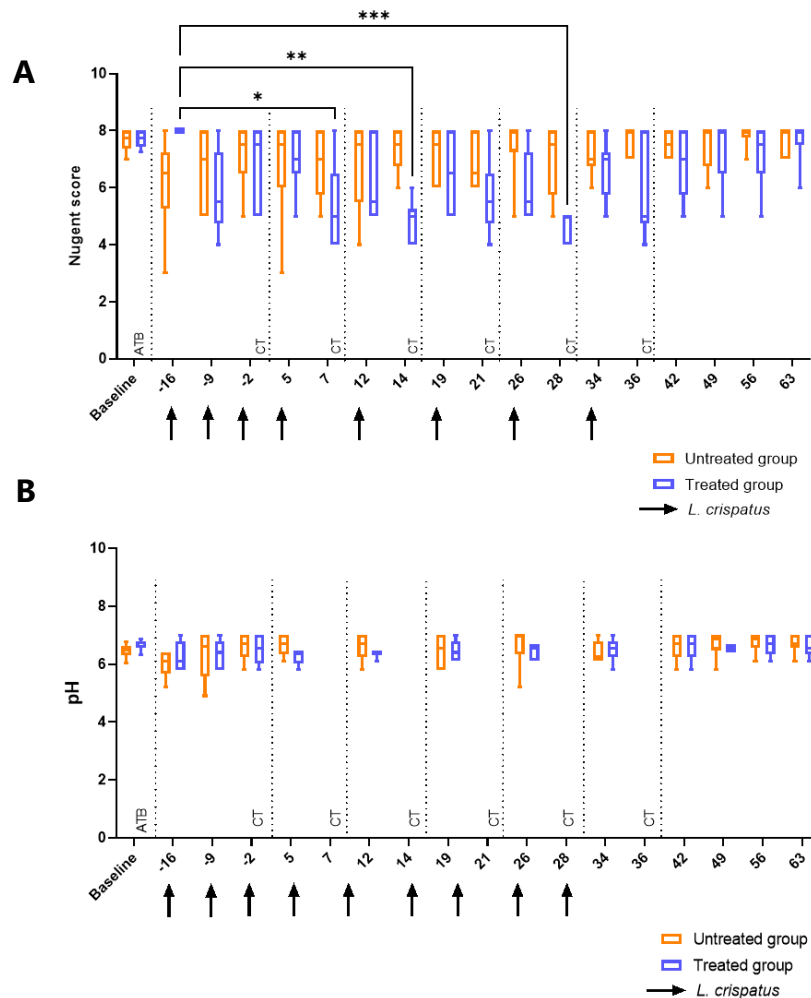
Sequence reads were converted into FASTQ files. A quality using NanoFilt was applied. Reads were filtered on quality metrics (Q > 10) and on read length (reads between 1200-1800 pb were kept). SILVA138_16S_pintail_100 ([Index of /frogs_databanks/assignation/SILVA/16S_\(inra.fr\)](https://index.of/frogs_databanks/assignation/SILVA/16S_(inra.fr))) was used for assignation using Minimap2 for alignment. A supplementary filter was applied on alignment metrics and of the percentage of identity. Species abundance was obtained per sample (reads

assigned to one species/total reads of the sample). Raw abundance file was converted to a biom file and Phyloseq object was created using FROGS pipeline (Find Rapidly OTU with Galaxy Solution) [28] implemented on a galaxy instance (<http://migale.jouy.inra.fr/galaxy/>). Finally, to study differentially abundant species in different conditions, we used DESeq2 [29]. Heatmaps representing fold change of cytokine/chemokine concentration in cervicovaginal fluids or plasma were obtained by Tableau software (Seattle, USA). GraphPad prism software version 9 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was used for graphical representation of the vaginal microbiota, cytokine concentration, and neutrophil subpopulation. Significant differences between groups were confirmed using either a Mann Whitney, a two way ANOVA test with p value adjustment with Turkey test or Kruskal-Wallis test with p values adjustment with Dunn's test.

RESULTS

***L. crispatus* inoculations trigger a reduction of dysbiosis**

To follow variations of Gram-positive rod-shaped enrichment and of the pH, associated with *Lactobacillus spp*, Nugent score and vaginal pH measurements were performed. The Nugent score baselines ranged between 7 and 8 and pH between 5.8 and 7 for all animals, suggesting a dysbiotic environment. Metronidazole treatment induced no significant differences in term of Nugent score or pH measurement in each group. However, repeated inoculation of *L. crispatus* in the treated group induced a significant decrease of Nugent score at day 7 (p value: 0.0498), day 14 (p value: 0.0011) and day 28 post CT inoculation (p value: 0.0002) [Suppl fig 2A]. No significant impact on pH was noticed [Suppl fig 2B].



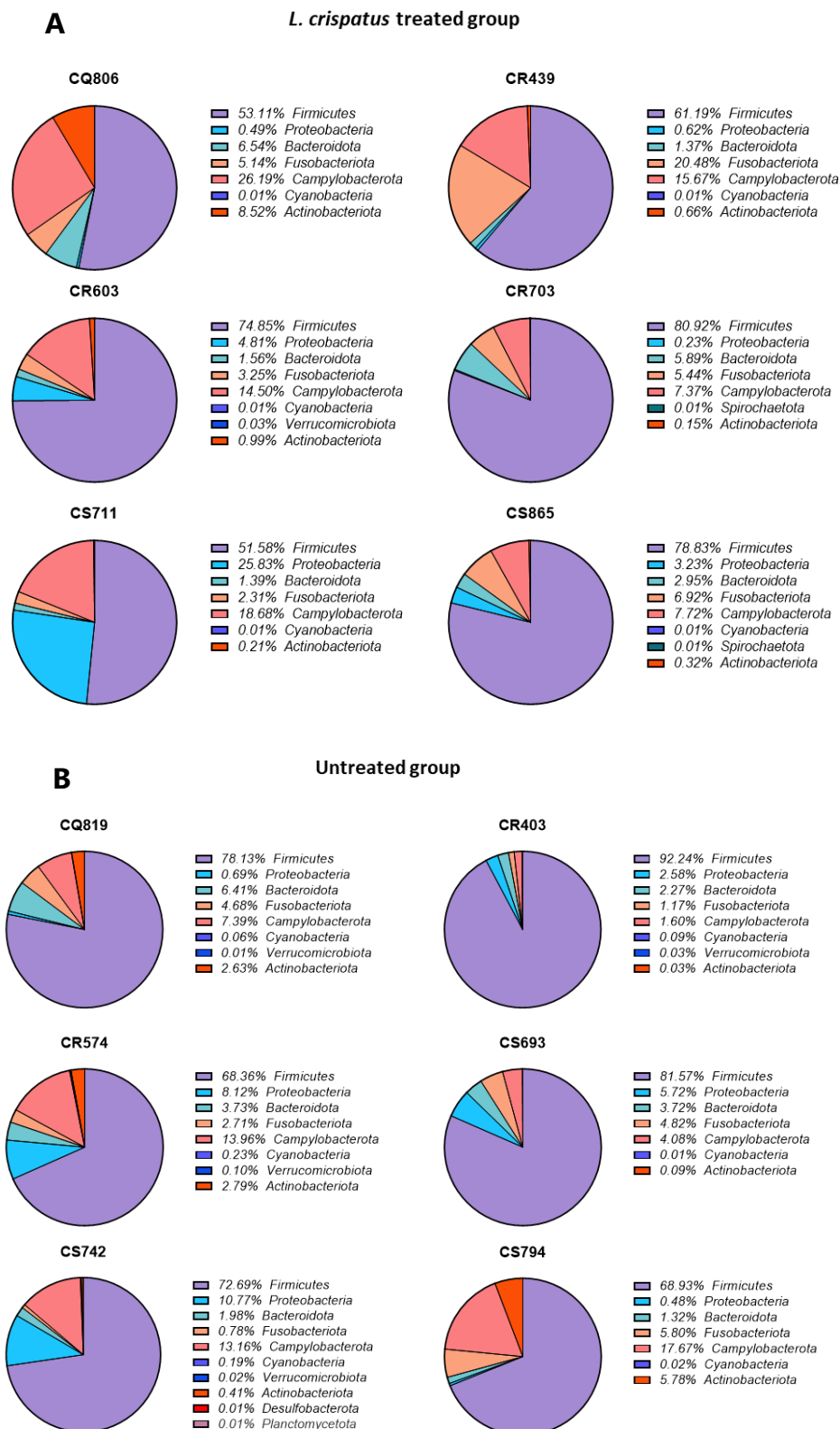
Supplementary figure 2 : Nugent scores of each female during the three months of follow up.

Metronidazole treatment increases the abundance of natural *Lactobacillus spp*

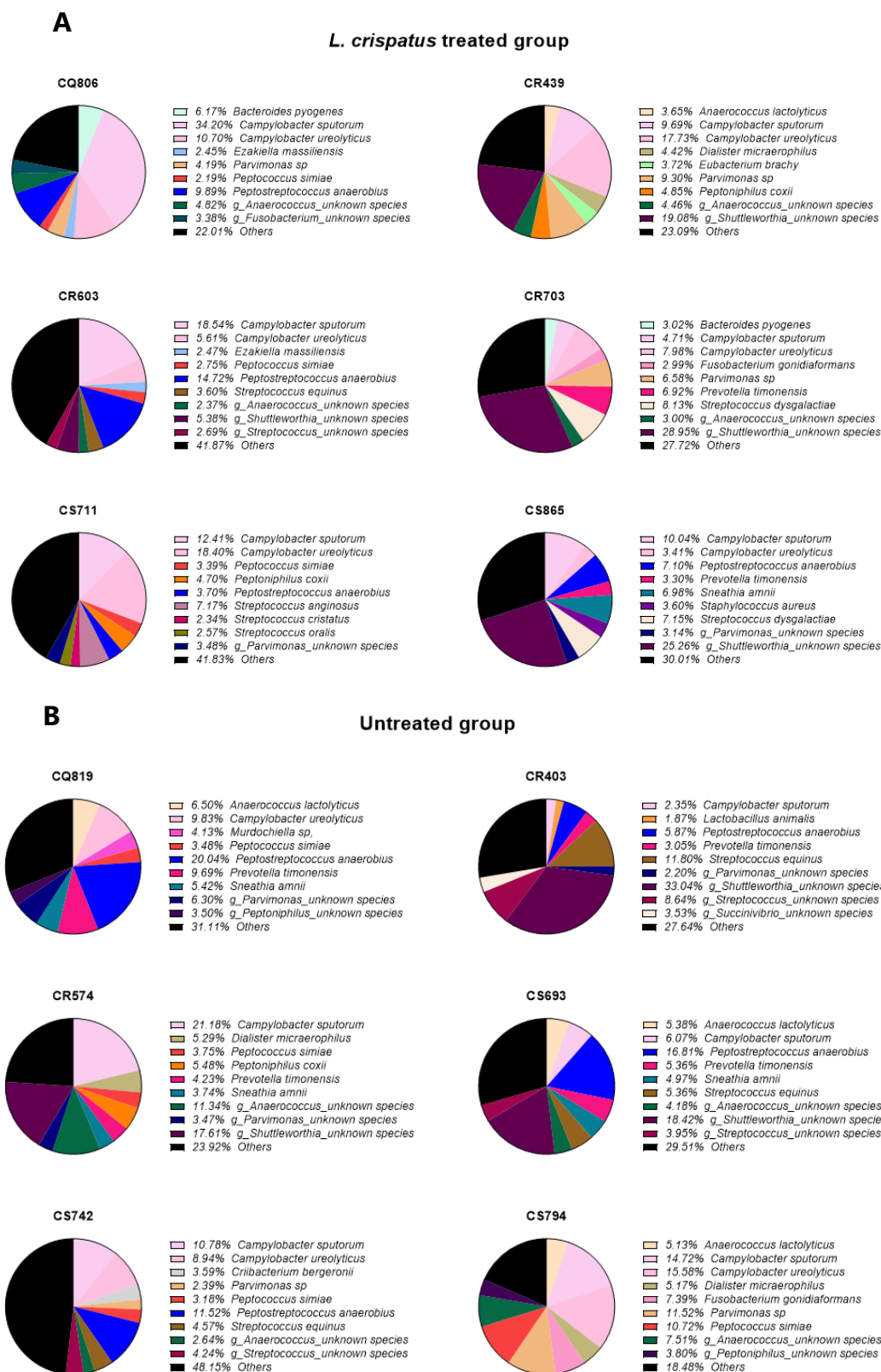
To study vaginal microbiota composition, we then characterized the vaginal microbiota at each time point for all females by 16S rRNA sequencing. At the baseline, six dominant phyla were present in all animals (total mean abundance ranging from maximum to minimum): *Firmicutes* (51.58% to 92.24%), *Campylobacterota* (1.60% to 26.19%), *Proteobacteria* (0.23% to 25.83%), *Fusobacteriota* (0.78% to 20.48%), *Bacteroidota* (1.32% to 6.54%), *Actinobacteriota* (0.03% to 8.52%). The other phyla represented less than 1% of the total abundance [Suppl fig3]. A representation of the top 9 most abundant species detected in each animal during baseline is shown in [Suppl fig4]. This representation highlights differences in terms of bacterial composition

at the species level. However, many other species are observed in several animals including *Campylobacter spurotum*, *Campylobacter ureolyticus*, *Peptococcus simae*, *Peptostreptococcus anaerobius*, species from the genus *Anaerococcus* and *Shuttleworthia* for instance. Then, to evaluate the impact of antibiotic treatment as well as *L. crispatus* treatment on bacterial species composition, a DEseq2 analysis was performed. A heatmap representing the log10 expression of differentially abundant species between the baseline timepoints and the point after antibiotic treatment was generated. The main species (*Prevotella timonensis*, *Peptoniphilus grossensis*, *Peptostreptococcus anaerobius*, *Fusobacterium gonidiaformans*, *Parvimonas sp*, *Peptococcus simae*, *Campylobacter ureolyticus*, *Dialister micraerophilus*, *Campylobacter sputorum*...) detected at baseline significantly decreased after antibiotic treatment. In contrast, some species were increased after antibiotic treatment including *Fastidiosipila sanguinis*, *Streptococcus dysgalactiae*, *Staphylococcus aureus*, *Actinotignum Shaalii* in addition to several *Lactobacillus spp.* (*L. murinus*, *L. animalis*, *L. prophage*, *L. johnsonii*) [Fig3 ; Table5]. Decreased abundance of several anaerobic species as well as an increase of endogenous *Lactobacillus spp* in addition to opportunistic bacteria such as *S. aureus* was demonstrated. To determine the effect of *L. crispatus* treatment or not on microbiota composition, a heatmap representing the log10 expression of differentially abundant bacteria between samples collected after antibiotic treatment and two weeks after *L. crispatus* inoculations or not was generated. *L. crispatus* was detected in the treated group only. In addition, *L. crispatus* treatment induced an increase of several bacteria including *L. reuteri*, *Gemella haemolysans*, *Peptoniphilus grossensis*, *Enterococcus faecalis*, *Porphyromonas sp.* [Fig4A ; Table 6]. Without *L. crispatus* treatment, animals show an increase in several bacterial taxa associated with bacterial vaginosis in women such as *E. coli*, *Helcococcus sueciensis*, *Peptoniphilus grossensis*, *Proteus spp.*, *S. aureus* as well as *Streptococcus spp.* In contrary, a slight decrease of *Lactobacillus murinus* and *L. animalis* was detected [Fig4B ; Table 7]. Therefore, the effect of metronidazole is transient and species associated with women bacterial

vaginosis are detected two weeks after antibiotic treatment in *L. crispatus* untreated animals.



Supplementary figure 3: Vaginal microbiota composition at the phylum level of *L. crispatus* treated (A) and untreated animals (B) at baseline. Percentage of mean relative abundance of phylum are represented in pie chart for each animal.



Supplementary figure 4 : Vaginal microbiota composition at the species level of *L. crispatus* treated (A) and untreated animals (B) at baseline. Percentage of mean relative abundance of the top 9 most represented species in each animal are represented in pie chart.

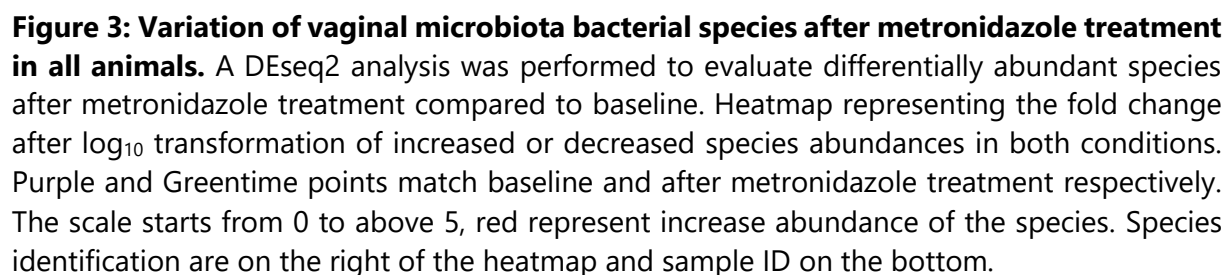
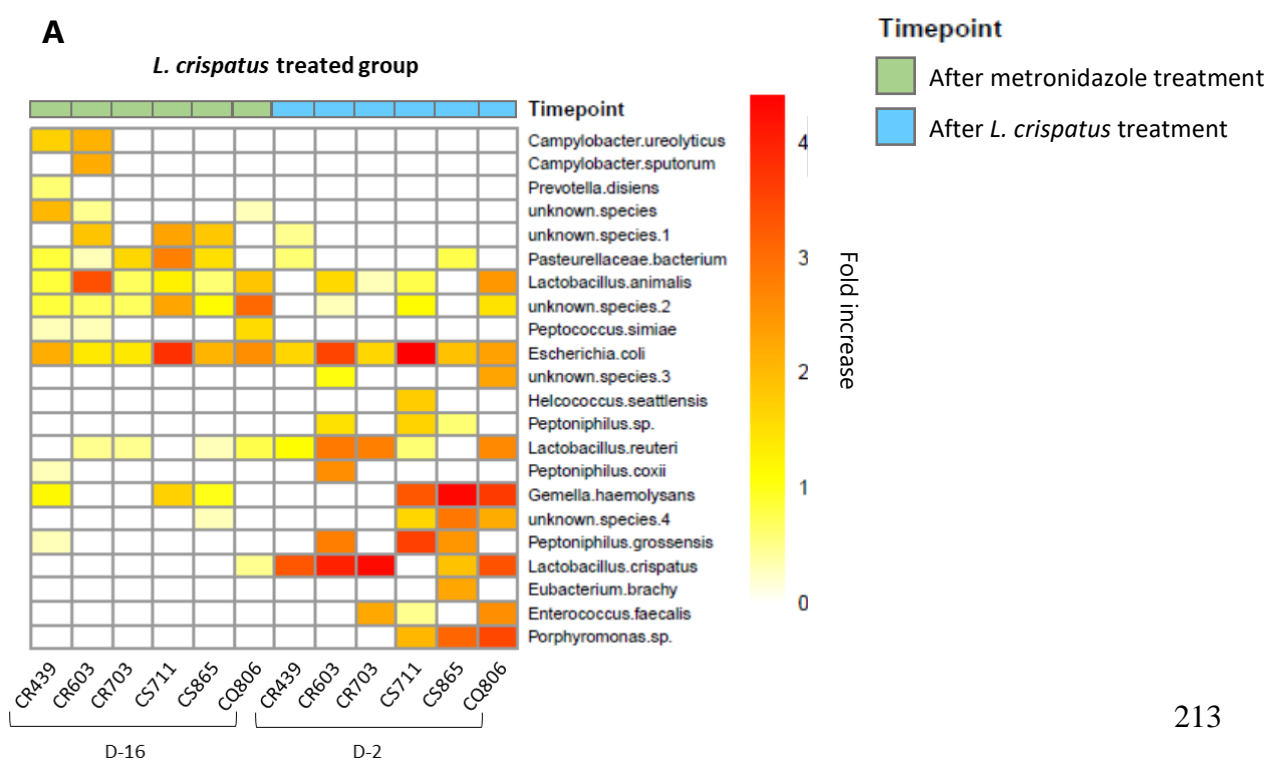


Table 5: Affiliation of unknown species at the genus or family level associated with the Figure 3.

Species	Genus	Family
Unknown species	<i>Catonella</i>	
Unknown species 1	<i>Peptoniphilus</i>	
Unknown species 2	<i>Anaerococcus</i>	
Unknown species 3	<i>Parvimonas</i>	
Unknown species 4	<i>Fusobacterium</i>	
Unknown species 5	<i>Shuttleworthia</i>	
Unknown species 6	<i>Peptostreptococcus</i>	
Unknown species 7	<i>Facklamia</i>	
Unknown species 8	<i>Terrisporobacter</i>	
Unknown species 9	<i>Sarcina</i>	
Unknown species 10	<i>Clostridium sensu stricto 1</i>	
Unknown species 11	<i>Porphyromonas</i>	
Unknown species 12	<i>Fastidiosipila</i>	
Unknown species 13	<i>Negativibacillus</i>	
Unknown species 14	<i>Helcococcus</i>	
Unknown species 15	<i>Escherichia-Shigella</i>	
Unknown species 16	<i>Aerococcus</i>	
Unknown species 17	<i>Actinobacillus</i>	
Unknown species 18	<i>Unknown genus</i>	<i>Actinomycetaceae</i>
Unknown species 19	<i>Staphylococcus</i>	
Unknown species 20	<i>Aggregatibacter</i>	
Unknown species 21	<i>Lactobacillus</i>	
Unknown species 22	<i>Haemophilus</i>	
Unknown species 23	<i>Anaerobiospirillum</i>	



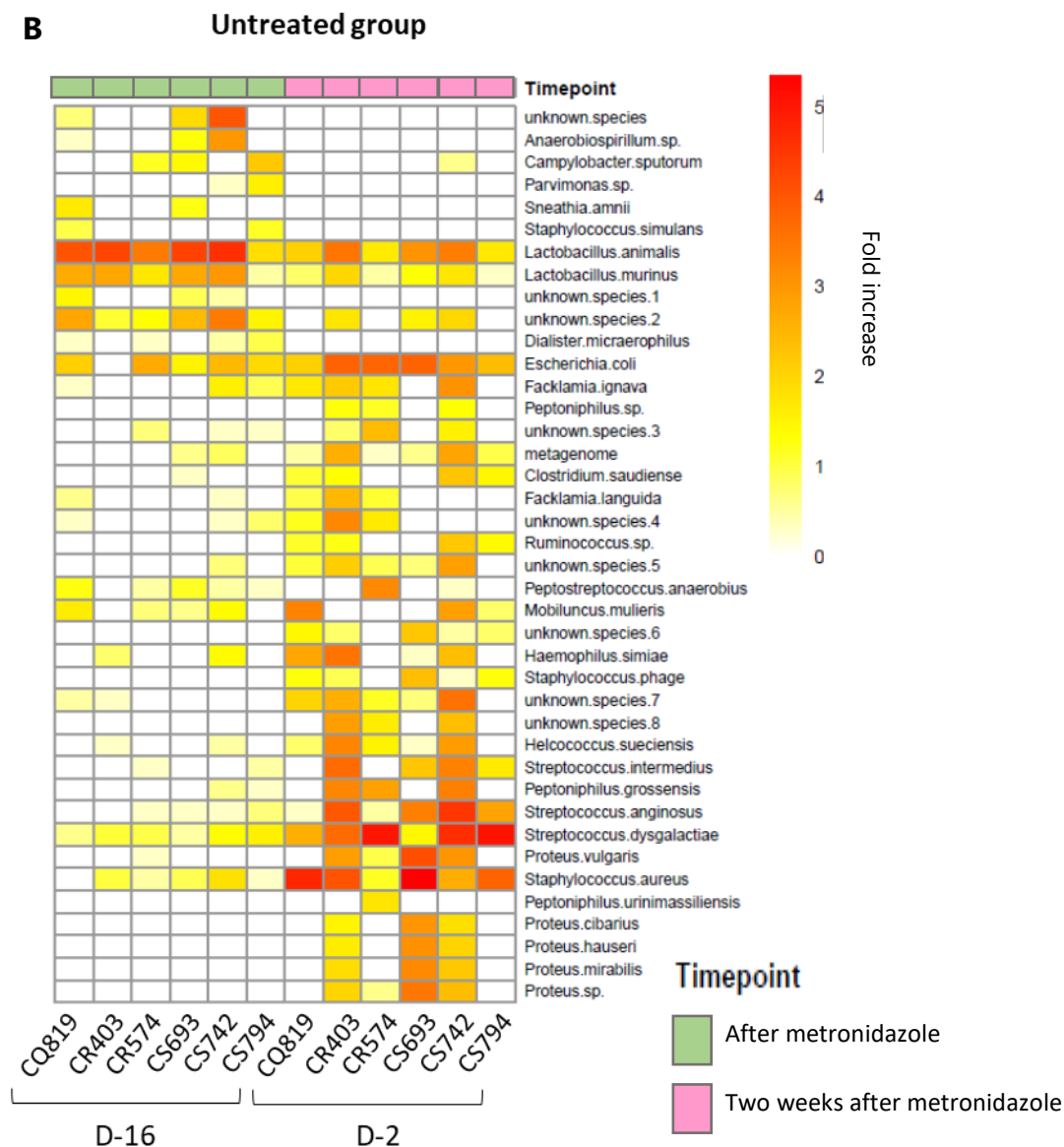


Figure 4: Variation of vaginal microbiota bacterial species after four inoculations of *L. crispatus* or not. A DEseq2 analysis was performed to evaluate differentially abundant species after *L. crispatus* inoculations compared to after metronidazole treatment. Heatmap representing the fold change after \log_{10} transformation of increased or decreased species abundances in both conditions in the *L. crispatus* treated group (A) and in the untreated group (B). Blue and pink time points match after *L. crispatus* inoculation (A) or no treatment (B) respectively. After metronidazole treatment are in green. The scale starts from 0 to above 5, red represent increase abundance of the species. Species identification are on the right of the heatmap and sample ID on the bottom.

Table 6: Affiliation of unknown species at the genus or family level associated with the figure 4A.

<i>L. crispatus</i> treated group		
Species	Genus	Family
Unknown species	<i>Shuttleworthia</i>	
Unknown species 1	<i>Succinivibrio</i>	
Unknown species 2	Unknown genus	<i>Actinomycetaceae</i>
Unknown species 3	<i>Sarcina</i>	
Unknown species 4	<i>Gemella</i>	

Table 7: Affiliation of unknown species at the genus or family level associated with the figure 4B.

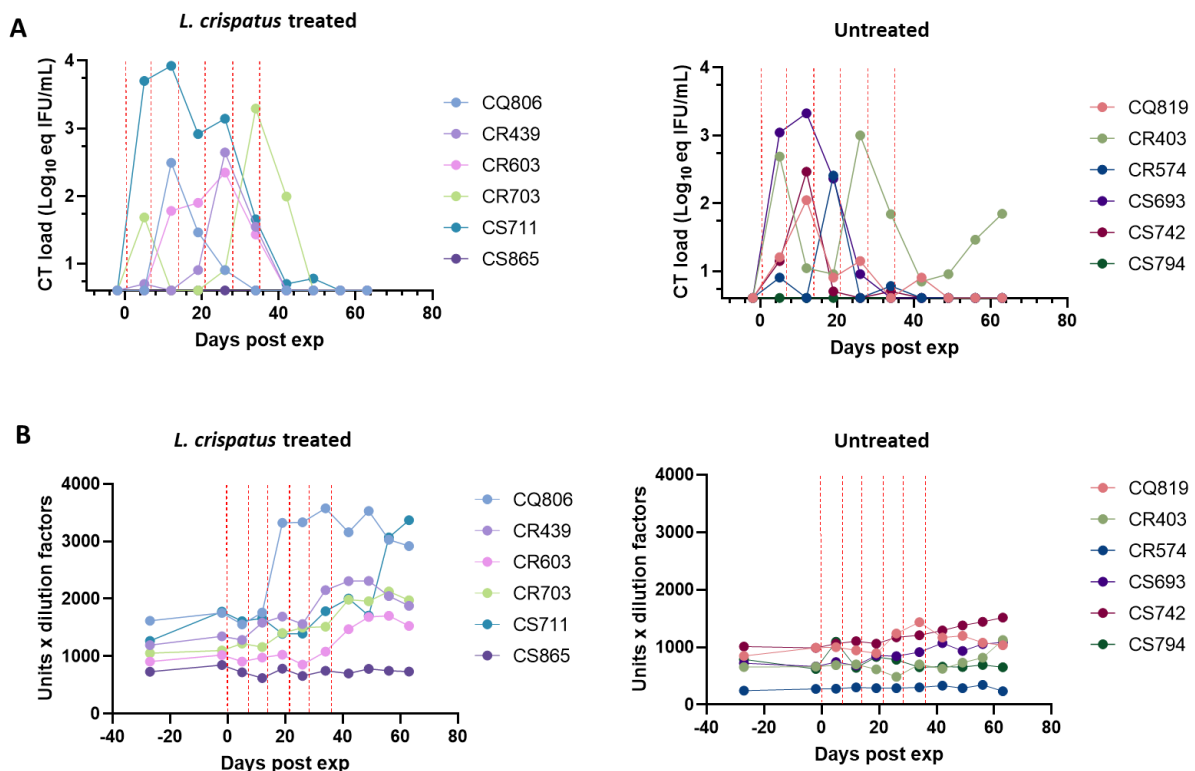
Untreated group	
Species	Genus
Unknown species	<i>Anaerobiospirillum</i>
Unknown species 1	<i>Parvimonas</i>
Unknown species 2	<i>Ruminococcus</i>
Unknown species 3	<i>Anaerococcus</i>
Unknown species 4	<i>Facklamia</i>
Unknown species 5	<i>Terrisporobacter</i>
Unknown species 6	<i>Staphylococcus</i>
Unknown species 7	<i>Helcococcus</i>
Unknown species 8	<i>Peptoniphilus</i>

No differences for CT infection were observed between the *L. crispatus* treated and untreated groups but specific anti-CT IgG production was higher in *L. crispatus* treated infected animals

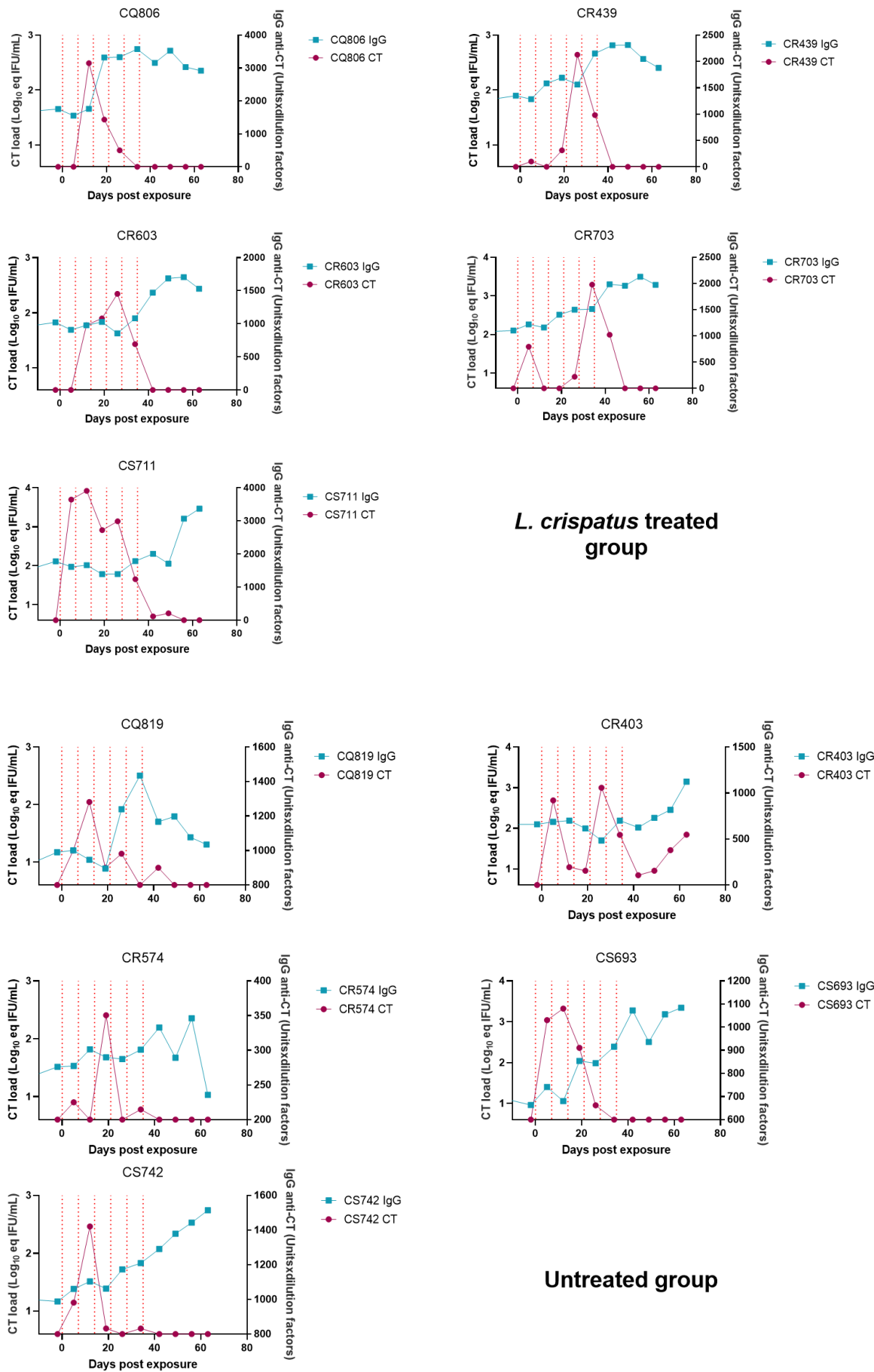
CT infection was monitored by quantifying CT load in cervicovaginal swabs and by measuring anti-CT IgG production in serum of all the females once a week. Two females, CS865 and CS794, one in each group neither were infected nor developed anti-CT IgG [Fig5 A & B]. The other females displayed a non-persisting infection independently of their group (*L. crispatus* treated or untreated) with one or more peaks of CT load. Individuals were mostly infected after the first or second inoculation [Fig5C]. Only CR439 from the treated group was infected after the third inoculation. Area under

the curve (AUC) for CT load was calculated for all individuals, and no significant differences was observed between the two groups [Fig5D, right]. Specific anti-CT IgG in the serum were detected between one to three weeks after a peak of CT load [Fig5C]. Graphical representation of CT specific IgG in both groups highlighted a higher production of antibodies in the serum of animals from the *L. crispatus* treated group [Fig5B]. AUC determination for anti-CT IgG curves showed a significant difference between both groups [Fig5C, right].

To conclude, ten animals out of twelve were infected but none of them developed a persistent infection. There was no difference of susceptibility to CT between the *L. crispatus* treated and untreated groups suggesting that *L. crispatus* probiotic treatment once a week does not protect from CT infection. However, animals that received *L. crispatus* treatment developed more anti-CT specific IgG.



C



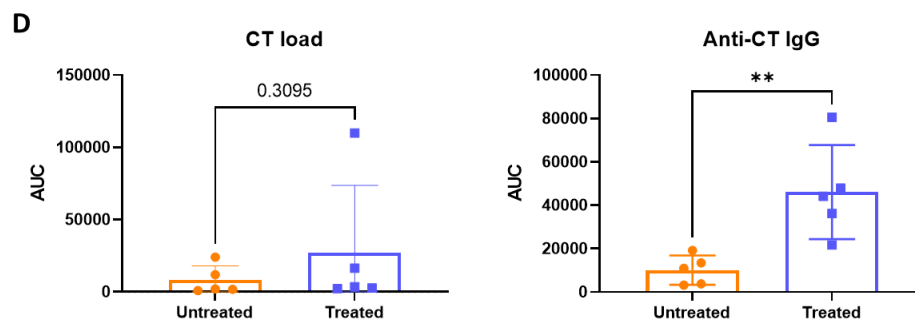


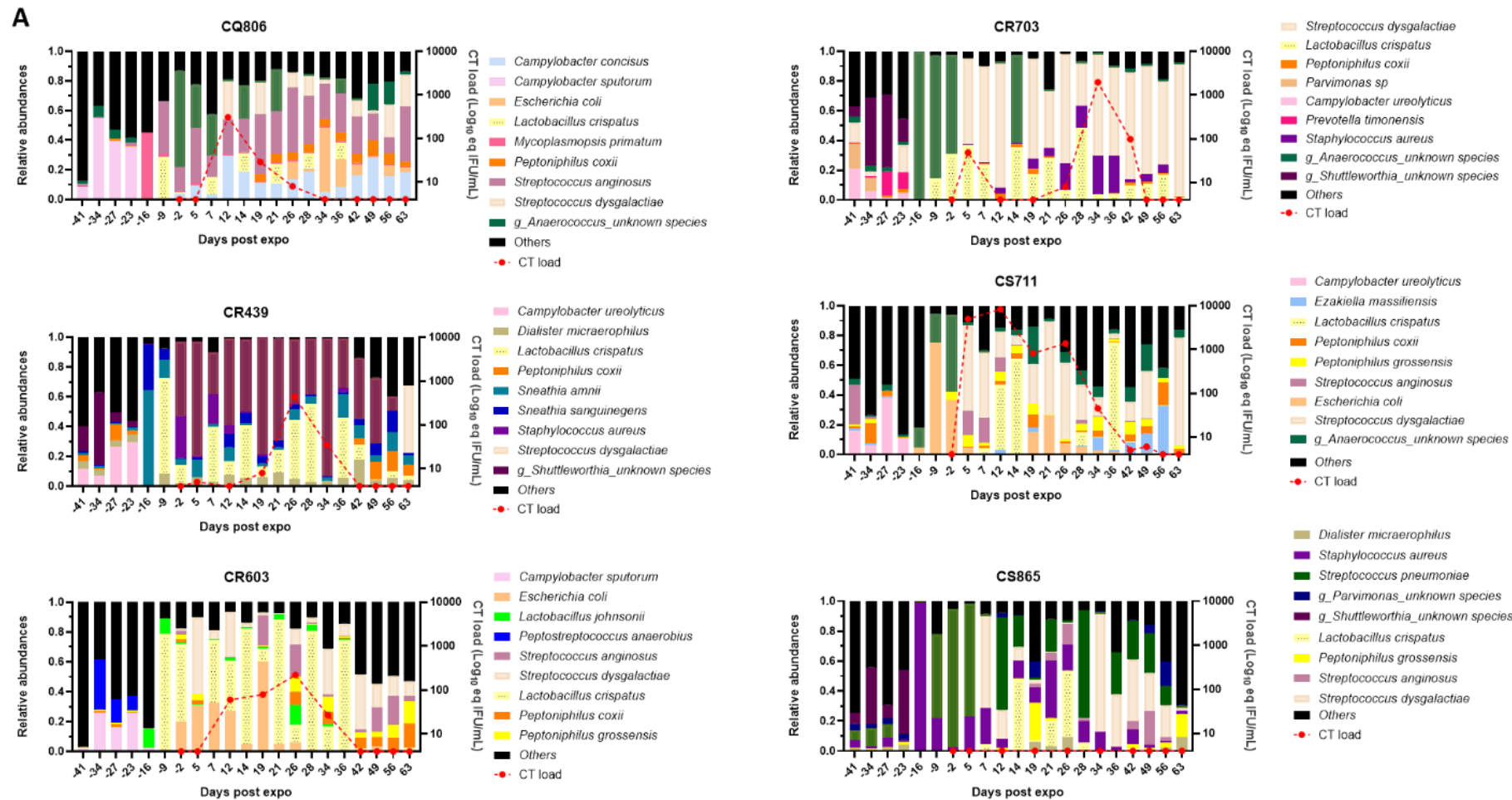
Figure 5: CT load and anti-CT IgG production in both groups. Graphical representation of (A) \log_{10} transformation of CT load and (B) anti-CT IgG production (Units x dilution factors) in *L. crispatus* treated (left) and untreated (right) animals throughout the study. One color represents one animal. (C) Individual representation of CT load (\log_{10} transformation) and anti-CT IgG production in each CT infected animal throughout the study. Blue and red curves match anti-CT IgG and CT load respectively. Left y-axis correspond to CT load and right y-axis correspond to anti-CT IgG. (D) Area under the curve (AUC) calculation of CT load (left) and anti-CT IgG (right). A Mann-Whitney test was performed to compare AUC of CT load or anti-CT IgG between groups. Asterisks indicate a statistically significant p value (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

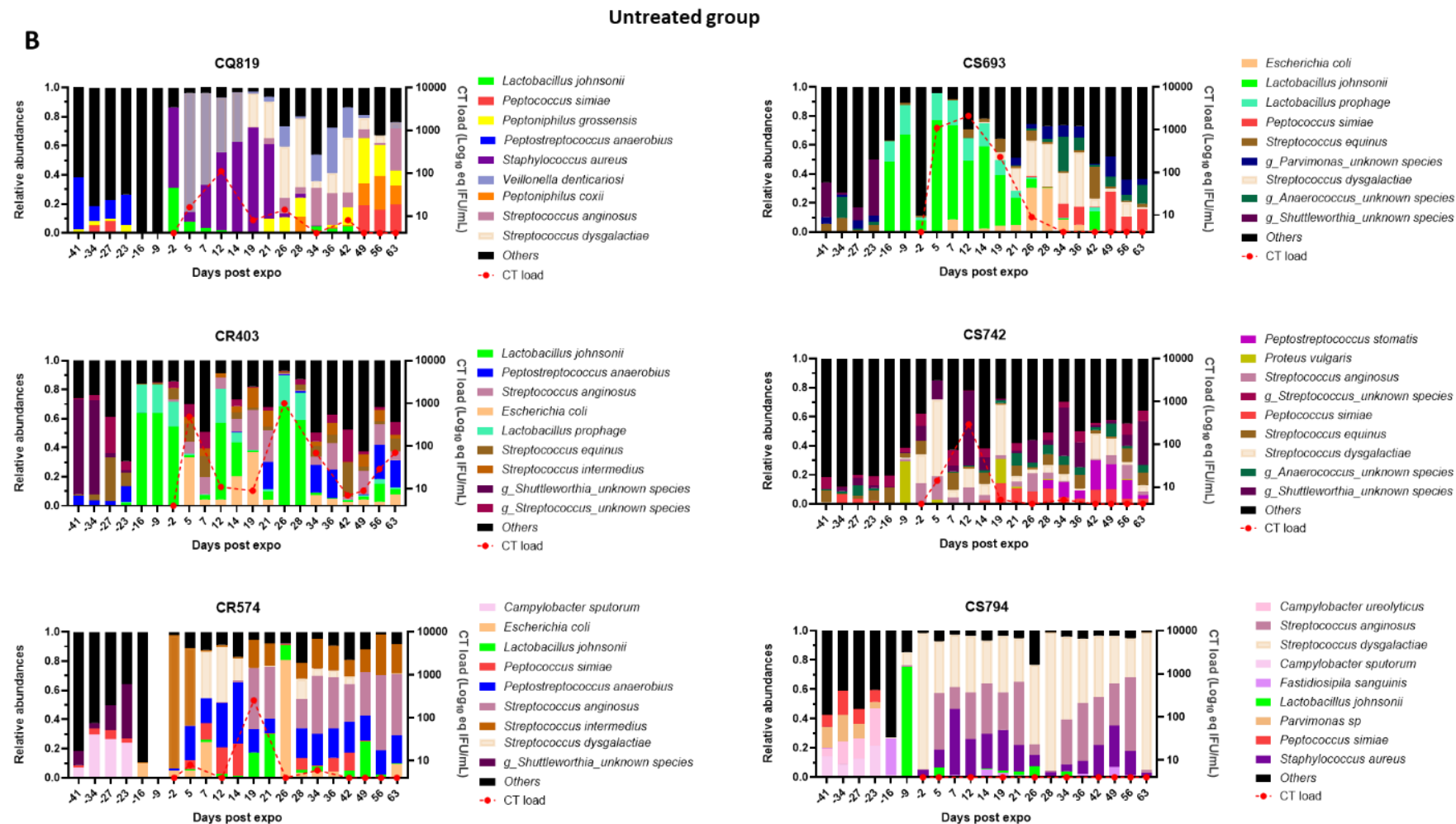
***L. johnsonii* abundance was associated with CT load**

To evaluate if CT load can be associated with a specific vaginal microbiota composition, individual bar plot of the top 9 most represented species in each animal was represented with CT load curves [Suppl fig5 A and B]. Variation of relative abundance was observed in each individual without highlighting a clear association between species abundances and CT load. Then, to study the impact of *Lactobacillus spp.* on CT susceptibility, individual bar plot representation of *Lactobacillus spp* abundance overlaid with the bacterial load was made [Fig6 A and B]. In the treated group, the main species of *Lactobacillus* observed was *L. crispatus* [Fig6A]. It's abundance varied according to animals and time points. In the untreated group, *L. johnsonii* was the main *Lactobacillus* species observed [Fig6B]. It's abundance varied according to animal but two females (CR403 and CS693) had high abundance of *L. johnsonii* at most of the time points. Interestingly, peaks of CT matched with high abundance of *L. johnsonii*. To further investigate if a specific bacterial composition at CT inoculation could facilitate

or protect CT infection, the vaginal microbiota composition was compared in each group between CT inoculations that induced an infection or not. In the treated group, rather similar bacterial abundances were observed between CT inoculation that triggered an infection or not. However, in the untreated group, a higher abundance (abundance at CT inoculation leading or not to infection) of *L. johnsonii* (4.73% vs 27%) and *L. prophage* (1.13% to 7.94%) was observed [Suppl fig6A]. Indeed, a positive association was determined, in the untreated group, between *L. johnsonii* (Spearman r: 0.43 ; p value: 0.0084) or *L. prophage* (Spearman r: 0.45 ; p value: 0.0084) with CT [Suppl fig6B]. Simple linear regression analysis also demonstrated an association between relative abundance of *L. johnsonii* or *L. prophage* with CT load [Suppl fig6C]. No positive or negative association was noticed in the *L. crispatus* treated group with *Lactobacillus spp* and the CT load [Data not shown]. Therefore, CT load seems to be positively associated to higher abundance of *L. johnsonii* and *L. prophage*.

L. crispatus treated group





Supplementary Figure 5: Vaginal microbiota composition at the species level of *L. crispatus* treated (A) and untreated animals (B) throughout the study. Percentage of relative abundance of the top 9 most represented species in each animal are represented in bar plot. A red dotted line represents CT load in each animal.

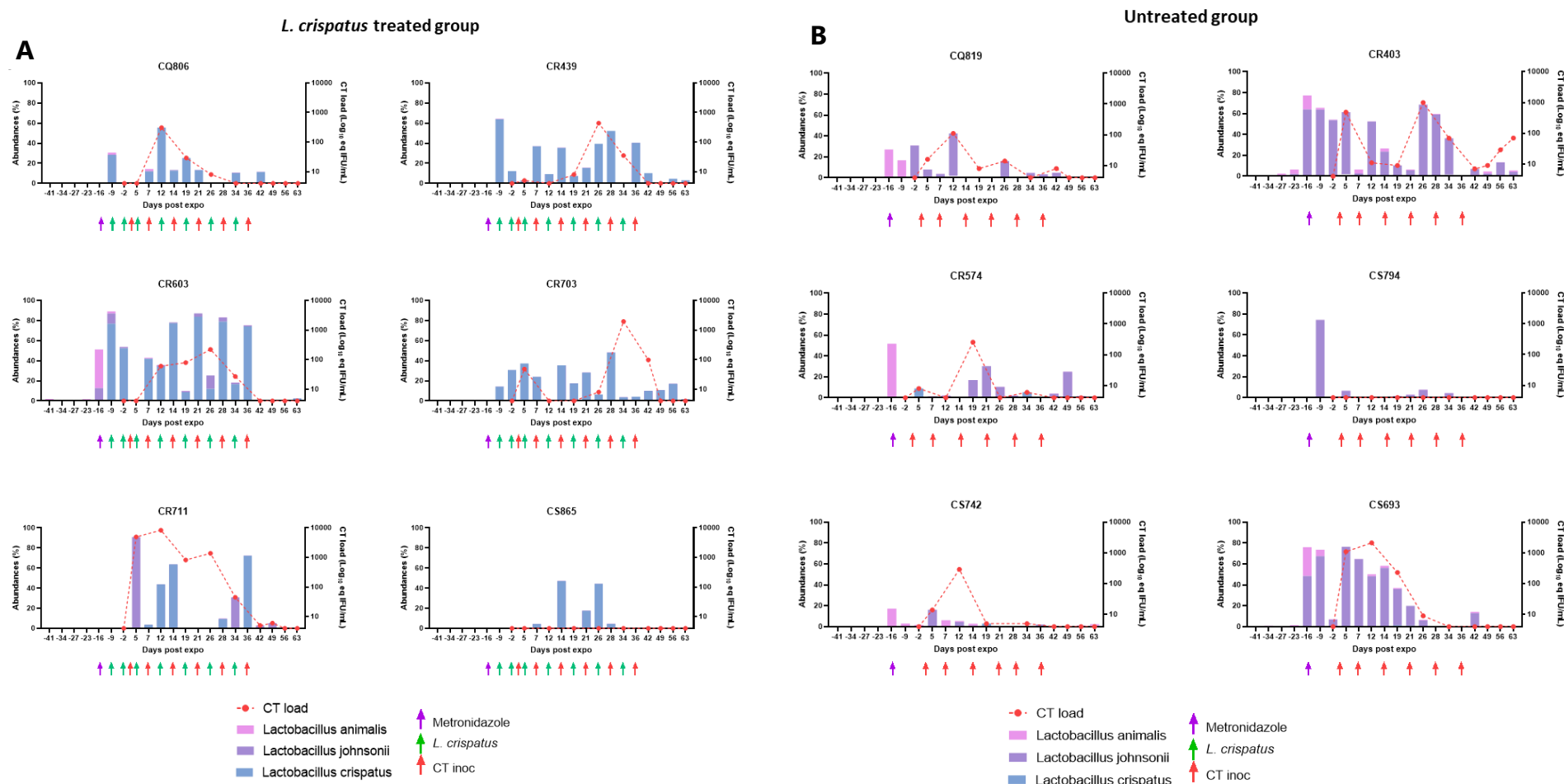
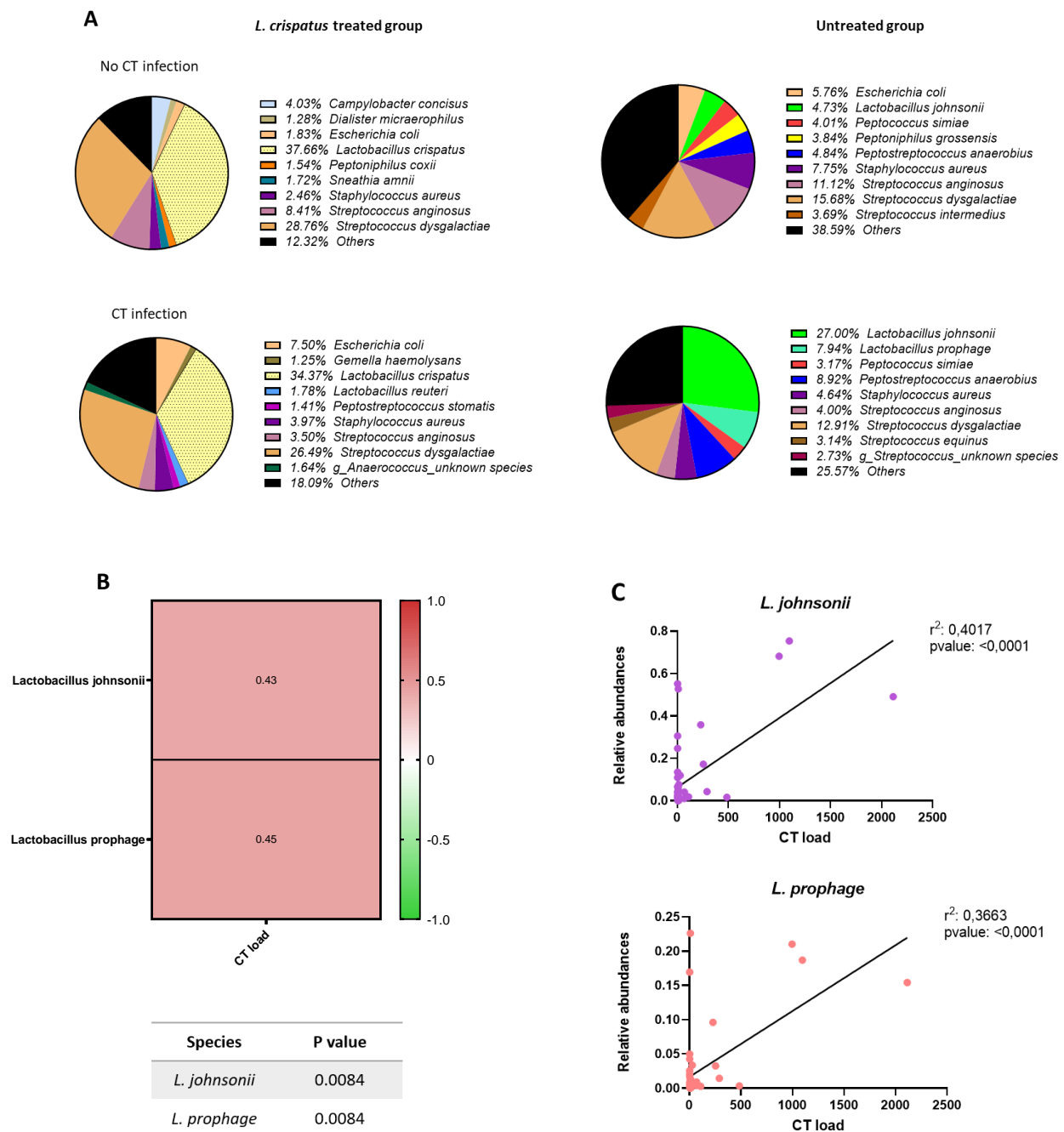


Figure 6: Vaginal *lactobacillus* spp abundances and CT load in each animal. Individual bar plot representing *Lactobacillus* spp abundances (%) in each animal from the *L. crispatus* treated group (A) and untreated group (B) superposed with CT load after log₁₀ transformation throughout the study. Red dotted line represent CT load. Bar plot color of *L. animalis* is pink, *L. johnsonii* is purple and *L. crispatus* is blue. Purple, green and red arrows represent metronidazole treatment, *L. crispatus* inoculation and CT inoculation respectively.



Supplementary figure 6: Association between vaginal microbiota bacterial species and CT infection. (A) Top 9 most represented species at CT inoculations that trigger a peak of CT load or not in *L. crispatus* treated animal (left) or untreated animals (right) are represented in pie chart. (B) Spearman correlation test was performed to determine association between *L. johnsonii* or *L. prophage* relative abundance and CT load. Heatmap representing the Spearman correlation coefficient was generated. Green represents a negative association and red a positive association. A table with the p value obtained was also generated. (C) Simple linear regression was performed to compared the relative abundance of *L. johnsonii* or *L. prophage* with CT load.

Microbiota changes upon CT infection

Secondly, to determine the impact of CT infection on the vaginal microbiota composition, the samples of each animal during CT inoculation were divided into three groups: before, during or after CT infection, independently of their group affiliation [Fig7A and B]. Samples collected during follow up thus after CT inoculations were excluded of the analysis to avoid the effect of CT inoculation on the vaginal microbiota. For those analyses, the two females (CS865 and CS794) that were not infected were removed from the analyses. The microbiota composition was altered by CT infection in most of the individuals. To investigate bacterial taxa that were differentially abundant according to CT infection, a heatmap representing the log10 expression of bacterial taxa abundance was generated between the time points before CT and during CT infection (CT+) in all individuals. CT+ samples exhibited an increase abundance of many bacteria taxa including bacteria from the genus *Anaerococcus*, *Blautia* and *Succinivibrio* for instance [Fig8A ; table 8]. Other bacteria were increased in some individuals during CT infection (*Peptostreptococcus spp.*, *Parvimonas spp...*). A second heatmap representing the log10 expression of bacterial taxa that are differentially abundant before and after CT infection was generated [Fig8B ; table 9]. After CT infection, there is an increased abundance of many bacterial taxa such as *Peptoniphilus spp.* and bacteria from the genus *Succinivibrio*, among others [Fig8B ; table 9]. CT infection might triggered significant modifications of the vaginal microbiota at the infection but also after CT infection.

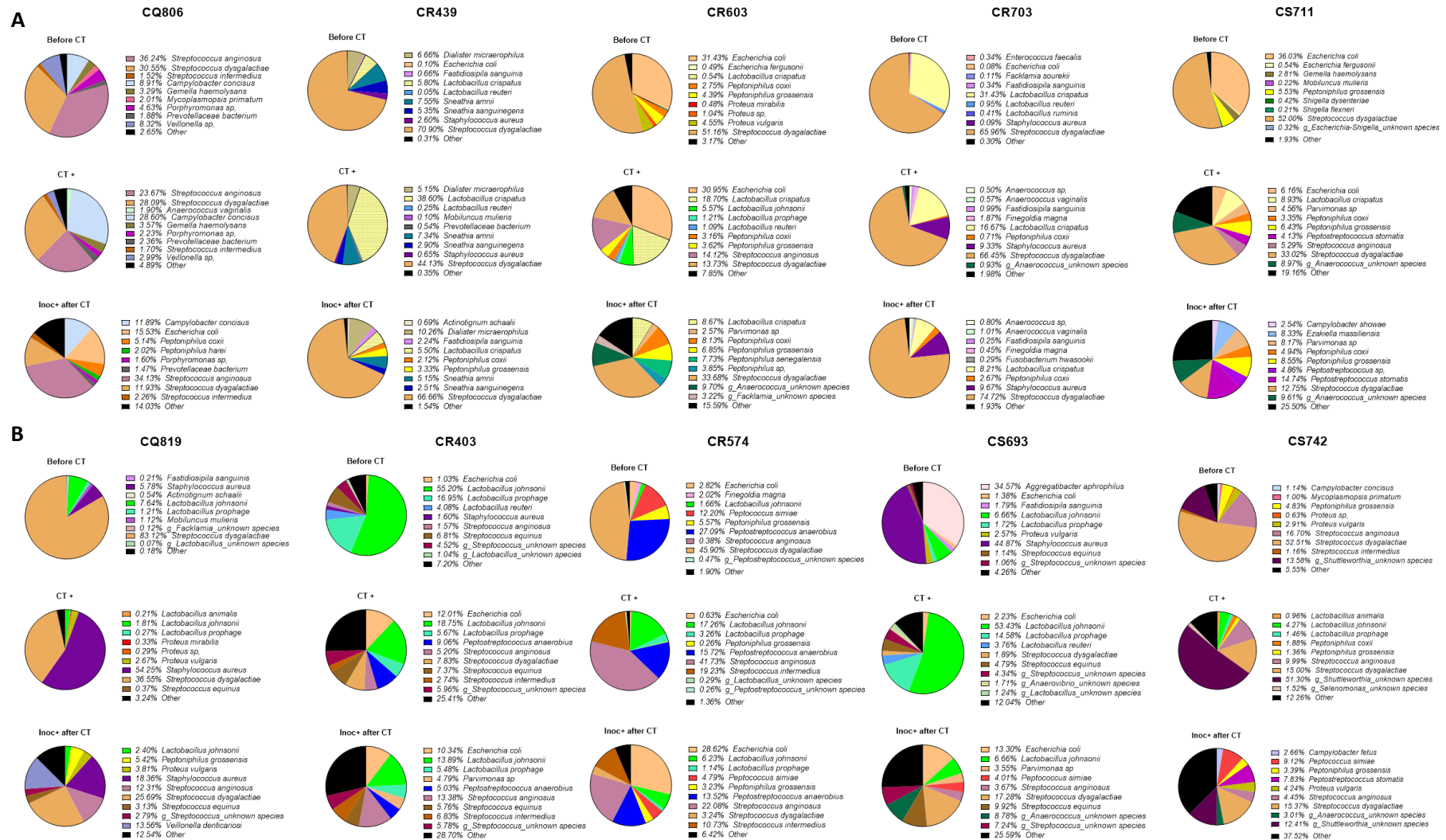
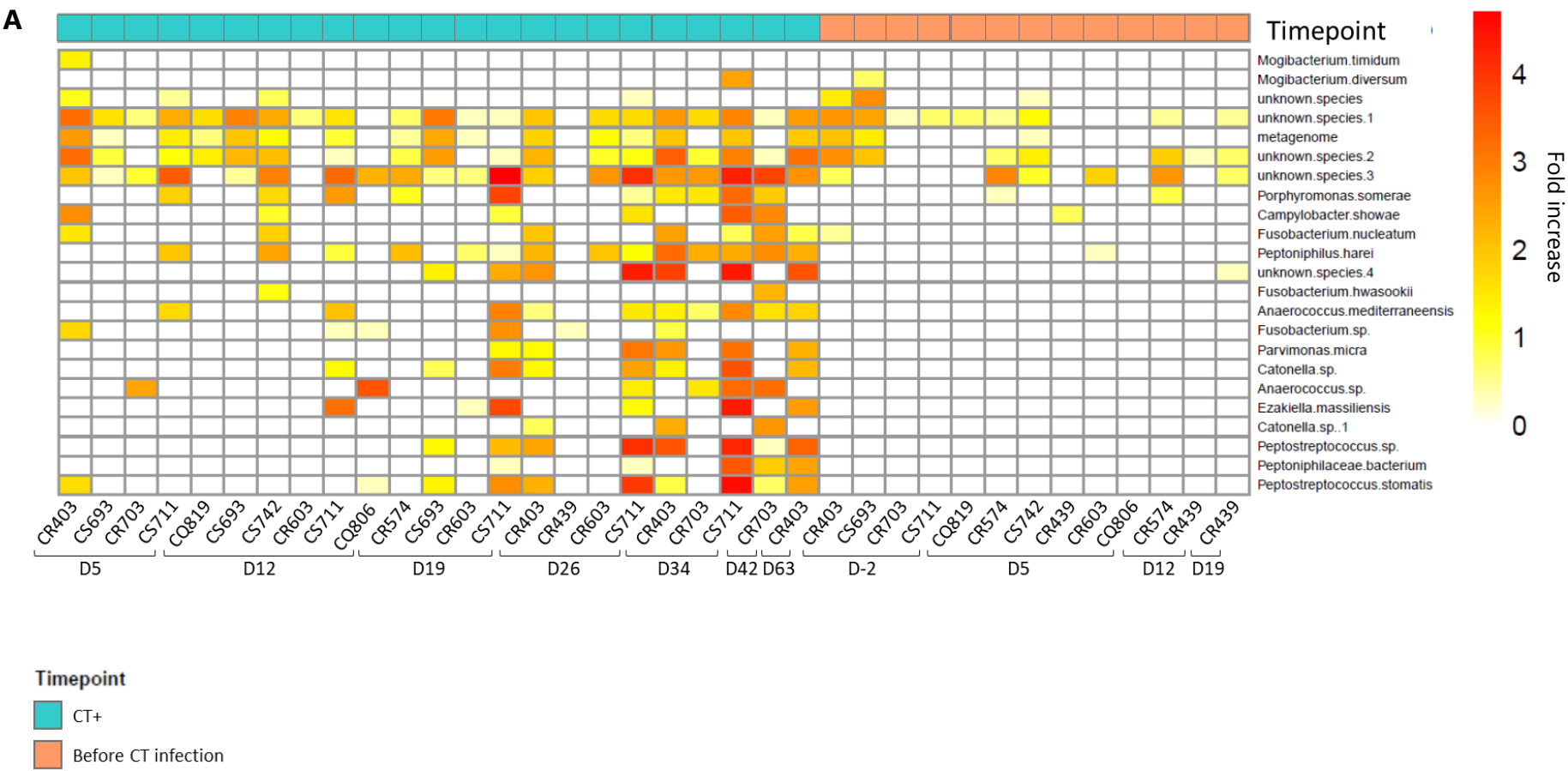


Figure 7: Alteration of vaginal microbiota composition by CT infection for each animal. Pie chart representing the top 9 most represented species in each animal before CT infection (before CT), at the infection (CT+) and during inoculation but after CT infection (inoc + after CT) in the *L. crispatus* treated group (A) and in the untreated group (B).



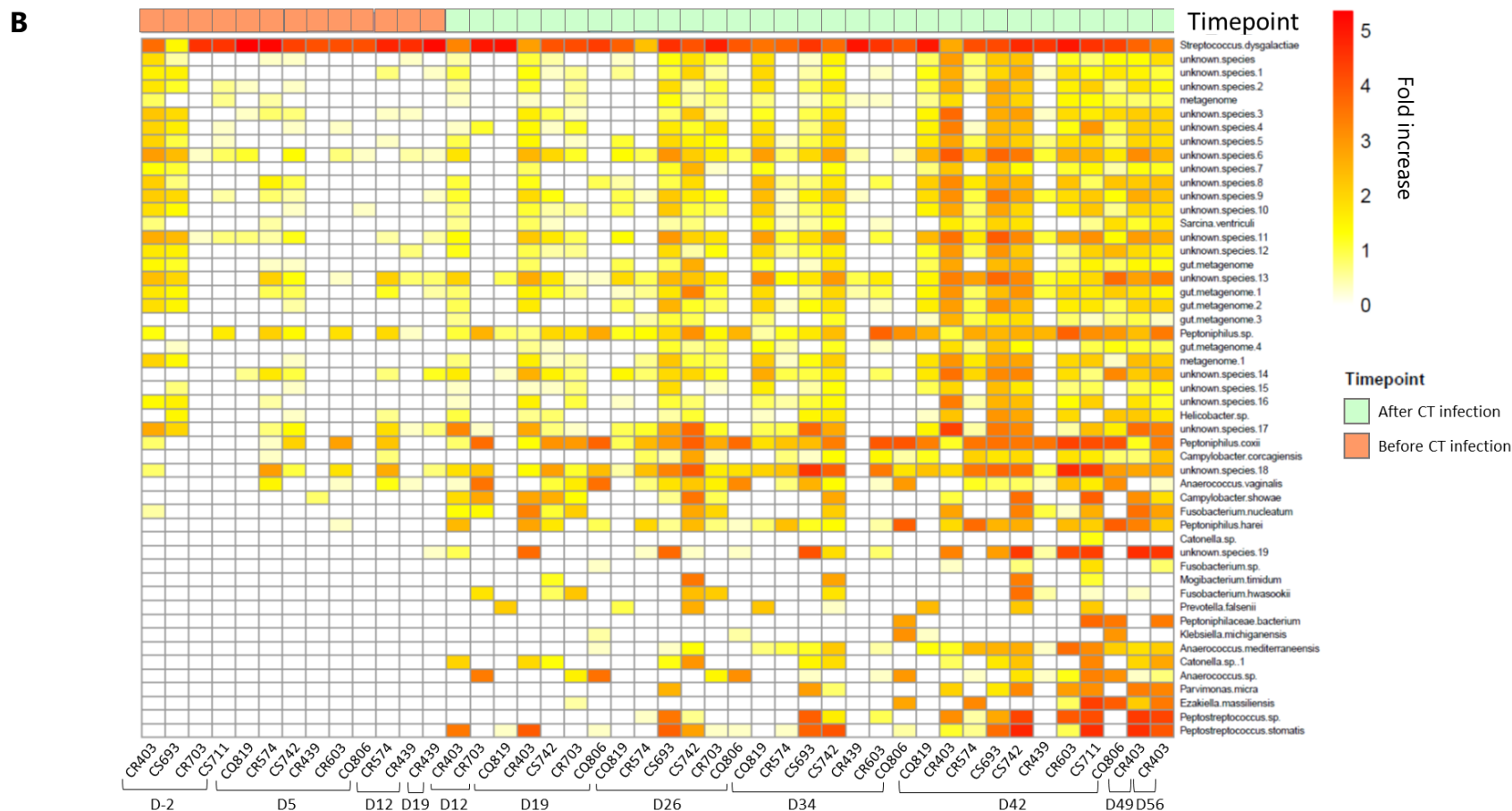


Figure 8: Variation of vaginal microbiota bacterial species at CT infection and after CT infection in all animals. A DESeq2 analysis was performed to evaluate differentially abundant species at the CT infection or after CT infection compared to before CT infection. Heatmap representing the fold change after \log_{10} transformation of increased or decreased species abundances in both conditions at CT infection (CT+) (A) and after CT infection (B) in comparison to before CT exposition. Turquoise, light green and orange time points match CT+, after CT and before CT infection respectively. The scale starts from 0 to above 5, red represents increase abundance of the species. Species identification are on the right of the heatmap and sample ID on the bottom.

Table 8: Affiliation of unknown species at the genus or family level associated with the figure 8A.

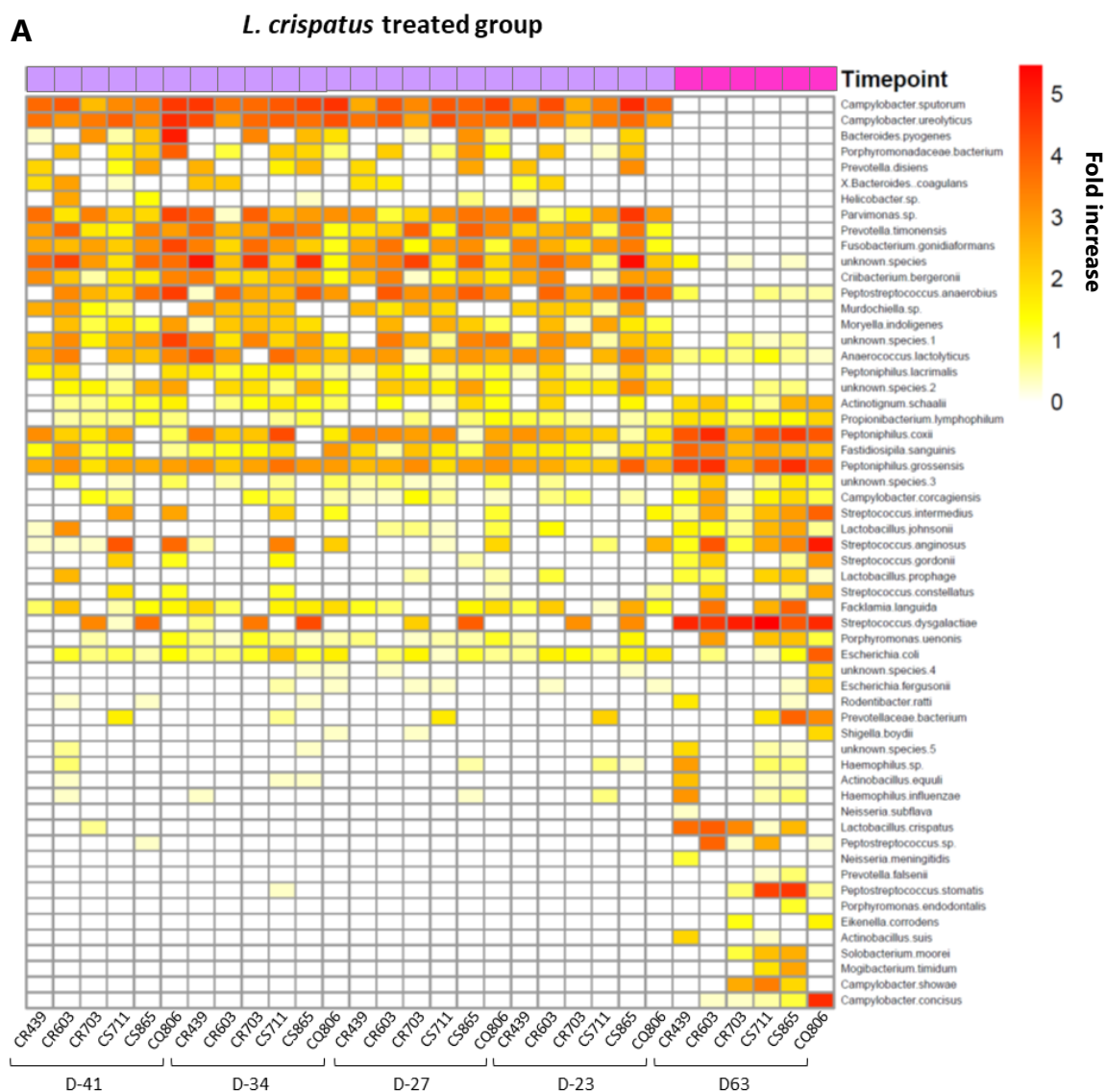
Before CT vs after CT		
Species	Genus	Order
Unknown species	<i>Christensenellaceae R7 group</i>	
Unknown species 1	<i>UCG-002</i>	
Unknown species 2	<i>Marvinbryantia</i>	
Unknown species 3	<i>Rikenellaceae RC9 gut group</i>	
Unknown species 4	<i>Peptococcus</i>	
Unknown species 5	<i>[Ruminococcus] gauvreauii group</i>	
Unknown species 6	<i>UCG-008</i>	
Unknown species 7	<i>Oribacterium</i>	
Unknown species 8	<i>Terrisporobacter</i>	
Unknown species 9	<i>Subdoligranulum</i>	
Unknown species 10	<i>Dorea</i>	
Unknown species 11	<i>Blautia</i>	
Unknown species 12	<i>Unknown genus</i>	<i>Clostridia UCG-014</i>
Unknown species 13	<i>Sarcina</i>	
Unknown species 14	<i>Clostridium sensu stricto 1</i>	
Unknown species 15	<i>Fournierella</i>	
Unknown species 16	<i>Prevotella_9</i>	
Unknown species 17	<i>Succinivibrio</i>	
Unknown species 18	<i>Anaerococcus</i>	
Unknown species 19	<i>Parvimonas</i>	

Table 9: Affiliation of unknown species at the genus or family level associated with the figure 8B.

Before CT vs CT	
Species	Genus
Unknown species	<i>Aggregatibacter</i>
Unknown species 1	<i>Blautia</i>
Unknown species 2	<i>Succinivibrio</i>
Unknown species 3	<i>Anaerococcus</i>
Unknown species 4	<i>Parvimonas</i>

Persistent alteration of the vaginal microbiota after metronidazole with or without *L. crispatus* treatment

Finally, to determine if the vaginal microbiota composition return to baseline after CT inoculations, a differential analysis was performed in both groups at the last time point of the study (D63) compared to baseline [Fig9 A and B ; table 10 and 11]. The vaginal microbiota at the last time point was different compared to baseline. Treated animals had more *Peptoniphilus spp*, *Streptococcus spp*. As for the untreated group, *Streptococcus spp*, *Proteus spp* and *Peptostreptococcus spp* were increased at the end of follow up timepoint compared to the baselines. Therefore, a persistent modification of the vaginal microbiota occurred in all animals.



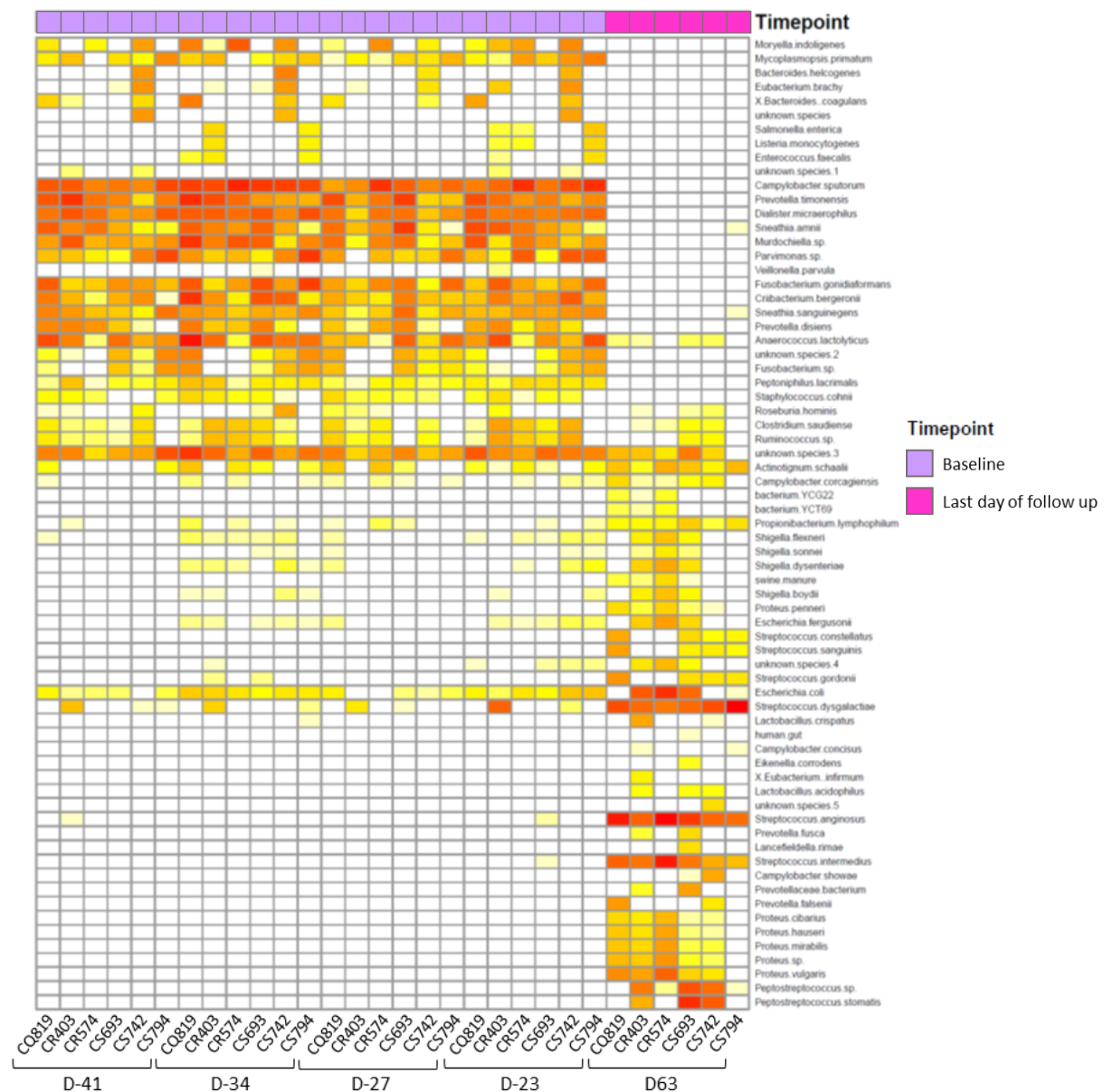
B**Untreated group**

Figure 9: Variation of vaginal microbiota bacterial species at the last time point of the protocol in each group. A DEseq2 analysis was performed to evaluate differentially abundant species at the last time point of the protocol compared to baseline. Heatmap representing the fold change after \log_{10} transformation of increased or decreased species abundances in both conditions in *L. crispatus* treated group (A) or untreated group (B). Fuchsia and purple time points match last time point of the protocol and baseline respectively. The scale starts from 0 to above 5, red represents increase abundance of the species. Species identification are on the right of the heatmap and sample ID on the bottom.

Table 10: Affiliation of unknown species at the genus or family level associated with the figure 9A.

<i>L. crispatus</i> treated group	
Species	Genus
Unknown species 1	<i>Shuttleworthia</i>
Unknown species 2	<i>Fusobacterium</i>
Unknown species 3	<i>Peptostreptococcus</i>
Unknown species 4	<i>Ezakiella</i>
Unknown species 5	<i>Escherichia-Shigella</i>
Unknown species 6	<i>Haemophilus</i>

Table 11: Affiliation of unknown species at the genus or family level associated with the figure 9B.

Untreated group	
Species	Genus
Unknown species 1	<i>Catonella</i>
Unknown species 2	<i>Alloprevotella</i>
Unknown species 3	<i>Fusobacterium</i>
Unknown species 4	<i>Peptoniphilus</i>
Unknown species 5	<i>Escherichia-Shigella</i>
Unknown species 6	<i>Anaerosporebacter</i>

Cytokines induced by CT infection were different according to groups and compartments

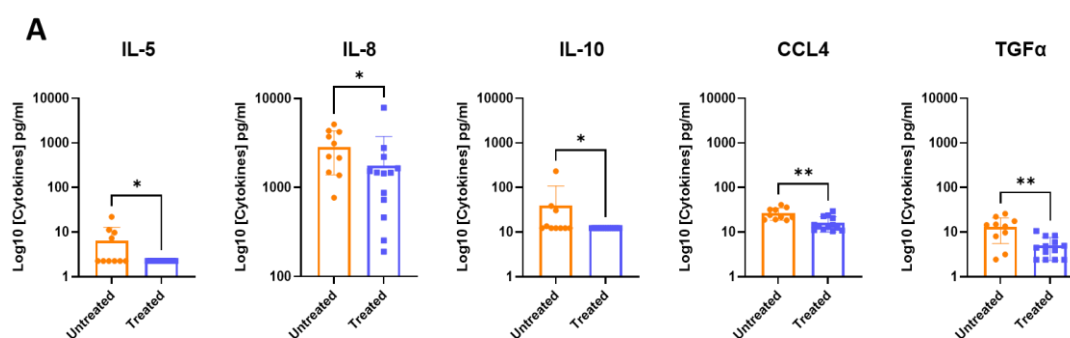
Cytokine and chemokine expressions were measured in the serum from peripheral blood and in cervicovaginal fluids of all animals once a week throughout the study.

Cytokine concentration in peripheral blood

In peripheral blood serum, basal expression of IL1-RA, IL-2, IL-8, CCL2, CCL4, TGF α , VEGF and G-CSF were detected in each animal. No significant differences were observed in terms of cytokine production between baseline and two weeks after antibiotic in all animals.

The impact of CT infection on peripheral blood cytokine concentration was then determined by comparing cytokine expression during CT inoculation but before CT

infection, during CT infection and after CT infection (all samples collected after CT infection including samples during inoculation and follow up). For those analyses, the two females (CS865 and CS794) that were not infected were removed from the analyses. No significant modification of cytokine expression within each group during CT infection was observed [Data not show]. However, differences of cytokine concentration between groups were observed. IL-5, CCL4, IL-10, TGF α and IL-8 concentration was higher in untreated animal compared to *L. crispatus* treated animal during CT infection [Fig10A]. Subsequently, kinetics of cytokine concentration was analysed throughout the study. Heatmap representing median fold change of cytokine expression based on the concentration determined two days before CT inoculation was generated. Higher median expression of several cytokines was noticed in untreated compared to treated animals after CT infection [Fig10B]. Indeed, cytokine concentration (IFN γ , IL-1RA, IL-2, IL-10, IL-12/23, IL-15, CCL2, CCL4, CCL3, TGF α , TNF α and VEGF) were significantly higher in untreated animals compared to treated animals after CT infection [Fig10C]. These results demonstrate an increased cytokine production after CT infection in untreated animals compare to treated animals. This higher level of cytokines after CT infection can be partially attributed to two females, CR403 and CR574 [Suppl figure 7].



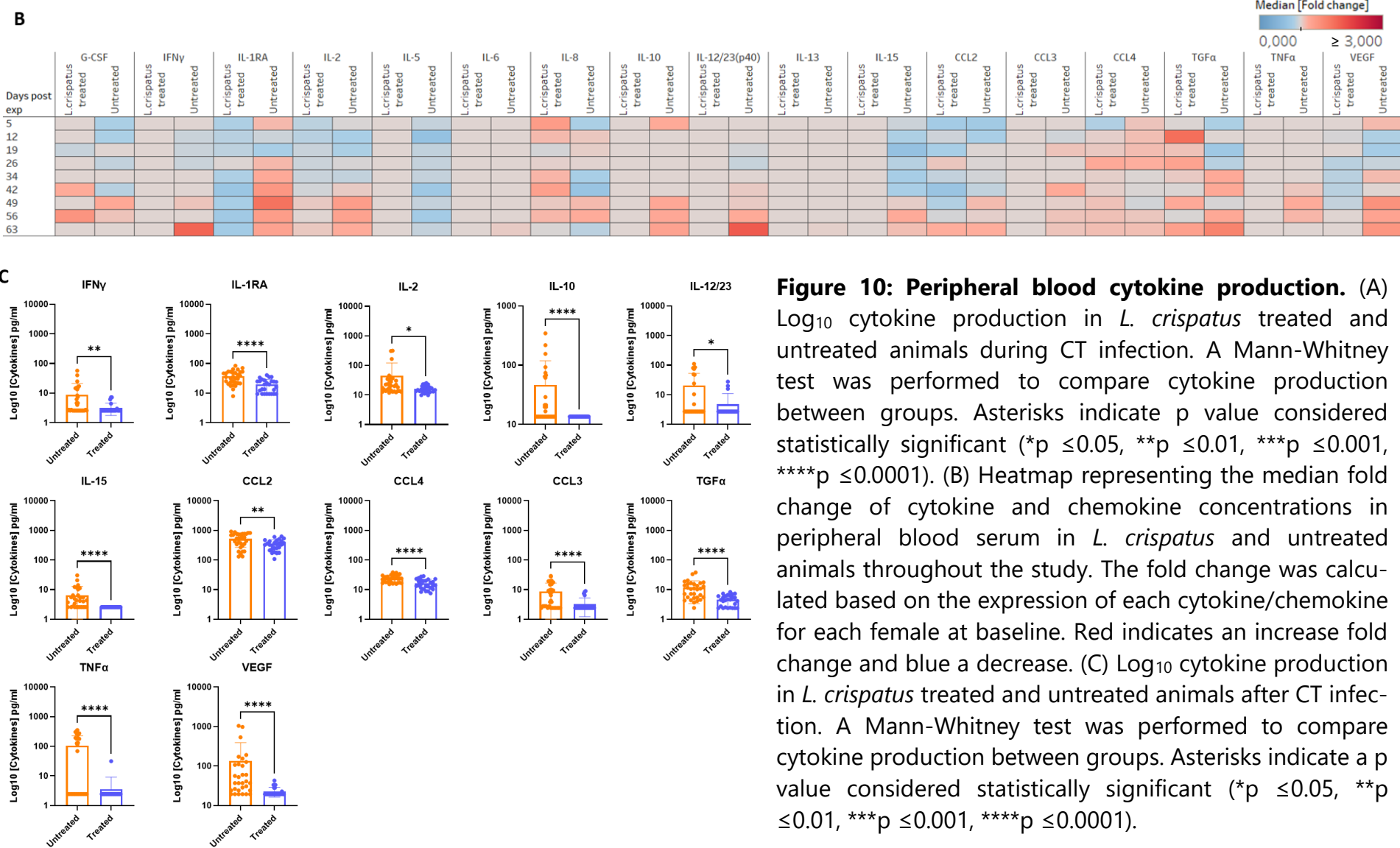
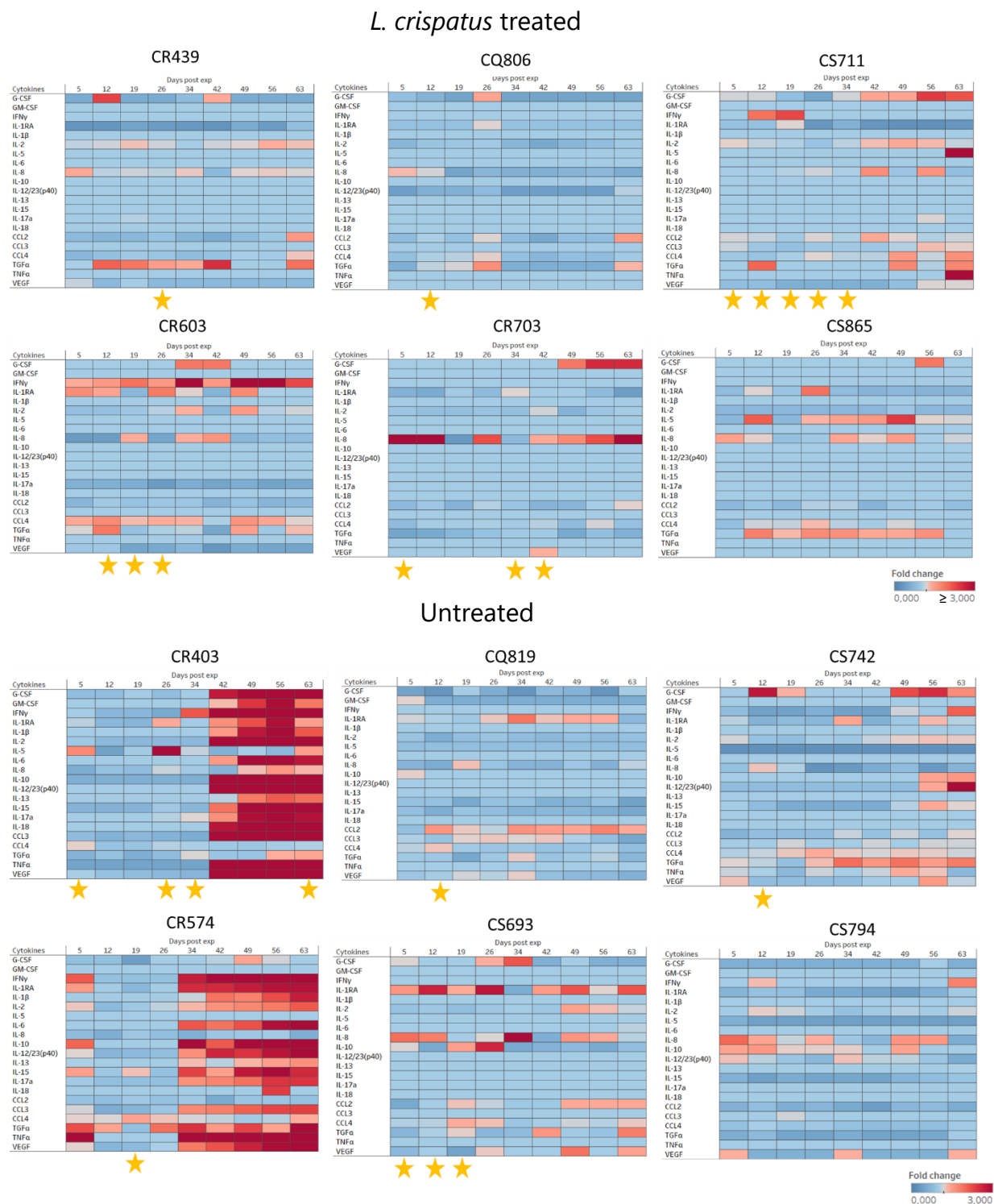


Figure 10: Peripheral blood cytokine production. (A) Log₁₀ cytokine production in *L. crispatus* treated and untreated animals during CT infection. A Mann-Whitney test was performed to compare cytokine production between groups. Asterisks indicate p value considered statistically significant (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). (B) Heatmap representing the median fold change of cytokine and chemokine concentrations in peripheral blood serum in *L. crispatus* and untreated animals throughout the study. The fold change was calculated based on the expression of each cytokine/chemokine for each female at baseline. Red indicates an increase fold change and blue a decrease. (C) Log₁₀ cytokine production in *L. crispatus* treated and untreated animals after CT infection. A Mann-Whitney test was performed to compare cytokine production between groups. Asterisks indicate a p value considered statistically significant (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).



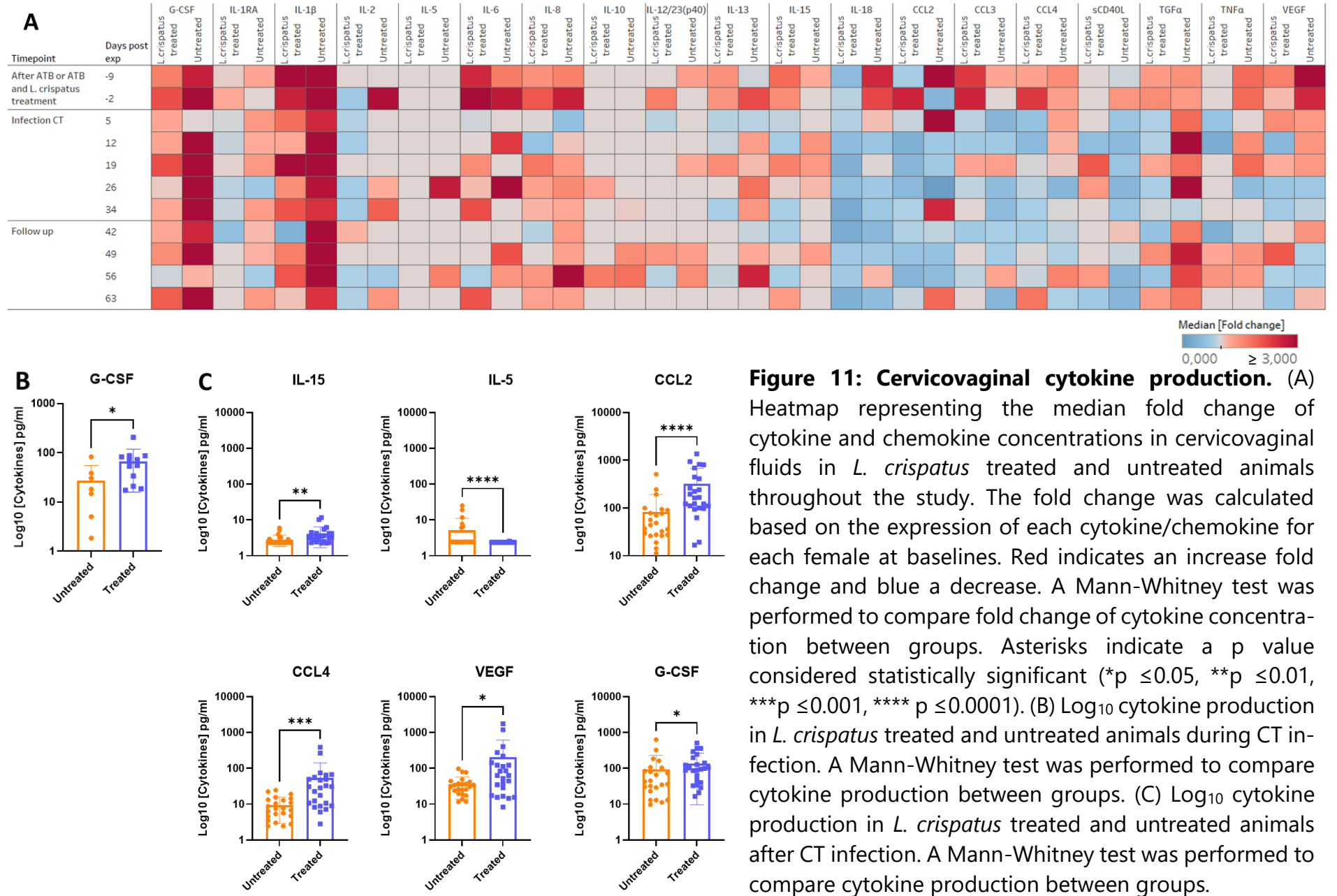
Supplementary figure 7: Peripheral blood cytokine concentration in each animal. Heatmap representing the fold change of cytokine and chemokine concentrations in peripheral blood serum in each animal throughout the study. The fold change was calculated based on the expression of each cytokine/chemokine for each female at baselines. Red indicates an increase fold change and blue a decrease. Yellow stars represent CT infection.

Cytokine expression in cervicovaginal fluids

Within cervicovaginal fluids, various cytokines including IL-1 β , IL-6, CCL2, G-CSF, IL-1RA, IL-8, IL-18 and VEGF, were detectable at baseline. To study the effect of antibiotic treatments on cytokine concentration in cervicovaginal fluids, each concentration was compared to baseline value. In all analysis evaluating cervicovaginal cytokine production, samples collected during menstruation were excluded since we have previously shown (C. Adapen, submitted manuscript) that during the menstruation cervicovaginal cytokine concentrations are increased. Analysis of cytokine production after antibiotic treatment in each individuals demonstrated cytokine concentration alterations that were female specific [Data not shown].

To study the local production of cytokines in each group throughout the study, a heatmap was generated, representing the median fold change expression for cytokines quantified after baseline, compared to the basal concentration [Fig11A]. Higher concentration of G-CSF, IL-1 β , IL-13, CCL2, TGF α , TNF α and VEGF was observed in the untreated group compared to the treated group. These differences between the two groups were observed starting from one week after metronidazole treatment and throughout the whole study, however the differences are not significant [Data not show]. We then, studied the impact of CT inoculation and infection on cervicovaginal cytokine production. The two uninfected animals (CS865 and CS794) were removed from the analysis. No significant differences in terms of cytokine expression were observed during CT infection in all animals or in each group [Data not show]. However, an increased expression of G-CSF was noticed in treated animals compared to the untreated during CT infection [Fig11B]. In addition, treated animals exhibited higher level of CCL2, G-CSF, VEGF, CCL4 and IL-15 after CT infection whereas untreated individuals had an increased expression of IL-5 compared to treated animal [Fig11C].

Overall, those results demonstrated an effect of antibiotic treatment on cytokine and chemokine concentrations. Moreover, CT infection induced higher cytokine expression in cervicovaginal fluids of treated compared to untreated animals.



Increase of mature and activated neutrophils after CT infection in peripheral blood but not in cervicovaginal cytobrushes

Neutrophil phenotype was performed on whole blood and on cervicovaginal cells at five time points: two baselines, after antibiotic treatment, one week after the six CT inoculations and four weeks after the six CT inoculations. In the blood, two populations were observed: CD11b⁺ CD101⁺ CD10⁺ CD32a⁺ and CD11b⁺ CD101⁺ CD10⁻ CD32a⁺ characterized as mature and immature subsets respectively [Fig12]. Mean mature subset (Mean \pm SD) of baselines in the treated group represents $42.5\% \pm 24.5$ and the immature subset represents $54.7\% \pm 24.9$ of the total neutrophil population respectively. Similar percentage were observed in the untreated group (mature: 39.4 ± 18.7 ; immature: 57.8 ± 19). No impact of metronidazole treatment was noticed neither on subpopulations nor on the activation status on total neutrophils [Fig 12 and 13]. However, CT inoculations significantly increased the mature subset thus decreasing the immature one in both groups. Interestingly, for the treated group, these modifications were observed one week and four weeks after the six CT inoculations [Fig12A]. On the contrary, the alteration of neutrophil subpopulations in the untreated group was significant only one week after the six CT inoculations [Fig12B]. To study the expression of activation and maturation markers, the mean fluorescence intensity on total neutrophil populations was analysed [Fig13]. In both groups, CD32a, HLA-DR and CD10 expression was significantly increased after CT inoculations whereas CD101 and CD62L expression was decreased. CD101 expression was decreased in both groups at four weeks after CT inoculations [Fig13A and C]. Furthermore, HLA-DR expression was significantly increased in both group one and four weeks after CT inoculation [Fig13B and D]. In *L. crispatus* treated animals, CD32a and CD10 expression were increased at one and four weeks after CT inoculations whereas in the untreated group CD32a and CD10 were significantly increased only one week after CT inoculations [Fig13]. Finally, untreated individuals exhibited a decreased expression of CD62L at both time points after CT inoculations whereas it was significantly decreased only four weeks after CT inoculation in treated animals [Fig13B and D]. Overall, CT infection induced an accumulation of mature (CD10⁺) and activated (CD32⁺, HLA-DR⁺) neutrophils in peripheral blood in both groups.

Interestingly, the activation status (CD32a expression) and maturation (CD10) of neutrophils last longer in treated animal compared to untreated animals.

In cervicovaginal cytobrushes, three main subsets of neutrophils were observed: CD11b^{high} CD101⁺ CD10⁺ CD32⁺ (population 1); CD11b^{high} CD101⁺ CD10⁻ CD32⁺ (population 2) and CD11b^{low} CD101^{low} CD10⁻ CD32a⁻ (population 3). In all analysis evaluating cervicovaginal neutrophil subpopulations, samples collected during menstruation were excluded since we have previously shown (C.Adapen, submitted manuscript) that during the menstruation cervicovaginal neutrophil subpopulations are modified. Population 1 represents 60.3% \pm 18.6, population 2: 13.6 \pm 12.8, population 3: 7.6 \pm 7.2 of total neutrophils at baselines in *L. crispatus* treated group. In the untreated group, population 1 represents 47.9% \pm 21.1, population 2: 17.9 \pm 16.6, and population 3: 20.1% \pm 20.1 of total neutrophils at baselines. No significant impact neither of antibiotic treatment nor of CT inoculations was noticed on neutrophil subpopulations [Fig14A]. Moreover, very few impact was observed on surface marker expression after CT inoculations. Indeed, only a significant decrease of the mean fluorescence intensity of CD11b on the total number of neutrophils was observed in the treated (p value: 0.017) and untreated group (pvalue: 0.029) at four and one week after CT inoculation respectively [Fig14B]. Moreover, no significant increase of neutrophils expressing activation markers including CD32a, HLA-DR and PD-L1 was noticed [Data not shown].

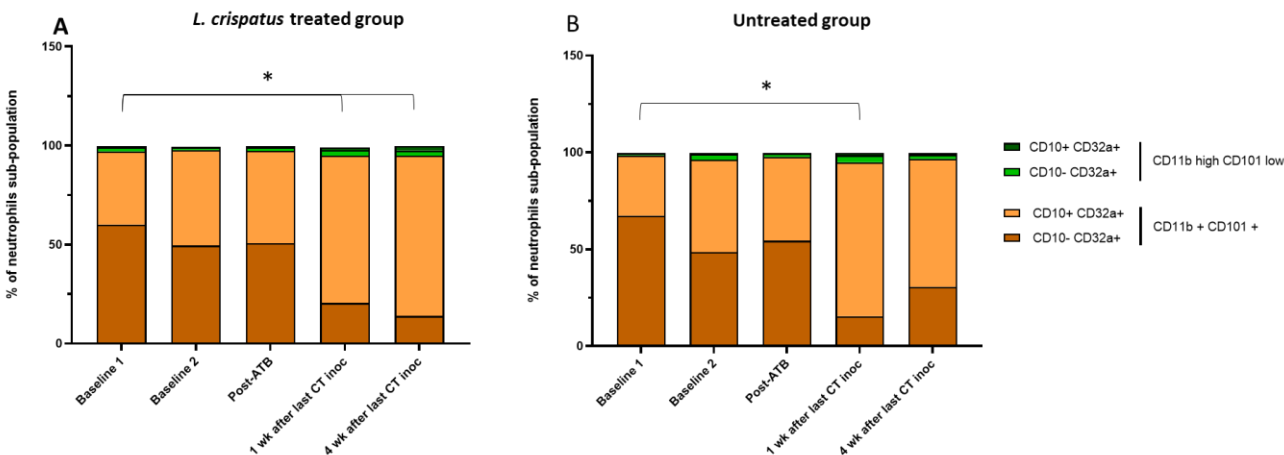


Figure 12: Neutrophil subpopulations in peripheral blood. Percentage of neutrophil subpopulations among $CD66^+ Lin^- CD14^-$ in *L. crispatus* treated (A) and untreated animals (B) at baselines, after metronidazole treatment or metronidazole treatment and *L. crispatus* inoculation (Post-ATB), one week after the last CT inoculation and four weeks after the last CT inoculation. A two way ANOVA test with the Turkey test to adjust p value was performed to compare baseline value to other time points. Asterisks indicate a p value considered statistically significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

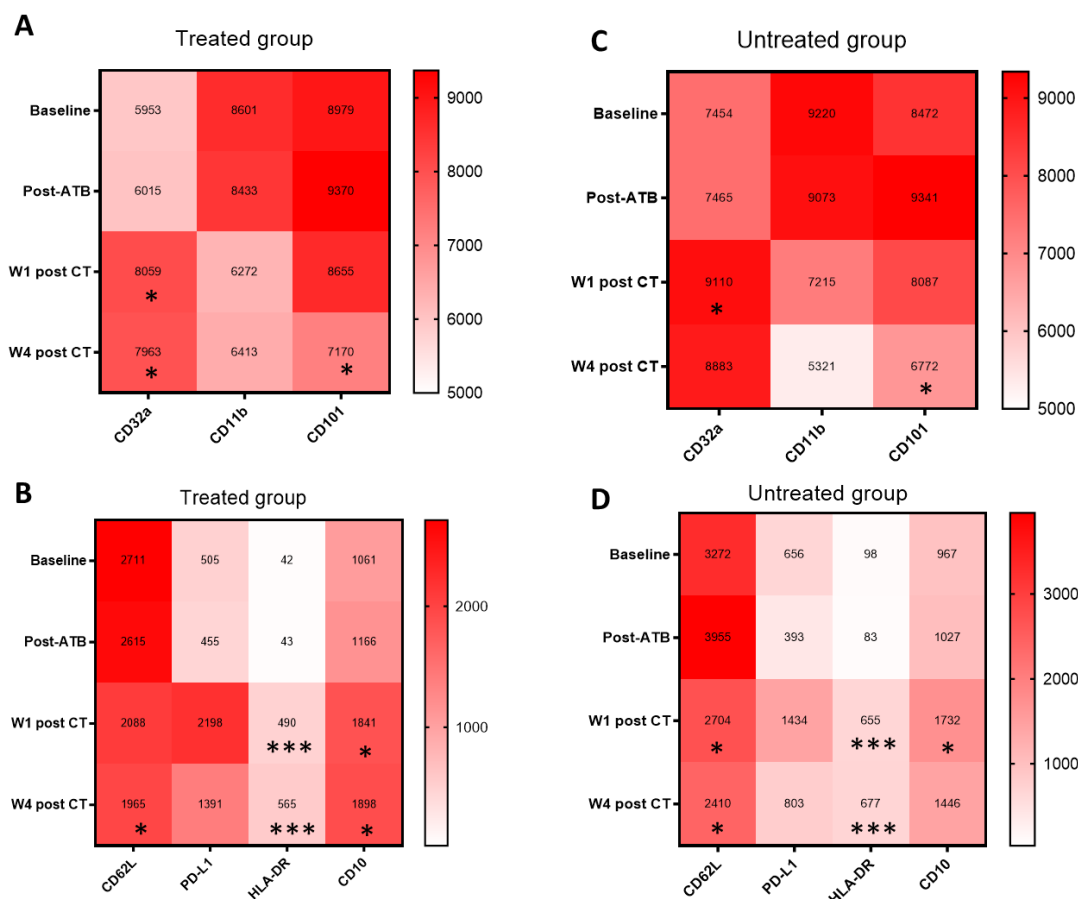


Figure 13: Expression of markers of activation and maturation on peripheral blood neutrophils. Heatmap representing the mean fluorescence intensity (MFI) of CD32a, CD11b CD101 (A, C), or CD62L, PD-L1, HLA-DR, CD10 (B, D) expression on neutrophils ($CD66^+ Lin^- CD14^-$) at all time points. Heatmap of *L. crispatus* treated (A, B) and untreated animals (C, D). A two way ANOVA test with the Turkey test to adjust p value was performed to compare baseline value to other time points. Asterisks indicate p values considered statistically significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

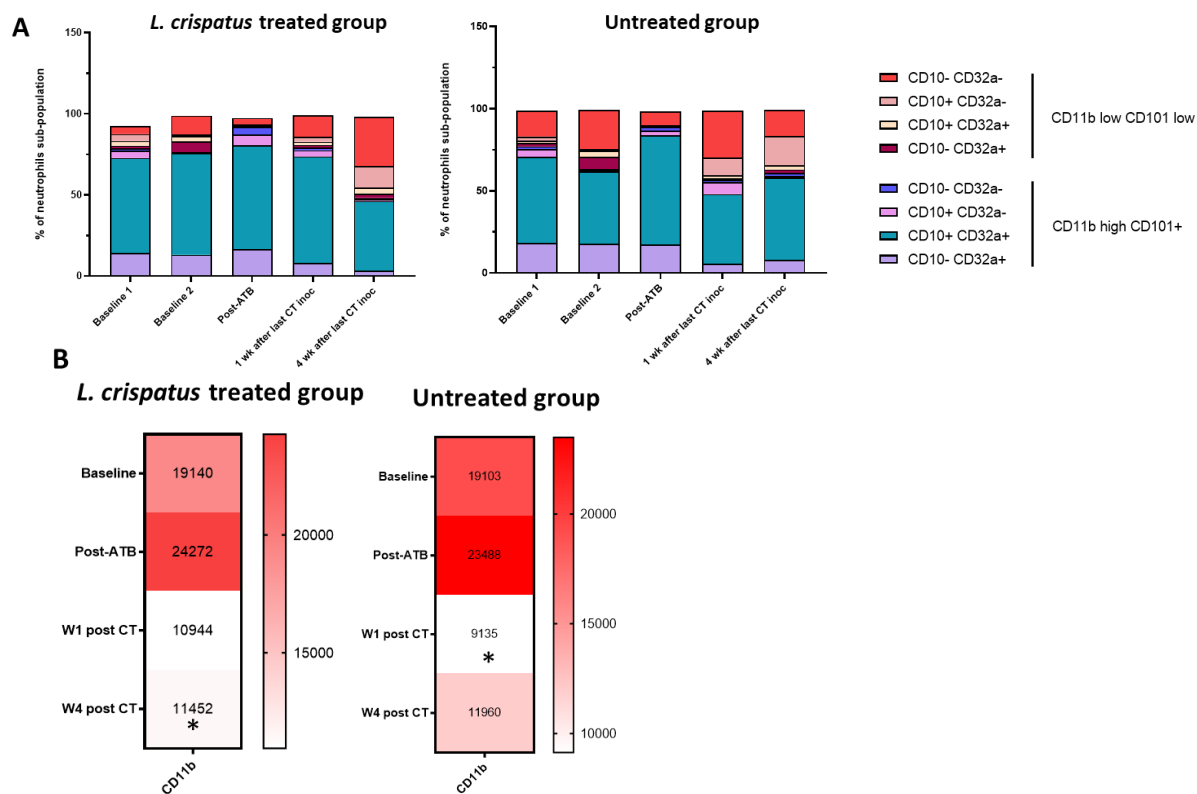
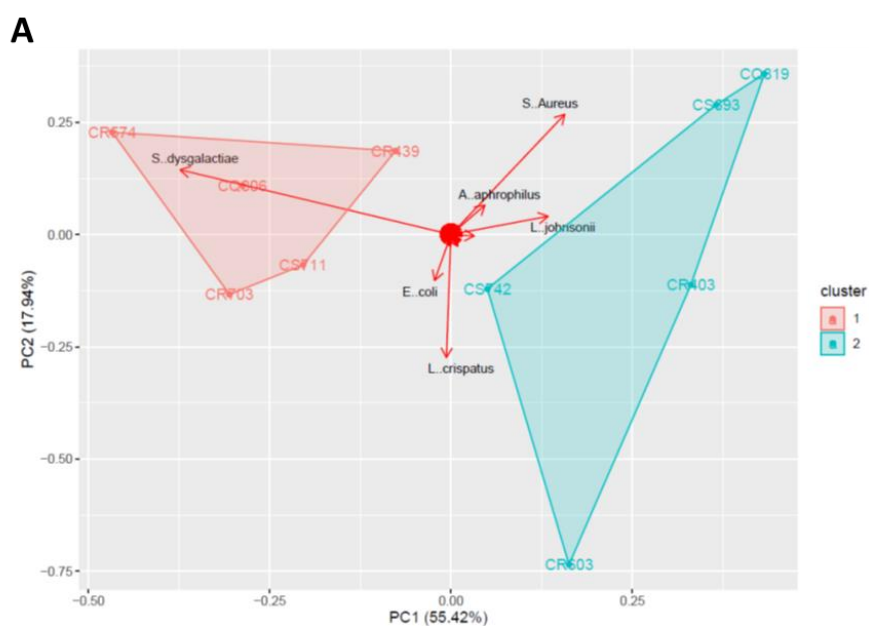


Figure 14: Neutrophil subpopulations in cervicovaginal cytobrushes. Percentage of neutrophil subpopulations among $CD66^+ Lin^- CD14^-$ in *L. crispatus* treated (A) and untreated animals (B) at baselines, after metronidazole treatment or metronidazole treatment and *L. crispatus* inoculation (Post-ATB), one week after the last CT inoculation and four weeks after the last CT inoculation. (B) Heatmap representing the mean fluorescence intensity (MFI) of CD11b expression on neutrophils ($CD66^+ Lin^- CD14^-$) at all time points in *L. crispatus* treated (left) and untreated animals (right).

High *S. dysgalactiae* abundance before CT inoculations is associated to better production of specific antibodies against CT as well as increased production of chemokines after CT infection.

To determine if a specific bacterial composition can be attributed to an increase CT susceptibility or inflammation, new groups were determined based on the microbiota composition detected before the first CT inoculation. A principal component analysis was performed on the microbiota results at day-2 (two days before the first CT inoculation). Animals that were not infected were removed of the analysis (CS865 and CS794). An automatic clustering in two groups were added, highlighting two separated groups composed of 5 animals each: (1) CQ806, CR439, CR703, CS711, CR574; (2) CR603, CQ819, CR403, CS693, CS742. Groups were split mainly based on the abundance of five bacterial species: *S. dysgalactiae*, *S. aureus*, *L. johnsonii*, *E. coli* and *L. crispatus*. The group one has a

high abundance of *S. dysgalactiae* and a low abundance of the other mentioned bacteria [Fig15A]. CT load and IgG anti-CT production were compared in both groups to study if one group had a higher CT load or a better antibody production [Fig15B and C]. No significant differences for CT load were observed, however anti-CT IgG production was significantly higher in the group 1 compared to group 2. We can notice that four animals out of five were treated with *L. crispatus* gel in the group 1 suggesting that the treatment as induced microbiota changes associated with *S. dysgalactiae* increase and a better anti-CT antibody response. To determine if cytokine production was different according to both groups, cytokine concentrations before CT infection, during CT infection and after CT infection were compared between each group [Fig15D]. The samples collected during menstruation were removed of the analysis. Higher concentration of CCL4 and CCL2 was noticed in the group 1 compared to the group 2 after CT infection whereas sCD40L was significantly decreased in the group 1 compare to group 2 after CT infection. Moreover, CCL2 concentration was also higher in the group 1 during CT infection. No significant differences were observed in terms of neutrophil subpopulations or activation. Overall, the group that had a high abundance of *S. dysgalactiae* before CT inoculation had a higher specific IgG production as well as chemokine (CCL4 and CCL2) concentrations after CT infection.



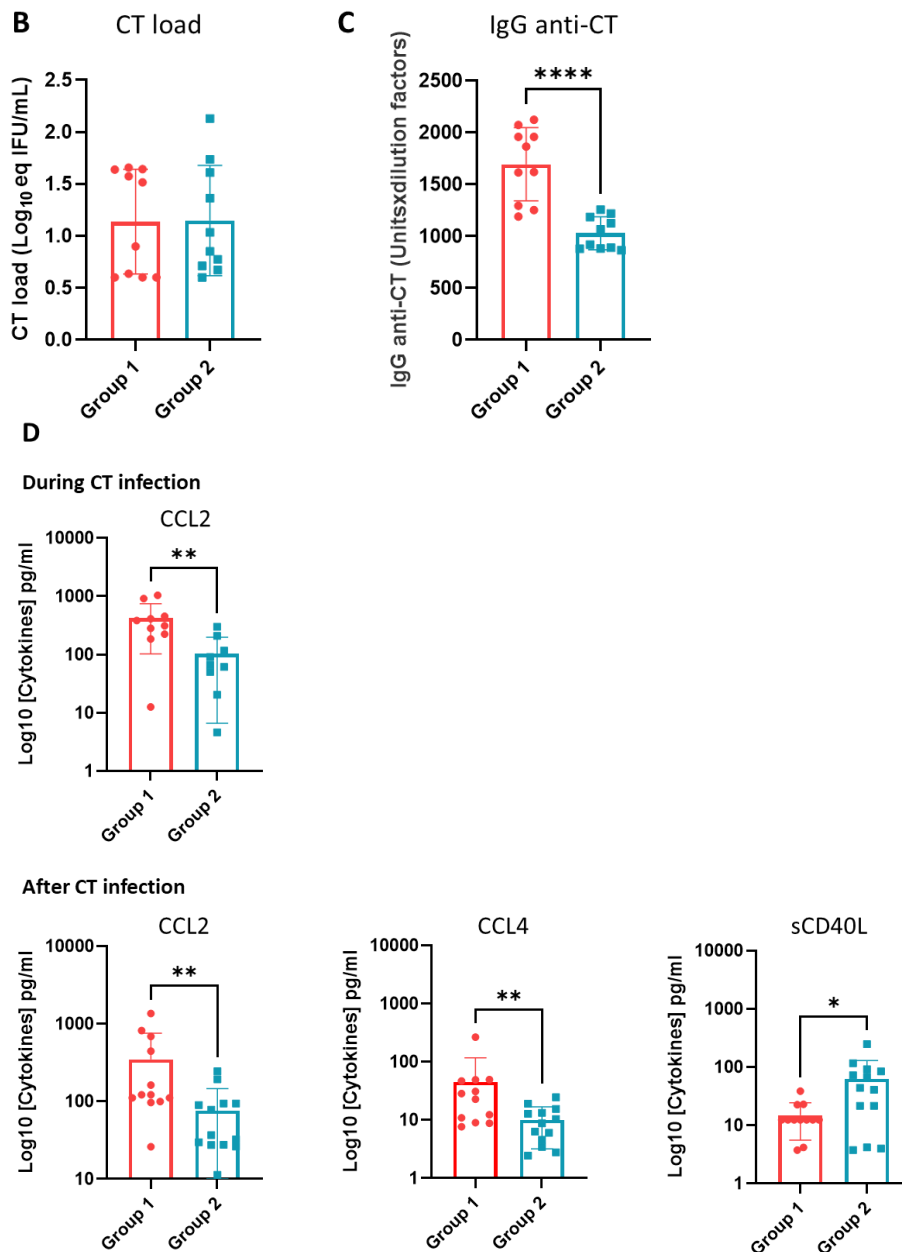


Figure 15: Clustering before CT inoculations exhibits differences in CT immune responses. (A) Principal component analysis on microbiota data collected two days before the first CT inoculation. Animals that were not infected (CS865 and CS794) were excluded of the analysis. A clustering in two groups was generated distributing the animals in two groups: group 1: CQ806, CR439, CR703, CS711, CR574 ; group 2: CR603, CQ819, CR403, CS693, CS742. Bar plot representation of CT load (B), anti-CT IgG (C) in each group. (C) Bar plot representation of CCL2 concentration during CT infection (top) as well as CCL2, CCL4, sCD40L measured in cervicovaginal fluids after CT infection (bottom) in each group was generated. A Mann Whitney test was performed to compare both groups. Asterisks indicate p values considered statistically significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Discussion

During this study, we have analysed the impact on the vaginal microbiota composition on *Chlamydia trachomatis* infection and on the cervicovaginal and peripheral blood inflammation induced by CT in female cynomolgus macaques. We have observed that females with an *L. crispatus* enriched vaginal microbiota have a higher cervicovaginal cytokine production but a better control of the systemic inflammation as well as a higher specific anti-CT IgG production in peripheral blood.

We have observed a non-persisting CT infection in ten animals out of twelve and two animals remained uninfected independently of their group affiliation. CT reinfection was observed in some females that did not produce peripheral blood specific anti-CT IgG after a first peak of infection. However, two animals, one in each group, were not infected and did not produce specific anti-CT IgG. These results demonstrate that animals clear rapidly the infection and that specific antibodies protect against reinfection. In women, seropositivity was associated with a decrease in cervical bacterial burden but not endometrium bacterial burden [30]. In the pig-tailed macaques, a single cervicovaginal infection of 10^6 IFU allow the detection of CT during 9 weeks post-infection before clearing the infection [22]. We did not observe this sustained CT infection despite the repeated inoculation of CT. However, in a previous experiment performed in our laboratory, the repeated cervicovaginal inoculations of 10^6 IFU of two cynomolgus macaques (untreated with antibiotic and *L. crispatus*) induced a sustained presence of CT in cervicovaginal fluids 56 days after the first inoculation [Data not show]. Metronidazole does not have a direct effect on CT infection; however, it is prescribed in addition to azithromycin or doxycycline for the treatment of pelvic inflammatory disease, an upper genital tract poly-microbial infection involving CT and anaerobic bacteria associated with bacterial vaginosis (BV) [31]. In addition, a study on CT infected women suffering from BV treated with metronidazole demonstrated that metronidazole treatment, by inducing changes in the microbiome, was able to facilitate the eradication of CT by the immune response [32]. As observed in cynomolgus macaques, a study described that 22% of women spontaneously resolved CT infection whereas 16.5% suffered from reinfection. Reinfection

was observed to be more frequent in women that suffered from persisting infection compared to the ones that spontaneously resolve CT infection, suggesting an attenuated protective immunity in antibiotic treated women [4]. Moreover, antibiotic treatment in pig-tailed macaques infected with CT exhibited a decrease in peripheral antibody level [33]. Those studies highlight a significant impact of antibiotic treatment on CT susceptibility and CT related immune responses. Thus, we cannot exclude that the metronidazole treatment in all the females of our study had an impact on the persistence of the CT infection.

We then studied the production of specific anti-CT antibodies in the serum of all animals. We described that animals with treated with *L. crispatus* had a significantly higher level of specific IgG against CT compared to other animals. The bacterial load was not different according to group, suggesting that the impact observed on IgG production is not linked to the amplitude of the infection. In addition, we have observed that anti-CT IgG is higher in a group of animals that had a high abundance of *S. dysgalactiae* right before CT inoculations. This group were composed of four animals out of five that were treated with *L. crispatus* gel. Therefore, *L. crispatus* treatment is able to modify the vaginal microbiota composition inducing a better anti-CT IgG production. A review on the relationship between antibodies and gut microbiota suggests that IgG plasmocytes are close to the lamina propria, allowing the passage of anti-commensal IgG in the blood [34]. We can hypothesise that local presence of a group of bacteria can prime the specific local immune response, inducing a better production of IgG locally that can then enter the bloodstream.

We also characterized the vaginal microbiota throughout the whole study. Antibiotic treatment induced drastic modifications of the vaginal microbiota in all animals, and these modifications were animal specific. We described a higher abundance of *Staphylococcus aureus*, *Streptococcus* and endogenous *Lactobacillus spp* for instance. The proportion of natural *Lactobacillus spp.* was higher in animals from the untreated group. In Rwandan women, BV treatment with metronidazole induced a cure rate of only 54.5% suggesting strong variabilities among women. They observed a higher concentration of *Lactobacillus spp* including *L. iners* mainly and no modification of the concentration of *Streptococcus*, *Staphylococcus*, *Escherichia* and *Shigella* species was observed. However, they observed an

increase relative abundance of pathobionts (*Proteobacteria*, *Streptococci*, *Enterococci*, *Spirochaetaceae*, *Listeria*, CT and *N. gonorrhoeae*) that seems to be associated with treatment failure [35]. *Lactobacillus spp.* abundance was studied throughout our study. *L. crispatus* was present only in treated animals whereas untreated animals had high abundance of *L. johnsonii* and *L. prophage*. Interestingly, *L. johnsonii* and *L. prophage* abundance was increased during CT infection in untreated animal. Their relative abundance was positively associated with CT load. This result suggests that endogenous *Lactobacillus spp.* in untreated cynomolgus macaques are associated with increased CT infection. *Lactobacillus spp.* are present in women vaginal microbiota and are associated with anti-inflammatory effect and protection against STI [16, 17, 36, 37]. *L. johnsonii* was described to restrain vaginal colonization by pathobionts and to have anti-inflammatory effect [38, 39]. However, a recent paper highlights different inflammatory properties of *Lactobacillus spp.* strains depending if they were isolated from a *Lactobacillus spp.* dominant vaginal microbiota or a dysbiotic vaginal microbiota [40]. Interestingly, *L. johnsonii* has been described to inhibit IDO1 *in vitro*, leading to a decrease concentration of kynurenine [41]. We hypothesise that *L. johnsonii* strain, present in the vaginal microbiota of cynomolgus macaque, by inhibiting IDO1 might promote CT infection. Cytokine and chemokine production was monitored once a week to evaluate local and systemic inflammation after CT infection. In peripheral blood serum, there was an increased production of cytokines after CT infection in the untreated group compared to the *L. crispatus* treated group. This result suggests a higher systemic inflammation in the untreated group compared to *L. crispatus* treated animal. In the untreated group, two animals CR403 and CR574 exhibit a higher production of cytokines after CT inoculations. CT infection of CR574 weakly induces specific anti-CT IgG. As for CR403, specific anti-CT IgG are increased after the second peak of infection but interestingly CT can still be detected at this end of the four weeks follow up (day 63). In cervical fluids of women suffering from upper genital tract infection or from fertility disorders there is an increased level of IL-6, IL-8, IL-10, IFN γ , CXCL10, TNF α , IL-17A, CXCL9, CXCL11, CCL4 and CXCL13 [7, 8]. Higher level of IL-6, IL-8, IL-10, IL-12/23, IL-13, TGF α and TNF α in cervicovaginal fluids of CR403 during animal follow up (day 49 and 56) were noticed [Suppl fig7]. We supposed that this important increased production

of cytokines in the peripheral blood serum might result from an upper tract dissemination of CT. We could not verify this hypothesis since no euthanasia was performed.

The analyses of cytokine production in cervicovaginal fluids demonstrated difference in *L. crispatus* and untreated groups before CT infection [Fig11A] suggesting an impact of *L. crispatus* treatment on cytokine production. Statistical analysis on cytokine production during baseline and after antibiotic treatment was also performed to compare both groups and confirmed that the differences between groups are not due animals [Data not show]. No significant differences according to groups were noticed before *L. crispatus* treatment. In contrast to the blood, cervicovaginal cytokine production was increased after CT infection in treated animals compared to untreated animals suggesting a better stimulation of the immune response in treated animals compared to untreated. The increase of cytokine production in *L. crispatus* treated animals involved: (1) chemokines (CCL2 and CCL4) involved in monocyte, lymphocyte and NK cell recruitment [42, 43]; (2) IL-15 involved in T and NK cell proliferation and activation [44, 45]; (3) growth factor including VEGF but also G-CSF, a key cytokine involved in neutrophil proliferation [46]. In addition, analysis of cytokine production in groups based on principal component analysis before CT inoculation exhibit differences. Indeed, animals that have a high abundance of *S. dysgalactiae* have an increase production of CCL2, CCL4, TNF α after CT infection and CCL2 during infection. This group is composed of four animals out of five that have received *L. crispatus* inoculation suggesting that the treatment can be linked to the variation of cytokine production observed.

In terms of inflammation, neutrophil phenotype was studied in peripheral blood and in cervicovaginal cytobrushes. In peripheral blood, neutrophils were more mature and activated after CT infection in both groups but the maturation and activation were sustained longer in the treated group compared to untreated. In cervicovaginal cytobrushes, no impact on maturation or activation was noticed in opposition to peripheral blood neutrophils. Sampling was performed only after the six CT inoculations, in order to avoid inflammation induced by cervicovaginal cytobrushes, therefore the accumulation/activation or maturation of neutrophils might have been missed and only a more tardive immune response in the blood compartment could be seen. However, a decrease in CD11b on total neutrophils was

observed after CT infection in both groups. CD11b is an integrin family member expresses in many cells including neutrophils. Its increase in the blood has been associated with neutrophil priming as well as CD62L shedding [27]. Several hypothesis could be made for the decrease of CD11b and/or absence of CD11b increase: (1) It could be a tissue specific phenotype since no information on CD11b expression on neutrophils after CT infection is documented; (2) neutrophil phenotyping was performed one and four weeks after CT infection so we might have missed the increase of CD11b expression on local neutrophils; (3) it could be resident neutrophils with increase survival that might have lost the expression of some markers including CD11b. This is the first study describing neutrophils in peripheral blood and cervicovaginal cytobrushes in a context of CT infection.

Overall, this study demonstrates that female cynomolgus macaques with a *L. crispatus* enriched vaginal microbiota develop a better specific antibody response against CT in peripheral blood compared to *L. crispatus* untreated animal. Moreover, the increase presence of *L. johnsonii* in untreated animals, after metronidazole treatment, is associated with higher bacterial load thus a higher CT susceptibility. Unfortunately, we were not able to link an increased production of cytokines with *L. johnsonii* abundance. However, we could observe a higher systemic production of cytokines in untreated animal compared to treated animal. In contrary, treated animal have an increase production of cervicovaginal cytokines after CT infection compared to untreated animal suggesting a better local immune response. Our study has some limitations. Significant inter-individual differences enable us to evaluate the effect of a specific vaginal microbiota on CT infection, therefore a higher number of animals should be investigated for further studies. This animal model was able to reproduce an asymptomatic CT infection observed in women but it would be interesting to evaluate the impact of the vaginal microbiota composition on a sustained infection *ie* inflammation. Contraception has been described to modify the FRT environment [47, 48] therefore control of menstrual cycle by injecting hormone was not performed during this study. Progesterone concentration as well as menstrual bleeding was monitored and menstruation effect on the local compartment was taken into account for the analysis.

In conclusion, this study demonstrates a significant impact of the vaginal microbiota composition on the local and systemic immune response induced by CT infection. It highlights a need for a better understanding of the effect of vaginal microbiota composition on STI acquisition.

Acknowledgments:

The authors would like to thank all members of the ASW (Raphaël Ho Tsong Fang; Jean-Marie Robert, Sebastien Langlois, Maxime Potier, Quentin Sconosciuti, Nina Dhooge), L2I (Laetitia Bossevot, Julie Morin) and LFC (Anne-Sophie Gallouet, Jérôme Van Wassenhove, Mario Gomez-Pacheco, Wesley Gros) teams of the IDMIT infrastructure as well as the members of the Animalliance group (Thierry Prot, Marjorie Benfissa). The authors also would like to thank Dr Frank Follmann and his team in Statens Serum Institut (Copenhagen, Denmark) for their help and for providing us the strain of CT used for the infection as well as Eric Ginoux, Virginie Chesnais and Sabrina Lakoum from Life&Soft.

Bibliography

1. Sexually transmitted infections (STIs). [https://www.who.int/news-room/fact-sheets/detail/sexually-transmitted-infections-\(stis\)](https://www.who.int/news-room/fact-sheets/detail/sexually-transmitted-infections-(stis)). Accessed 13 Sep 2021.
2. Murthy AK, Li W, Ramsey KH. Immunopathogenesis of Chlamydial Infections. *Curr Top Microbiol Immunol*. 2018;412:183–215. doi:10.1007/82_2016_18.
3. Ziklo N, Huston WM, Hocking JS, Timms P. Chlamydia trachomatis Genital Tract Infections: When Host Immune Response and the Microbiome Collide. *Trends Microbiol*. 2016;24:750–65.
4. Geisler WM, Lensing SY, Press CG, Hook EW. Spontaneous Resolution of Genital Chlamydia trachomatis Infection in Women and Protection from Reinfection. *J Infect Dis*. 2013;207:1850–6. doi:10.1093/infdis/jit094.
5. Walker J, Tabrizi SN, Fairley CK, Chen MY, Bradshaw CS, Twin J, et al. Chlamydia trachomatis Incidence and Re-Infection among Young Women – Behavioural and Microbiological Characteristics. *PLoS One*. 2012;7. doi:10.1371/journal.pone.0037778.
6. Spear GT, Kendrick SR, Chen HY, Thomas TT, Bahk M, Balderas R, et al. Multiplex Immunoassay of Lower Genital Tract Mucosal Fluid from Women Attending an Urban STD Clinic Shows Broadly Increased IL1 β and Lactoferrin. *PLOS ONE*. 2011;6:e19560. doi:10.1371/journal.pone.0019560.
7. Poston TB, Lee DE, Darville T, Zhong W, Dong L, O'Connell CM, et al. Cervical Cytokines Associated With Chlamydia trachomatis Susceptibility and Protection. *J Infect Dis*. 2019;220:330–9. doi:10.1093/infdis/jiz087.
8. Agrawal T, Vats V, Salhan S, Mittal A. The mucosal immune response to Chlamydia trachomatis infection of the reproductive tract in women. *Journal of Reproductive Immunology*. 2009;83:173–8. doi:10.1016/j.jri.2009.07.013.
9. Lu H, Shen C, Brunham RC. Chlamydia trachomatis Infection of Epithelial Cells Induces the Activation of Caspase-1 and Release of Mature IL-18. *The Journal of Immunology*. 2000;165:1463–9. doi:10.4049/jimmunol.165.3.1463.

10. Buckner LR, Lewis ME, Greene SJ, Foster TP, Quayle AJ. Chlamydia trachomatis infection results in a modest pro-inflammatory cytokine response and a decrease in T cell chemokine secretion in human polarized endocervical epithelial cells. *Cytokine*. 2013;63:151–65. doi:10.1016/j.cyto.2013.04.022.
11. Lijek RS, Helble JD, Olive AJ, Seiger KW, Starnbach MN. Pathology after Chlamydia trachomatis infection is driven by nonprotective immune cells that are distinct from protective populations. *Proc Natl Acad Sci U S A*. 2018;115:2216–21. doi:10.1073/pnas.1711356115.
12. Lehr S, Vier J, Häcker G, Kirschnek S. Activation of neutrophils by Chlamydia trachomatis-infected epithelial cells is modulated by the chlamydial plasmid. *Microbes and Infection*. 2018;20:284–92. doi:10.1016/j.micinf.2018.02.007.
13. Naglak EK, Morrison SG, Morrison RP. Neutrophils Are Central to Antibody-Mediated Protection against Genital Chlamydia. *Infect Immun*. 2017;85. doi:10.1128/IAI.00409-17.
14. Ziklo N, Vidgen ME, Taing K, Huston WM, Timms P. Dysbiosis of the Vaginal Microbiota and Higher Vaginal Kynurenine/Tryptophan Ratio Reveals an Association with Chlamydia trachomatis Genital Infections. *Front Cell Infect Microbiol*. 2018;8. doi:10.3389/fcimb.2018.00001.
15. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, et al. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA*. 2011;108 Suppl 1:4680–7.
16. Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UME, Zhong X, et al. Temporal dynamics of the human vaginal microbiota. *Sci Transl Med*. 2012;4:132ra52.
17. Balle C, Lennard K, Dabee S, Barnabas SL, Jaumdally SZ, Gasper MA, et al. Endocervical and vaginal microbiota in South African adolescents with asymptomatic Chlamydia trachomatis infection. *Sci Rep*. 2018;8. doi:10.1038/s41598-018-29320-x.
18. Nardini P, Ñahui Palomino RA, Parolin C, Laghi L, Foschi C, Cevenini R, et al. Lactobacillus crispatus inhibits the infectivity of Chlamydia trachomatis elementary bodies, in vitro study. *Sci Rep*. 2016;6. doi:10.1038/srep29024.
19. Boyd KL, Muehlenbachs A, Rendi MH, Garcia RL, Gibson-Corley KN. Female Reproductive System. In: *Comparative Anatomy and Histology*. Elsevier; 2018. p. 303–34. doi:10.1016/B978-0-12-802900-8.00017-8.
20. Van Esch E, Cline JM, Buse E, Wood CE, de Rijk EPCT, Weinbauer GF. Summary Comparison of Female Reproductive System in Human and the Cynomolgus Monkey (*Macaca fascicularis*). *Toxicol Pathol*. 2008;36 7_suppl:171S–172S. doi:10.1177/0192623308327415.
21. Marlin R, Nugeyre M-T, Tchitchek N, Parenti M, Hocini H, Benjelloun F, et al. Modified Vaccinia Virus Ankara Vector Induces Specific Cellular and Humoral Responses in the Female Reproductive Tract, the Main HIV Portal of Entry. *J Immunol*. 2017;199:1923–32.
22. Patton DL, Sweeney YC, Baldessari AE, Cles L, Kari L, Sturdevant GL, et al. The Chlamydia trachomatis Plasmid and CT135 Virulence Factors Are Not Essential for Genital Tract Infection or Pathology in Female Pig-Tailed Macaques. *Infect Immun*. 2018;86. doi:10.1128/IAI.00121-18.
23. Le Grand R, Dereuddre-Bosquet N, Dispinseri S, Gosse L, Desjardins D, Shen X, et al. Superior Efficacy of a Human Immunodeficiency Virus Vaccine Combined with Antiretroviral Prevention in Simian-Human Immunodeficiency Virus-Challenged Nonhuman Primates. *J Virol*. 2016;90:5315–28.
24. Nugeyre M-T, Tchitchek N, Adapen C, Cannou C, Contreras V, Benjelloun F, et al. Dynamics of Vaginal and Rectal Microbiota Over Several Menstrual Cycles in Female Cynomolgus Macaques. *Front Cell Infect Microbiol*. 2019;9. doi:10.3389/fcimb.2019.00188.
25. Nguyen NDNT, Olsen AW, Lorenzen E, Andersen P, Hvid M, Follmann F, et al. Parenteral vaccination protects against transcervical infection with Chlamydia trachomatis and generate tissue-resident T cells post-challenge. *NPJ Vaccines*. 2020;5:7. doi:10.1038/s41541-020-0157-x.
26. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol*. 1991;29:297–301. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC269757/>. Accessed 15 Apr 2020.
27. Condliffe AM, Chilvers ER, Haslett C, Dransfield I. Priming differentially regulates neutrophil adhesion molecule expression/function. *Immunology*. 1996;89:105–11.
28. Escudié F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, et al. FROGS: Find, Rapidly, OTUs with Galaxy Solution. *Bioinformatics*. 2018;34:1287–94. doi:10.1093/bioinformatics/btx791.
29. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15:550. doi:10.1186/s13059-014-0550-8.

30. Russell AN, Zheng X, O'Connell CM, Taylor BD, Wiesenfeld HC, Hillier SL, et al. Analysis of Factors Driving Incident and Ascending Infection and the Role of Serum Antibody in Chlamydia trachomatis Genital Tract Infection. *J Infect Dis*. 2016;213:523–31.
31. Gradison M. Pelvic Inflammatory Disease. *AFP*. 2012;85:791–6. <https://www.aafp.org/afp/2012/0415/p791.html>. Accessed 12 Sep 2021.
32. Mott PD, Taylor CM, Lillis RA, Ardizzone CM, Albritton HL, Luo M, et al. Differences in the Genital Microbiota in Women Who Naturally Clear Chlamydia trachomatis Infection Compared to Women Who Do Not Clear; A Pilot Study. *Front Cell Infect Microbiol*. 2021;11:615770. doi:10.3389/fcimb.2021.615770.
33. Patton DL, Teng A, Randall A, Liang X, Felgner PL, de la Maza LM. Whole genome identification of *C. trachomatis* immunodominant antigens after genital tract infections and effect of antibiotic treatment of pigtailed macaques. *J Proteomics*. 2014;108:99–109.
34. Sterlin D, Fadlallah J, Slack E, Gorochoy G. The antibody/microbiota interface in health and disease. *Mucosal Immunology*. 2020;13:3–11. doi:10.1038/s41385-019-0192-y.
35. Verwijs MC, Agaba SK, Darby AC, van de Wijgert JHHM. Impact of Oral Metronidazole Treatment on the Vaginal Microbiota and Correlates of Treatment Failure. *American Journal of Obstetrics and Gynecology*. 2019. doi:10.1016/j.ajog.2019.08.008.
36. Mehta O, Ghosh TS, Kothidar A, Gowtham MR, Mitra R, Kshetrapal P, et al. Vaginal Microbiome of Pregnant Indian Women: Insights into the Genome of Dominant Lactobacillus Species. *Microbial Ecology*. 2020;80:487–99. doi:10.1007/s00248-020-01501-0.
37. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, et al. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity*. 2015;42:965–76. doi:10.1016/j.immuni.2015.04.019.
38. Teixeira GS, Carvalho FP, Arantes RME, Nunes AC, Moreira JLS, Mendonça M, et al. Characteristics of Lactobacillus and Gardnerella vaginalis from women with or without bacterial vaginosis and their relationships in gnotobiotic mice. *J Med Microbiol*. 2012;61 Pt 8:1074–81.
39. Bereswill S, Ekmekci U, Escher U, Fiebiger U, Stingl K, Heimesaat MM. Lactobacillus johnsonii ameliorates intestinal, extra-intestinal and systemic pro-inflammatory immune responses following murine Campylobacter jejuni infection. *Sci Rep*. 2017;7:2138. doi:10.1038/s41598-017-02436-2.
40. Manhanzva MT, Abrahams AG, Gamielien H, Froissart R, Jaspan H, Jaumdally SZ, et al. Inflammatory and antimicrobial properties differ between vaginal Lactobacillus isolates from South African women with non-optimal versus optimal microbiota. *Sci Rep*. 2020;10. doi:10.1038/s41598-020-62184-8.
41. Valladares R, Bojilova L, Potts AH, Cameron E, Gardner C, Lorca G, et al. Lactobacillus johnsonii inhibits indoleamine 2,3-dioxygenase and alters tryptophan metabolite levels in BioBreeding rats. *FASEB J*. 2013;27:1711–20.
42. Gschwandtner M, Derler R, Midwood KS. More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis. *Front Immunol*. 2019;10:2759. doi:10.3389/fimmu.2019.02759.
43. Maurer M, von Stebut E. Macrophage inflammatory protein-1. *The International Journal of Biochemistry & Cell Biology*. 2004;36:1882–6. doi:10.1016/j.biocel.2003.10.019.
44. Huntington ND, Legrand N, Alves NL, Jaron B, Weijer K, Plet A, et al. IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *J Exp Med*. 2009;206:25–34. doi:10.1084/jem.20082013.
45. Schluns KS, Williams K, Ma A, Zheng XX, Lefrançois L. Cutting Edge: Requirement for IL-15 in the Generation of Primary and Memory Antigen-Specific CD8 T Cells. *The Journal of Immunology*. 2002;168:4827–31. doi:10.4049/jimmunol.168.10.4827.
46. Demetri GD, Griffin JD. Granulocyte Colony-Stimulating Factor and Its Receptor. *Blood*. 1991;78:2791–808. doi:10.1182/blood.V78.11.2791.2791.
47. Balle C, Konstantinus IN, Jaumdally SZ, Havyarimana E, Lennard K, Esra R, et al. Hormonal contraception alters vaginal microbiota and cytokines in South African adolescents in a randomized trial. *Nat Commun*. 2020;11. doi:10.1038/s41467-020-19382-9.
48. Edfeldt G, Lajoie J, Röhl M, Oyugi J, Åhlberg A, Khalilzadeh-Binicy B, et al. Regular Use of Depot Medroxy-progesterone Acetate Causes Thinning of the Superficial Lining and Apical Distribution of Human Immunodeficiency Virus Target Cells in the Human Ectocervix. *The Journal of Infectious Diseases*. 2020. doi:10.1093/infdis/jiaa514.

In vitro study: Effect of *L. crispatus* and *G. vaginalis* on CT induced inflammation. Consequences on neutrophil function and phenotype as well as HIV-1 co-infection

I. Introduction and objectives

In parallel to our *in vivo* study and to evaluate the mechanisms involved in the regulation of the innate immune response by the vaginal microbiota, we set up a Human *in vitro* model. This model aims at better characterizing the mechanisms involved in inflammation upon a STI and how the vaginal microbiota can regulate it. CT preferentially infects columnar epithelial cells leading to the production of cytokines such as IL-1, IL-6, IL-8, IL-12, IL-18, TNF α , IFN γ , IL-10 as well as growth factors like VEGF, G-CSF, GM-CSF [34, 35, 38–40]. FRT inflammation is regulated by environmental factors including the vaginal microbiota (dominant in *Lactobacillus spp.* vs low in *Lactobacillus spp.*). A vaginal microbiota dominated by *Lactobacillus spp.* is known to be protective against STI including CT and HIV-1 [191, 192]. Moreover, *Lactobacillus crispatus* is the most protective strain due in part to its ability to produce both lactic acid isomers [207]. On the contrary, *Gardnerella vaginalis* has a central role in bacterial vaginosis acquisition by being a part of a group of organisms including *Atopium*, *Megashaera*, *Sneathia* and BV associated bacteria (BVAB1/2/3) involved in the induction of a dysbiotic environment [220]. It was described to induce a pro-inflammatory environment compared to *Lactobacillus spp.* [249]. Therefore, *L. crispatus* and *G. vaginalis* have opposite effect on the local inflammation. However, the effects of both bacteria on the inflammation induced by a STI is poorly studied. Moreover, this modified environment could also have an impact on the phenotype and function of immune cells and the susceptibility to co-infection. We have demonstrated *in vivo*, that cervicovaginal neutrophil phenotype is not altered after CT infection but we did not study the survival of neutrophils. Indeed, neutrophil survival was described to increase in presence of anaerobic bacteria [281]. The effect of vaginal microbiota composition on co-infection is poorly studied even though STI co-infection is a major public health issue especially in young women. A preexisting STI can favour the acquisition of a second STI. For instance, women suffering

from CT infection are more at risk of HIV-1 infection [92, 95, 96]. However, very few informations are available about the impact of vaginal microbiota composition after CT infection on the susceptibility to another STI.

We hypothesised that both bacteria from the microbiota will have opposite effect on the inflammation induced by CT where *G. vaginalis* will exacerbate and *L. crispatus* dampen the inflammation. Moreover, the modification of the environment induced either by *L. crispatus* or *G. vaginalis* in presence of CT infected epithelial cells might have an impact on neutrophil phenotype and survival but also on HIV-1 co-infection susceptibility.

In conclusion, we decided to develop an *in vitro* assay, to study the impact of *L. crispatus* and *G. vaginalis*, two antagonist bacteria, on the cytokine production of CT infected epithelial cells. We then investigated whether this environment had an impact on neutrophil phenotype and function and on HIV-1 infection [Fig38].

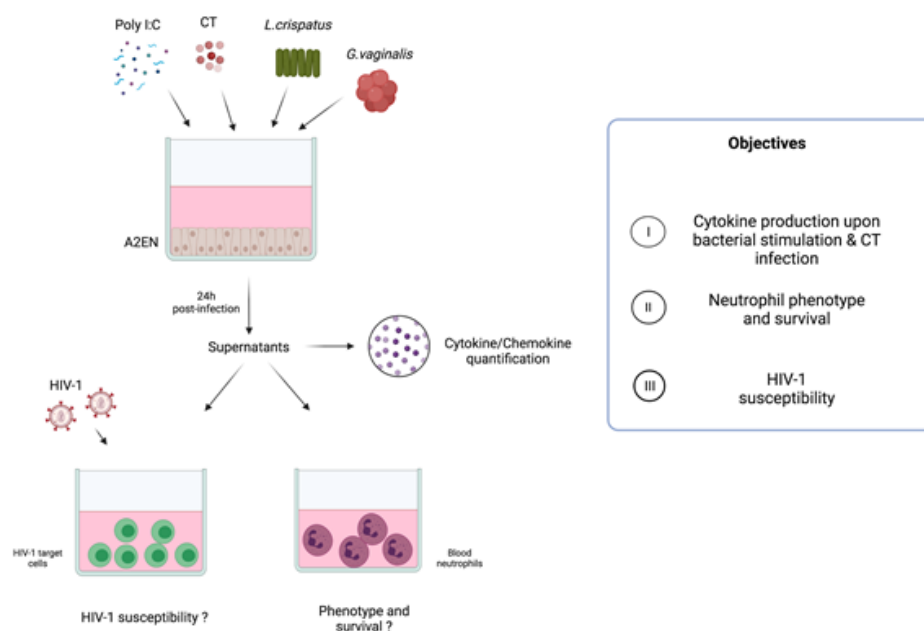


Figure 38: Experimental design.

A2EN cells were exposed to CT then *L. crispatus* or *G. vaginalis* were added or not and 24h post-CT infection supernatants were collected. Poly I:C was also used to ensure the good responsiveness of A2EN cells upon stimulation. Supernatants from each condition were analyzed for cytokine/chemokine production. Then supernatants of each conditions were added on (1) HIV-1 target cells exposed to the virus to study HIV-1 susceptibility or (2) isolated neutrophils from the blood to study their phenotype and survival. Created with BioRender.com.

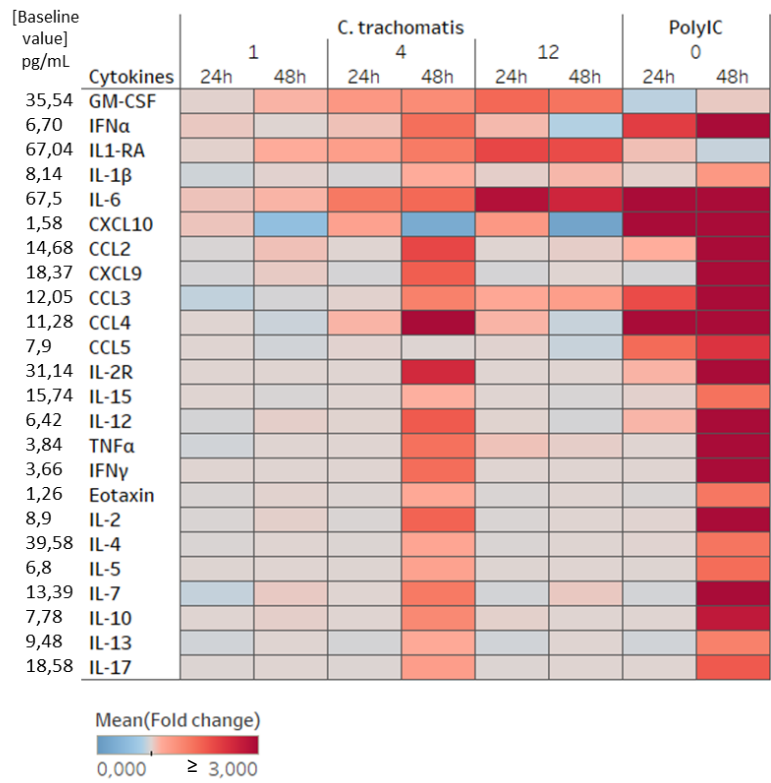
II. Results

a. *Chlamydia trachomatis* infection of epithelial cells

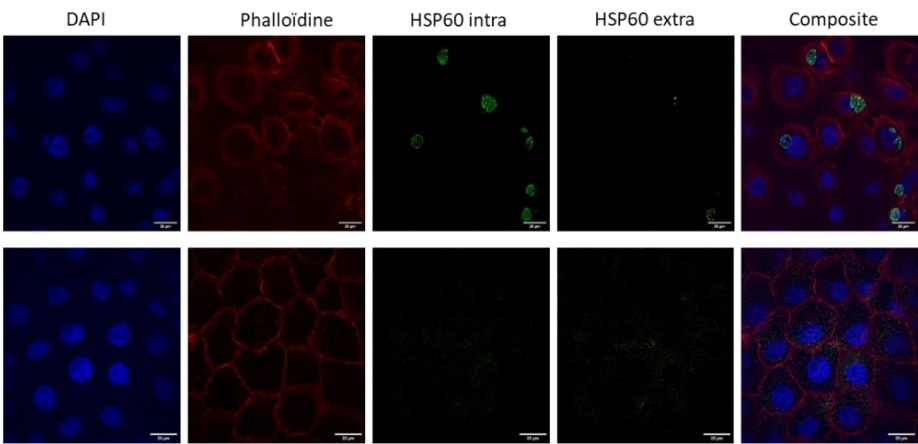
First of all, CT infection of a Human endocervical epithelial cell line (A2EN) [316] was established. CT infection was performed using a D serovar strain UW3/Cx obtained from Dr Follmann laboratory (Statens Serum Institut, Copenhagen, Denmark). A2EN cells at a concentration of $5 \cdot 10^4$ cells/well were seed in a 24 wells plate exposed to different MOI (1, 4, and 12) of CT serovar D or 100µg/mL of Poly I:C for 24h and 48h. Poly I:C is an agonist of Toll like receptor (TLR) 3, it was used to confirm the good responsiveness of cells to stimulation. On one hand, supernatants were collected to quantify the concentration of cytokines and chemokines by a multiplex assay. On a second hand, cells were fixed and stained with antibodies allowing the detection of cell membrane (Phalloïdine), intracellular or extracellular CT (anti-HSP60) and nucleus (Dapi). Cytokine concentrations are represented in a heatmap representing fold change expression calculated based on cytokine concentration observed in the uninfected control [Fig39A]. Cells exposed to Poly I:C induced a strong production of cytokines such as IFN α , IL-6, CXCL10, CCL3, CCL4, TNF α at 24h and 48h confirming the good responsiveness of the cells to stimulation. No increase of IL-8 was observed due to an already high basal production of IL-8 by uninfected A2EN cells [data not show]. CT MOI 1 exposition did not trigger an important cytokine increased expression at 24h or 48h post exposition with a percent of infection between 2% to 4% at 24h and 4% to 6% at 48h. CT MOI 4 exposition induced a higher increased concentration of cytokines compared to MOI 1 with a percent of infection between 13 to 14% at 24h and 23% to 24% at 48h post exposition. At 24h, MOI 4 exposition of epithelial cells induced a higher production of GM-CSF, IL-6, CXCL-10, IL-1RA which was amplified at 48h post exposition. The strong increase of cytokines observed at MOI 4 at 48h occurred in one experiment out of two. Twenty-two percent of infection was achieved at 24h with a MOI 12 [Fig39B] and 36% to 38% of infection were observed at 48h. Cytokine profile were quite similar between MOI 12 at 24h and 48h with an increase concentration of GM-CSF, IL-6, CXCL10, IL-1RA. The same cytokines were increased at MOI 4 however the increase was more significant at

MOI 12 compared to MOI 4. Significant increase of GM-CSF (p value: 0.012), IL-1RA (p value: 0.002) and IL-6 (p value: 0.005) was observed at MOI 12 after 24h of culture compared to uninfected cells. For further experiments the condition of CT infection at MOI 12 was kept.

A



B



DAPI : nucleus
Phalloïdine : actine
HSP60 intra : intracellular *Chlamydia trachomatis*
HSP60 extra : extracellular *Chlamydia trachomatis*

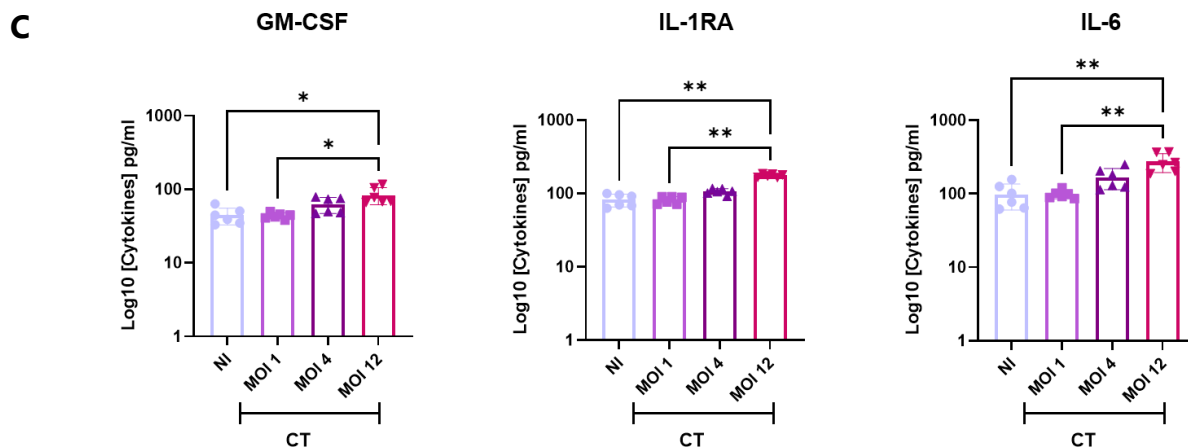


Figure 39: CT infection of A2EN cells.

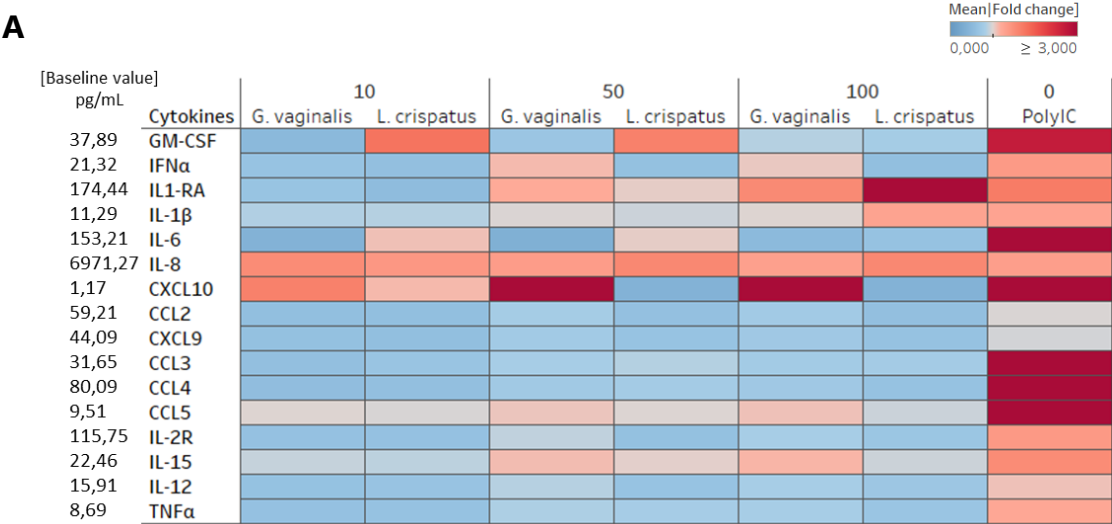
(A) Heatmap representing the mean fold change expression of cytokine concentration based on the cytokine concentration obtained in uninfected cells. Mean of two independent experiments (triplicates for each condition) was calculated. Different conditions are represented: *C. trachomatis* MOI 1 (1), MOI 4 (4), MOI 12 (12) and Poly I:C (0). Blue represents decrease expression and red an increase. Baseline values are presented on the left of the heatmap. (B) Staining of CT infected A2EN cells at MOI 12 after 24h of culture. Anti-HSP60 was used to stained intracellular (yellow) and extracellular CT (green). Phalloïdine was used to stain actin (red) and Dapi the nucleus. (C) GM-CSF, IL-1RA and IL-6 concentrations in different conditions: non-infected (NI), CT MOI 1, CT MOI 4, CT MOI 12. Log₁₀ of cytokine concentrations (pg/mL) are represented and a Kruskal-Wallis test was performed. p value was adjusted with Dunn's test. Asterisks indicate p values considered statistically significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

b. *L. crispatus* and *G. vaginalis* exposition of A2EN epithelial cells

Then, set up experiments were performed to find the best condition of coculture of A2EN cells with *L. crispatus* or *G. vaginalis*. *L. crispatus* or *G. vaginalis* were added at different MOI (10, 50 and 100) to A2EN cells for 24h to 48h. Supernatants were collected to quantify the concentration of cytokines and chemokines by a multiplex assay. pH in culture supernatants was monitored and cell viability was checked using the microscope as well as colorimetric technic (CellTiter®). Cytokine concentrations are represented in a heatmap representing fold change expression calculated based on cytokine concentration observed in the unexposed control [Fig40A]. Interaction of *L. crispatus* induced a low expression of

cytokines with a similar increase of GM-CSF and IL-8 at MOI 10 and 50. At MOI 100, a different cytokine profile was observed compared to the two other MOI. Indeed, we observed an induction of IL-1RA, IL-1 β and IL-8. *G. vaginalis* interaction with A2EN cells induced the expression of IL-8 and CXCL10 at MOI 10, those two factors were also increased at MOI 50 and MOI 100 in addition to IFN α and IL-1RA.

We then compared the concentration of cytokines observed in the conditions *L. crispatus* and *G. vaginalis* MOI 50. We showed an increase concentration of CCL5 (p value: 0.004), CXCL10 (p value: 0.002), IL-2R (p value: 0.039) and IFN α (p value: 0.002) in *G. vaginalis* MOI 50 conditions compared to *L. crispatus* MOI 50. In contrast, GM-CSF (p value: 0.002) and IL-6 (p value: 0.002) concentration was higher in *L. crispatus* MOI 50 compared to *G. vaginalis* MOI 50 conditions [Fig40B]. pH value was decreased in all conditions involving *G. vaginalis* and *L. crispatus*. The strongest decrease was observed in the supernatant of A2EN cells with *L. crispatus* MOI 100 [Fig40C below]. Cell viability was increased in all conditions except Poly I:C [Fig40C top]. To check that cell titer measurement was not impacted by the bacteria, supernatant containing only bacteria was mixed with cell titer. Optical density measurement was not increase in presence of bacteria without cell [Data not show]. Overall, we choose to keep the condition MOI 50 for *L. crispatus* and *G. vaginalis*.



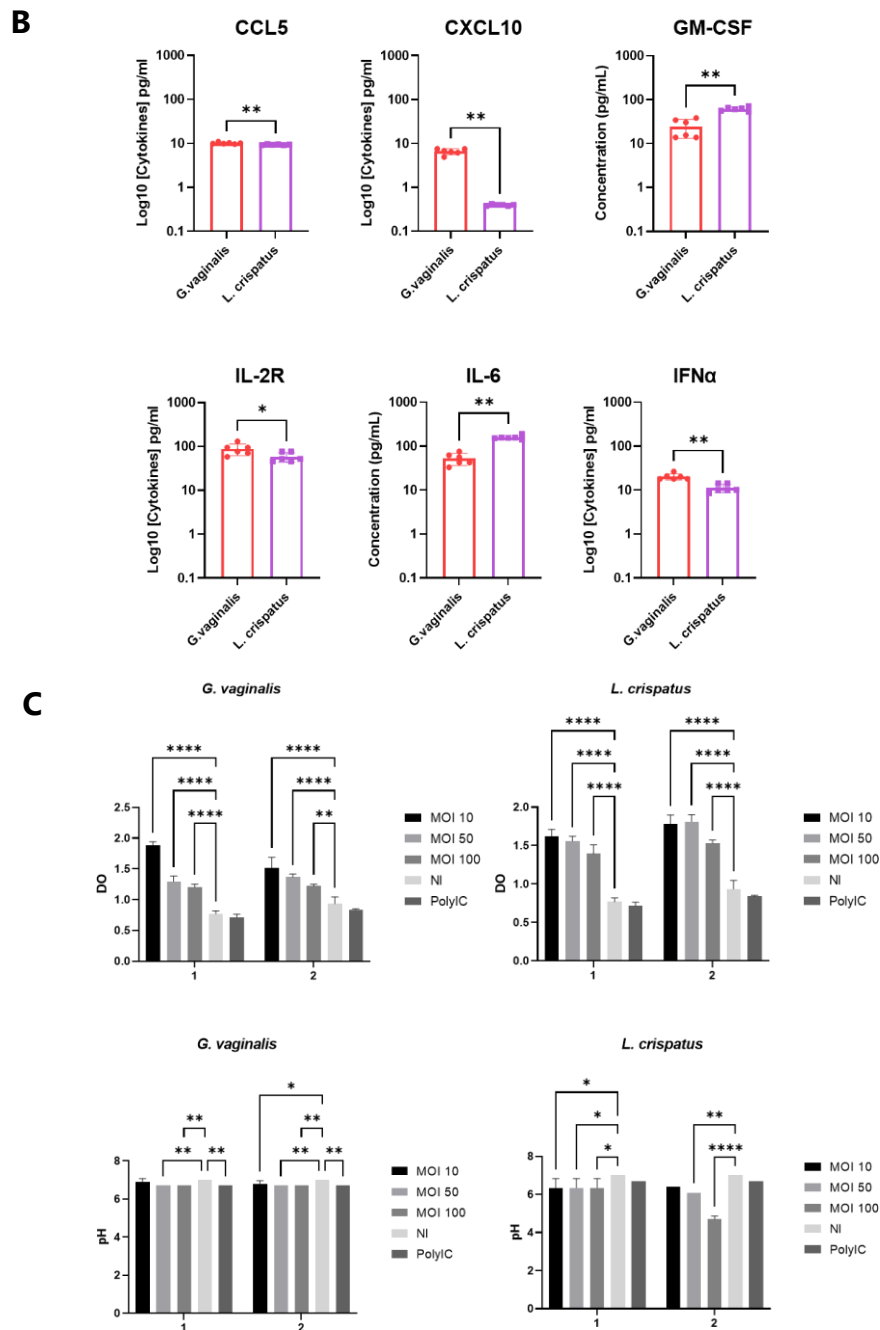


Figure 40: *G. vaginalis* and *L. crispatus* interaction with A2EN cells.

(A) Cytokine and chemokine concentration in supernatant of cells exposed during 24h with *L. crispatus* or *G. vaginalis*. Heatmap representing the mean fold change expression of cytokine concentration based on the cytokine concentration obtained in unexposed cells. Mean of two independent experiments (triplicates for each condition) was calculated. Different conditions are represented: MOI 10, MOI 50 and MOI 100 for each bacteria. Blue represents decrease expression and red an increase. Baseline values are presented on the left of the heatmap. (B) Cytokine and chemokine that are significantly different between the two conditions: *L. crispatus* MOI 50 and *G. vaginalis* MOI 50. Log10 of cytokine concentrations (pg/mL) are represented and a Mann-Whitney test was performed. Asterisks indicate p values considered statistically significant (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). (C) Cell viability (top) and pH measurement (bottom) were done in experiment 1 (triplicate) and 2 (triplicate). A two way ANOVA was used to compared mean value in each condition compared to the unexposed condition (NI).

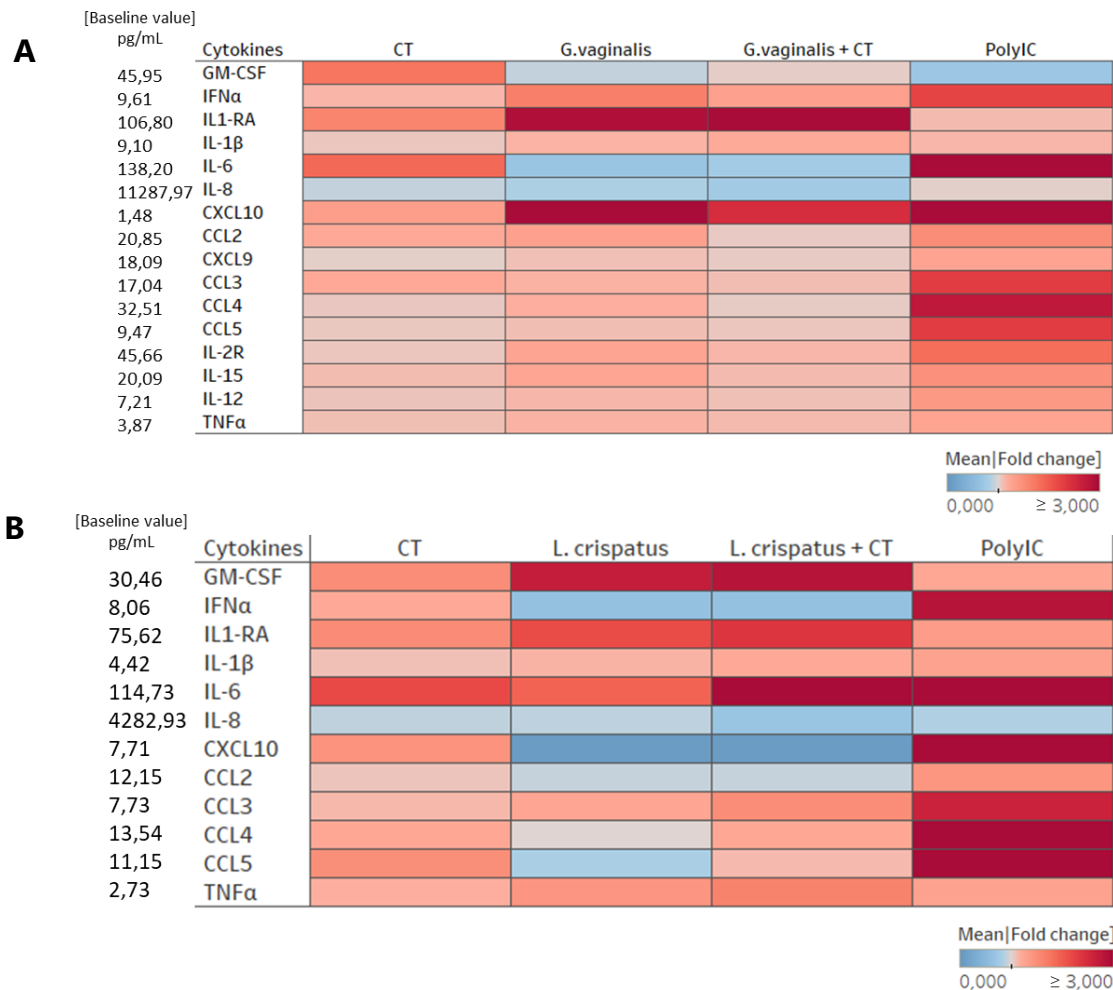
c. *L. crispatus* and *G. vaginalis* exposition on CT infected epithelial cells

After the optimal conditions established, interaction assays of CT infected A2EN cells with *L. crispatus* or *G. vaginalis* were performed. For those experiments, A2EN cells at a concentration of $5 \cdot 10^4$ cells/well were seed in a 24 wells plate and infected with CT svD MOI 12 for one hour then *L. crispatus* or *G. vaginalis* at MOI 50 was added to the cell culture for 24h. Supernatants were then collected and pool of 15 wells for each condition was made. The pool obtained for each condition was then used for cytokine and chemokine quantification. Five independent experiments was performed for *G. vaginalis* and *L. crispatus* interaction assays. Some experiments were performed with L. Réot, a PhD student in our team. The stimulation of A2EN cells with Poly I:C confirmed that A2EN cells were able to produce cytokines in response to a stimulation. Cytokine production of CT infected A2EN cells, in the experiments involving *L. crispatus* or *G. vaginalis* were similar. There was a higher production of GM-CSF, IL-1RA, IL-6 mainly and to a lesser extend IFN α , CXCL10, CCL5 etc. No modification of IL-8 concentration was observed due to a high basal concentration in uninfected cells [data not show]. *G. vaginalis* interaction with uninfected A2EN cells induced an increase production of IL-1RA, CXCL10, IFN α and IL-1 β mainly, as observed in previous experiments. *G. vaginalis* at MOI 50 co-cultured with CT infected cells induced the expression of IL-1RA, CXCL10, IFN α and IL-1 β [Fig41A]. Interestingly, the cytokine profile was more similar to the one observed in cells exposed to *G. vaginalis* alone compared to CT infected cells. pH was not impacted by the co-interaction CT/*G. vaginalis* with A2EN cells, however cell viability was reduced compared to control cells but this difference was not significant [Data not show]. On the contrary, the interaction between *L. crispatus* and A2EN cells induced a different inflammatory profile. Similar cytokine production were observed with *L. crispatus* alone and *L. crispatus* interaction with CT infected A2EN cells. Indeed, there was an increase production of GM-CSF, IL-1RA and IL-6 mainly [Fig41B].

We then compared the cytokine production in each experiment to CT condition. In *G. vaginalis* experiments, we observed a decreased production of IL-6 in *G. vaginalis* (p value: 0.011) and *G. vaginalis* + CT (p value: 0.049) conditions compared to CT alone. In addition,

CXCL10 (p value: 0.006) was increased whereas GM-CSF (p value: 0.014) was decreased in the condition *G. vaginalis* alone compare to CT infection alone. Finally, the concentration of IL-1RA (p value: 0.009) was increased in the condition *G. vaginalis* + CT compared to CT infection alone [Fig41C]. No significant differences were observed between the condition *G. vaginalis* and *G. vaginalis* + CT confirming that the profile of cytokines induced was similar according to both conditions.

In *L. crispatus* experiments, more significant differences were noticed between CT, *L. crispatus* alone or *L. crispatus* + CT. We observed an increase production of IL-1RA, GM-CSF and a decrease production of IFN α and CCL2 in *L. crispatus* as well as *L. crispatus* + CT conditions compared to CT. Compared to CT infection, TNF α (p value: 0.005) and CCL3 (p value: 0.006) were increased in *L. crispatus* + CT. Finally, CCL5 (p value: 0.011) concentration was decreased in *L. crispatus* condition compared to CT infection [Fig41D]. As observed in *G. vaginalis* experiments, no significant differences were observed between *L. crispatus* and *L. crispatus* + CT.



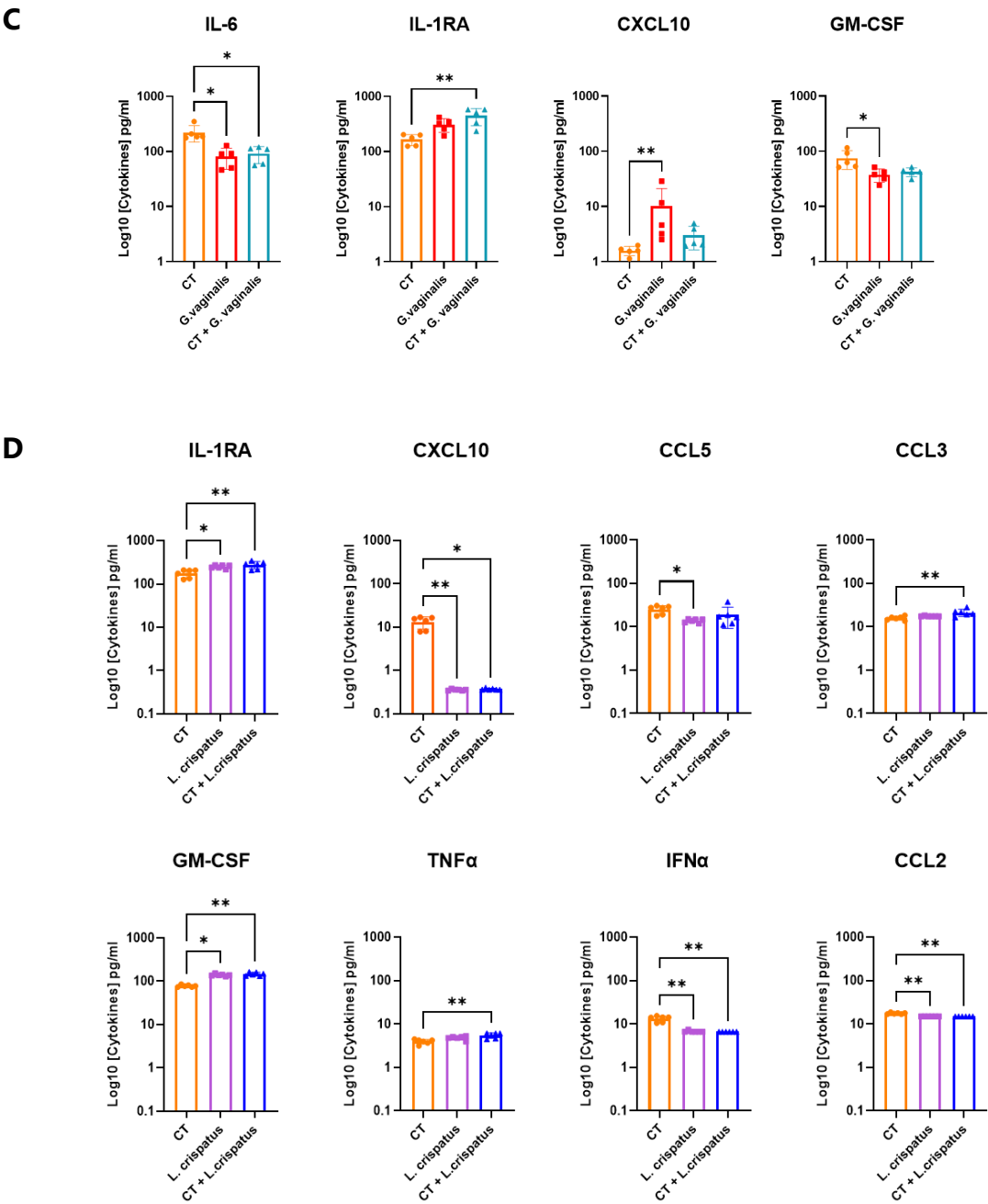


Figure 41: Interaction of *L. crispatus* or *G. vaginalis* with CT infected A2EN cells.

(A) Cytokine and chemokine concentrations in supernatants of CT infected cells exposed during 24h with *G. vaginalis* MOI 50. Heatmap representing the mean fold change expression of cytokine concentration based on the cytokine concentration obtained in unexposed cells. Mean of five independent experiments (triplicates for each conditions) was calculated. Different conditions are represented: CT, *G. vaginalis*, *G. vaginalis* + CT and Poly I:C. Blue represents decrease expression and red an increase. Baseline values are presented on the left of the heatmap. (B) Cytokine and chemokine concentrations in supernatants of CT infected cells exposed during 24h with *L. crispatus*. Heatmap representing the mean fold change expression of cytokine concentration based on the cytokine concentration obtained in unexposed cells. Mean of five independent experiments (triplicates for each conditions) was calculated. Different conditions are represented: CT, *L. crispatus*, *L. crispatus* + CT and Poly I:C. Blue represents decrease expression and red an increase. Baseline values are

presented on the left of the heatmap. (C) Cytokines and chemokines that are significantly different between the three conditions: CT, *G. vaginalis*, *G. vaginalis* + CT. (D) Cytokines and chemokines that are significantly different between the three conditions: CT, *L.crispatus*, *L.crispatus* + CT. (C) and (D) Log10 of cytokine concentrations (pg/mL) are represented and a Kruskal-wallis test was performed. p values were adjusted with Dunn's test. Asterisks indicate p values considered statistically significant (*P ≤0.05, **P ≤0.01, ***P ≤0.001, **** P ≤0.0001).

In conclusion, interaction of *G. vaginalis* or *L. crispatus* with A2EN cells induced distinct cytokine profiles. IL-6, IL-1RA and GM-CSF were increased by *L. crispatus* interaction whereas IFNα, IL-1RA, IL-1β and CXCL10 increased by *G. vaginalis* interaction with epithelial cells. Comparison of cytokines produced as well as their concentration according to both bacteria suggest that *G. vaginalis* is able to induce a stronger inflammatory environment compared to *L. crispatus* stimulation. Interestingly, in a context of CT infection, the cytokines produced are similar to the interaction of *L. crispatus* or *G. vaginalis* alone. IL-1RA and IL-1β increased in *G. vaginalis* + CT conditions compared to CT alone suggesting a slightly more inflammation in context of CT. Presence of *L. crispatus* with CT infected cells induce higher level of chemokine (CCL3) and TNFα suggesting similar conclusion as observed in *G. vaginalis* + CT condition.

The supernatants of these experiments were then tested on neutrophil phenotype and survival as well as on HIV-1 susceptibility (experiments performed by L. Réot, a PhD student in our team, thus only a summary of the results is presented below).

d. Effect of supernatants of *L. crispatus* or *G. vaginalis* in contact of CT infected A2EN cells on neutrophil phenotype and survival

Neutrophils have a role of sentinel and migrate within the tissue very quickly in case of infection. They are involved in antimicrobial immunity but are associated with increase pathology during CT infection [46]. Therefore, it was important to study if neutrophils are less activated and rapidly die in presence of *L. crispatus* suggesting a lower accumulation of neutrophils and a less inflammatory effect of neutrophils.

Neutrophils were isolated from Human peripheral blood and neutrophil phenotype and survival were attested at different times after exposition to supernatants. Different supernatants were used : (1) A2EN cells alone (2) Infected A2EN with CT (3) A2EN cells exposed to *L. crispatus* (4) A2EN cells exposed to *G. vaginalis* (5) A2ENs cells infected with CT and exposed to *L. crispatus* (6) A2ENs cells infected with CT and exposed to *G. vaginalis* (7) A2EN cells stimulated with Poly I:C. The phenotype was studied 6h post-supernatant exposition whereas survival was monitored at 24h post-supernatant exposition.

One main neutrophil population is present in the blood which is CD11+ CD101+ CD32a+ CD62L+. Neutrophils had a higher expression of CD11b and CD32a and a lower expression of CD62L when exposed to *L. crispatus* or *G. vaginalis* with or without CT infection. This suggest a higher activation of neutrophils. Moreover, a higher expression of CD101 was noticed in the same conditions previously described. CD101 is a marker of maturity, its expression allows to distinguish pre-neutrophils from immature neutrophils. Exposition of neutrophils to CT infected cell supernatant induced the expression of CD11b, CD32a and CD101 but no decrease of CD62L was observed. This suggests an activation of neutrophils that is lower than the one observed when stimulated with supernatants containing bacteria from the microbiota.

The survival assay is based on flux cytometry and allows the distinction between dead, alive and early apoptotic cells. At 24h of culture, the supernatants of A2EN cells cocultured with *G. vaginalis* or infected with CT significantly increased the survival of neutrophils. The supernatant of CT infected A2EN in presence of *G. vaginalis* significantly increased neutrophil survival compared to uninfected or CT infected A2EN supernatants. *L. crispatus* supernatant did not significantly modify neutrophil survival whereas the supernatant of CT infected A2EN cells with *L. crispatus* increased neutrophil survival.

Overall, the activation status of neutrophils was increased in presence of bacteria from the microbiota in a context of CT infection or not. However, neutrophil survival was increased in presence of CT, *G. vaginalis* +/- CT as well as *L. crispatus* + CT only. No significant modification of neutrophil survival was observed in the condition *L. crispatus* alone.

e. Effect of supernatants of *L. crispatus* or *G. vaginalis* in contact of CT infected A2EN cells on HIV-1 infection

The effect of each supernatant on HIV-1 infection was tested in a human glioblastoma cell line (U87) expressing CD4 and CCR5. Two HIV-1 pseudotypes were used: one with a VSV-G envelop (HIV-1_{VSV-G}) and a second one with HIV-1_{BaL} envelop (HIV-1_{BaL}). Pseudotype viruses are able to complete only one cycle of infection. HIV-1 pseudotype virus with the VSV-G envelop is able to enter the cells without binding to receptor (CD4) and co-receptor (CCR5). In contrast, CD4 and CCR5 binding is necessary to induce the entry in the cell of the pseudotype virus with HIV-1_{BaL} envelop. Both pseudotype viruses are coupled with luciferase. Luciferase activity, reflecting the infection, is read 48h post exposition.

The A2EN supernatants obtained previously were added on U87 cells 1h before/during and after HIV-1 exposition. Different supernatants were used : 1) A2EN cells alone (2) Infected A2EN with CT (3) A2EN cells exposed to *L. crispatus* (4) A2EN cells exposed to *G. vaginalis* (5) A2EN cells infected with CT and exposed to *L. crispatus* (6) A2EN cells infected with CT and exposed to *G. vaginalis* (7) A2EN cells stimulated with Poly I:C.

HIV-1_{BaL} infection decreased with supernatants of CT infected cells and Poly I:C stimulated cells. VSV-G infection decreased with supernatant of Poly I:C stimulated cells. *L. crispatus* supernatants in context of CT infection or not did not modify HIV-1_{BaL} or HIV-1_{VSV-G} infection compared to the supernatants of uninfected cells. In contrast, supernatants of *G. vaginalis* in context of CT infected or not decreased HIV-1_{BaL} or HIV-1_{VSV-G} infection compared to supernatants of uninfected cells.

Overall, we shown that supernatants of CT infected cells as well as supernatants of *G.vaginalis* with or without CT infection induced a decrease of HIV-1 pseudotype infection.

III. Data summary

Altogether, we set up a Human *in vitro* model permitting the evaluation of the effect of bacteria from the vaginal microbiota, in a context of CT infection, on cytokine and chemokine production but also on neutrophil phenotype/survival as well as HIV-1 co-infection. We have demonstrated that *L. crispatus* and *G. vaginalis* induce different cytokine profiles. Interestingly, the cytokines induced during CT infection, in presence of bacteria from the vaginal microbiota, are similar to a single interaction with *L. crispatus* or *G. vaginalis* without CT infection. These results demonstrated that bacteria from the vaginal microbiota have a strong impact on cytokine production of epithelial cells despite a preexisting CT infection. This observation could be due to the mild CT infection since the percentage of infection is only of 30% after 24 hours. Moreover, the stimulation with *L. crispatus* and *G. vaginalis* could be too early compared to CT infection development therefore explaining that the profile of cytokine produce are the same as a single stimulation with *L. crispatus* and *G. vaginalis*. Nevertheless, we can still observed a lower inflammation induced by *L. crispatus* compared to *G. vaginalis*.

Blood neutrophils activation was induced in all conditions but the presence of bacteria from the vaginal microbiota induced higher neutrophil activation. Higher neutrophil survival was also detected when the stimulation was performed with supernatants obtained during CT infection in presence of not of bacteria from the vaginal microbiota or with *G. vaginalis* alone. We did not observed the activation of neutrophils *in vivo* upon CT infection. We have described that neutrophil phenotype in peripheral blood and cervicovaginal neutrophils are different [C. Adapen et al., Local immune markers and vaginal microbiota composition are influenced by hormonal cycle phases]. Therefore, this difference in neutrophil activation status might be attributed to the use of peripheral blood neutrophils *in vitro* and not mucosal one. Low neutrophil survival in presence of *Lactobacillus spp* was also described in a previously published article of T. Hensley-McBain [281]. They also used peripheral blood neutrophils.

Surprisingly, we observed that CT infection can decrease HIV-1 susceptibility *in vitro* in opposition to the literature [100]. Moreover, we detected that *G. vaginalis* supernatants with or without CT infection reduced HIV-1 co-infection as well. These results might be explained by the fact that this *in vitro* model does not completely recapitulate the FRT mucosae. Only the first steps with the epithelial cells are studied. For further experiment, polarized cells might be a better model to study cytokine production. Moreover, an *in vitro* model including directly other immune cells should be used to study the impact on HIV-1 co-infection. Indeed, CT was observed to increase HIV-1 infection *in vitro* through an increased cell-associated virus migration [101].

Discussion

General discussion

During this thesis, I have studied environmental factors present within the FRT and their interplay in a context or not of an STI. This project focused on how we could modulate the local inflammation through the manipulation of the vaginal microbiota to decrease STI acquisition but also STI induced inflammation. Moreover, we were able to set up and validate an *in vivo* model essential to better understand the impact of FRT environmental factors on inflammation and on STI acquisition. Several objectives were achieved [Fig42]. First, we characterized the vaginal and rectal microbiota of cynomolgus macaques and a comparison between both microbiota was performed. Variation of the vaginal microbiota was analysed according to progesterone level. Moreover, vaginal microbiota was compared to human vaginal microbiota. This was, to our knowledge, the first study describing the vaginal microbiota of cynomolgus macaques. However, during this pilot study, we did not analyse the local and vaginal inflammation, thus a second study was developed. In this study, variations of vaginal microbiota and inflammation (cervicovaginal neutrophil phenotypes and cytokine production) according to menstrual cycle phases were evaluated and compared to systemic inflammation. The interplay between vaginal inflammatory markers and vaginal microbiota was also analysed. This study gave us important informations on the link between environmental factors and basal modulation of inflammation. Unfortunately we were not able to clearly determine an association between a group of bacteria from the microbiota and the local inflammation. To evaluate the impact of vaginal microbiota composition on local and systemic inflammation, a model of female cynomolgus macaques with a vaginal microbiota enriched with Human *Lactobacillus crispatus* strain was developed. Thanks to this model, we monitored the impact of vaginal microbiota composition 1) on the local and systemic inflammation, 2) on the susceptibility to CT infection and 3) on the inflammation induced by CT. So far, we were not able to directly link a bacteria or a group of bacteria with CT susceptibility and inflammation.

Finally, we developed a Human *in vitro* model to better characterize the mechanisms involving bacteria from the microbiota and inflammation induced by CT infection. Therefore, we studied the effect of bacteria from the Human vaginal microbiota on CT induced

inflammation. Then, this environment was tested to evaluate its ability to modify neutrophil phenotype and survival, as well as the susceptibility to HIV-1 acquisition.

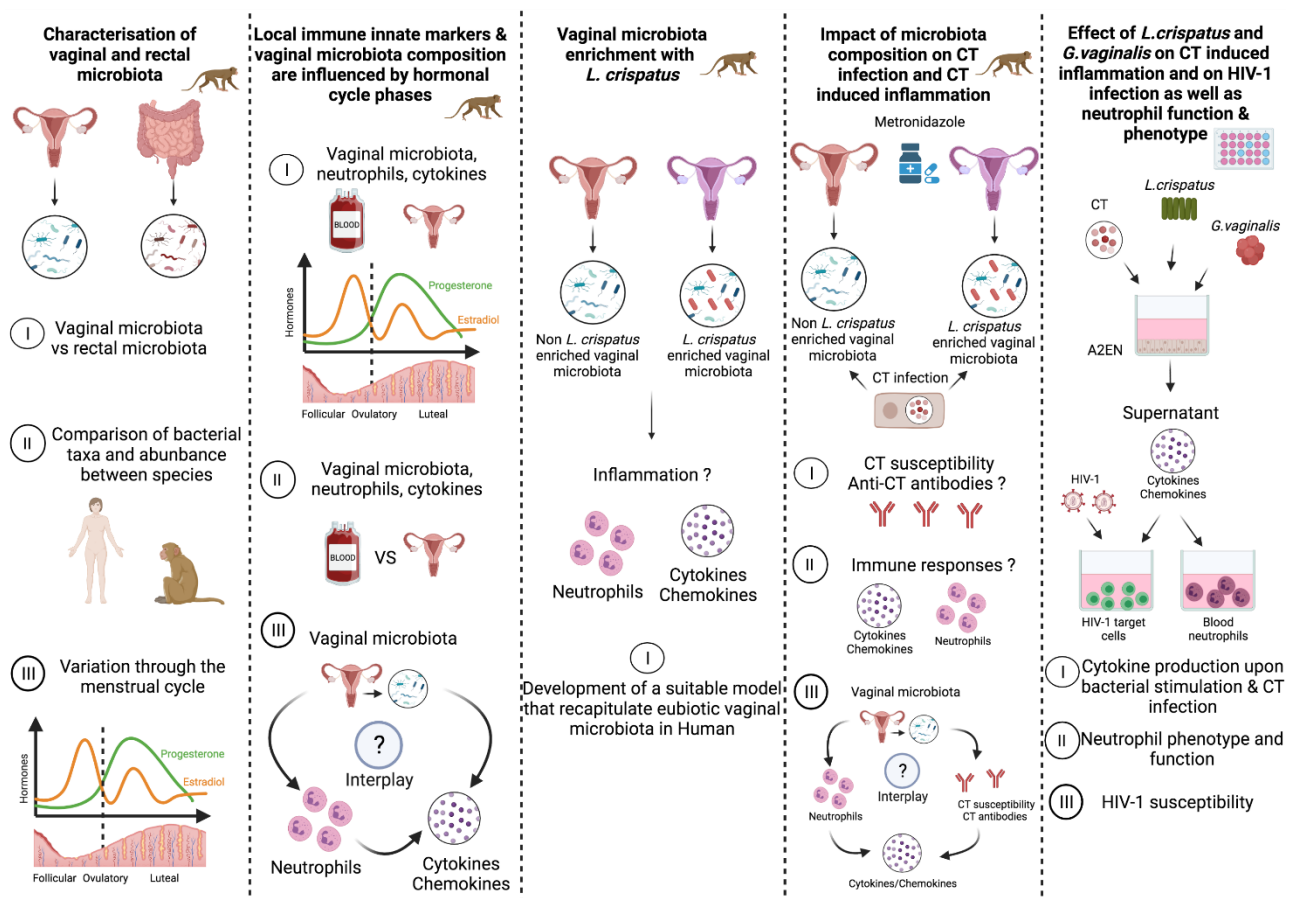


Figure 42: Thesis objectives.

Created with Biorender.com.

I. Establishment of a cynomolgus macaque model to study STI acquisition

The final aim of this part was to develop a suitable study model to evaluate the impact of FRT environmental factors on local inflammation and STI susceptibility. Cynomolgus macaques are used in different research fields including infectious, sexually transmitted diseases or vaccine and therapy testing. They are susceptible to a wide range of human pathogens [287, 288, 290] and have significant similarities with Human in terms of immune systems [285, 296]. Moreover, the FRT morphology, endocrine system and menstrual cycle are similar between the two species [298, 299]. These informations demonstrated that

cynomolgus macaques are a suitable model to study mucosal immune response to STI. However, little were known about their mucosal environment. Does the FRT environmental factors are similar and vary similarly to Human? Is there an association between vaginal microbiota composition and inflammation as observed in Human? Can the vaginal microbiota composition regulate the inflammation induced by STI acquisition?

a. Vaginal microbiota composition

The vaginal microbiota composition is well described in human [193]. However, it was never studied in cynomolgus macaques despite of its possible effect on immune responses induced by an infection or a vaccine [195]. We have thus conducted the first study describing the vaginal microbiota of cynomolgus macaques. We determined that vaginal microbiota was highly variable according to times and hormonal cycle, compared to rectal microbiota [Fig43]. Human vaginal microbiota comparison with cynomolgus macaque vaginal microbiota highlights similarities between both microbiota. We have shown that vaginal microbiota of cynomolgus macaques belong to CST IV-A in Human (highly diverse microbiota with few *Lactobacillus spp.*). Indeed, species such as *Sneathia*, *Porphyromonas*, *Prevotella*, *Peptoniphilus*, *Parvimonas*, *Peptoniphilus* or *Mobiluncus* often observed in BV positive bacteria [194] were in the top 9 most expressed genus in cynomolgus macaques [Fig43]. Similar species were detected in other macaque species including rhesus and pig-tailed macaques [302, 303]. All three species of macaques displayed very low abundances of *Lactobacillus spp.* compared to eubiotic vaginal microbiota of women. So far, it is not known exactly why human are the only mammal that have a vaginal microbiota dominated by *Lactobacillus spp.* Mirmonsef et al., hypothesized that the absence of *Lactobacillus spp.* in macaques might come from the low vaginal level of glycogen and lactic acid [306]. Moreover, Miller et al. suggest that an alimentation rich in starch have increased the level of glycogen in the vagina, therefore increasing the number of *Lactobacillus spp.* [307]. These differences of vaginal microbiota composition can raise question on our study model since it does not recapitulate an eubiotic vaginal microbiota composed of *Lactobacillus spp.* Interestingly, the vaginal microbiota of Hispanic and black women in US was observed to be

highly diverse compared to Asian and white women [193]. It was dominated by CST IV, demonstrated that a microbiota dominated by anaerobic bacteria is also common in Human and not always associated with a clinical condition. Therefore, the vaginal microbiota of cynomolgus macaques is quite comparable to the one of Hispanic and black women. Furthermore, we have shown that the relative abundance of some genera is associated with progesterone levels.

During this first pilot study, we did not analyze the cervicovaginal inflammation, but focused on the vaginal microbiota only. Therefore, we set up another study to analyze the variation of immune markers, as well as the composition of the vaginal microbiota according to hormonal phases in the female cynomolgus macaques.

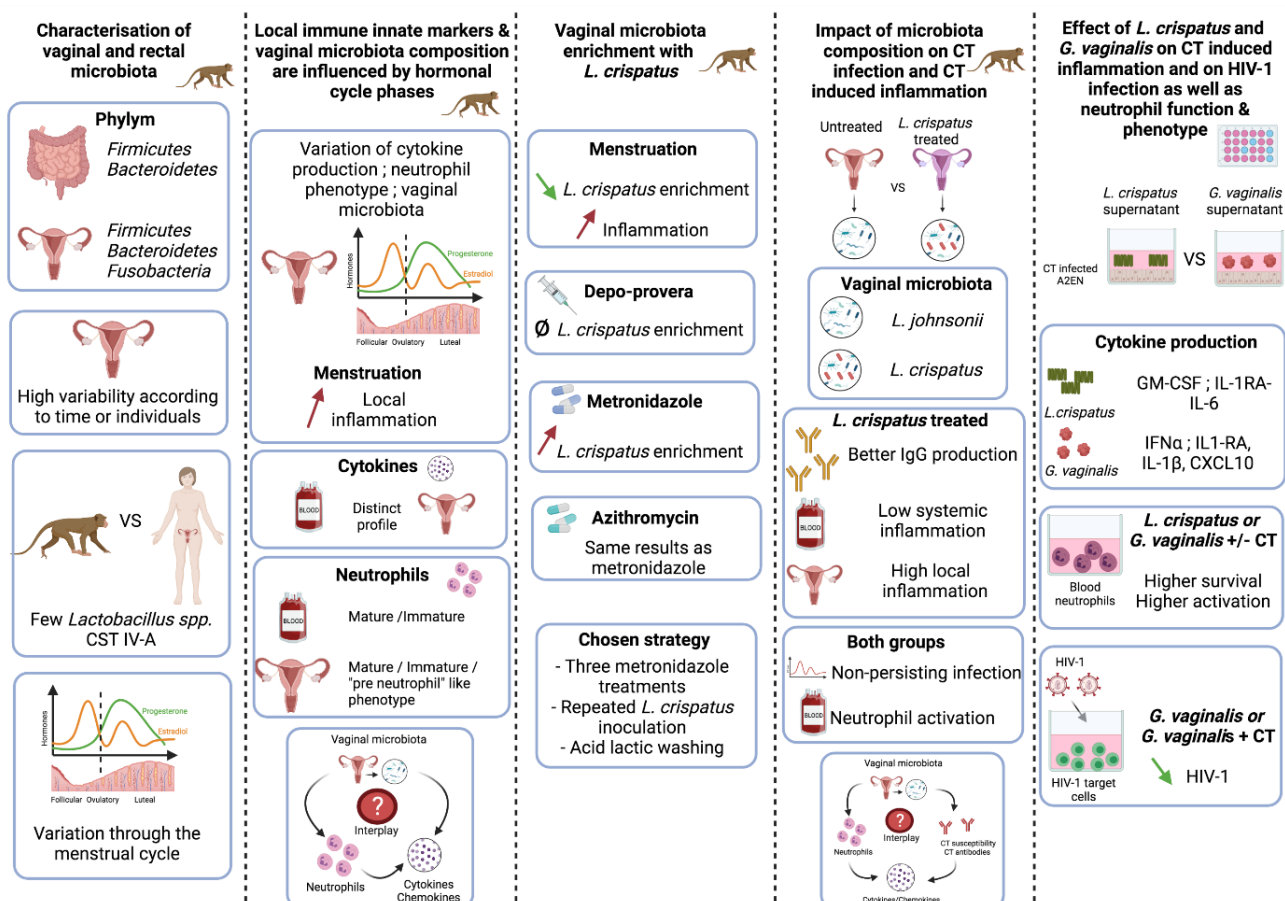


Figure 43: Thesis main results.

Created with Biorender.com.

b. Impact of menstrual phases on immune markers

We were able to determine that cervicovaginal immune markers including cytokine production and neutrophils were increased during the menstruation [Fig43]. We have characterized for the first time the phenotype of the cervicovaginal neutrophil subpopulations. Consequently, we have demonstrated that menstrual cycle has profound impacts on the local environment, but not on the systemic inflammation. Moreover, the profile and concentration of cytokine and the neutrophil phenotype were different in cervicovaginal fluids compared to blood [Fig43]. This conclusion was also drawn in human [110, 325]. The effect of menstrual cycle in the regulation of cytokine and chemokine concentration was also demonstrated in women [173, 174, 326]. However, cervicovaginal neutrophil phenotypes were never studied so far in women, so comparison between macaques and human is not yet possible.

This study clearly emphasizes that results obtained in women or female animal model focusing on cervicovaginal immune responses, without taking into consideration the menstrual cycle, might be biased. Indeed, longitudinal study involving repeated sampling is necessary. Moreover, our results support the importance of studying mucosal tissue specific responses and not only peripheral immune responses. Overall, this study demonstrates strong similarities between Human and cynomolgus macaques in terms of immune marker variation according to menstrual phases.

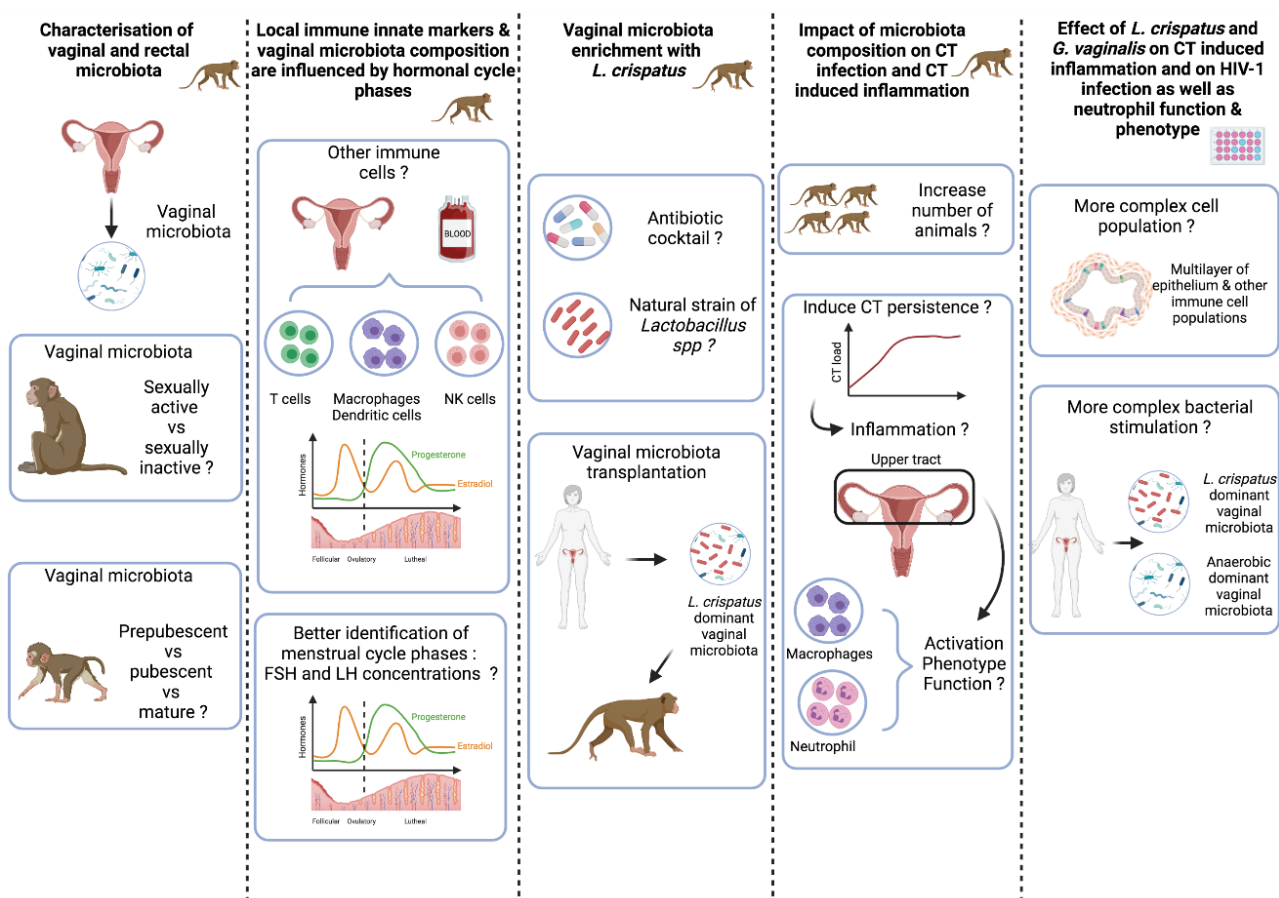
c. Study perspectives

In women of reproductive age, numerous factors influence vaginal microbiota composition, including hormones, STI, sexual activity, hygiene or ethnicity [245]. During our studies, only sexually mature animals from 3 to 5 years old were studied. Animals were housed with females only, so their vaginal microbiota is not the one of sexually active female cynomolgus macaques. M.C. Janiak et al., detected no age related significant difference in the vaginal microbiota composition of rhesus macaques in social group including males and females, but they grouped infant animals (<1 year) with juvenile animals (1-4 years).

Interestingly, M.C. Janiak et al. described that the bacteria, associated with BV in women, were not found in free ranging rhesus macaques in opposition to captive animal. Those differences might come from the fact that captive animals are usually sexually inactive since social groups include only females [327]. Moreover, sexual activity in women have been shown to modulate peripheral blood immune responses [328, 329]. The impact of heterosexual activity on women immune system is not clearly understood yet. It can be due to several factors, including sperm exposure. Sperm is a complex fluid composed of a cell fraction and acellular fraction (seminal fluid). Seminal fluid is able to trigger an inflammation (cytokine production, neutrophil and T cell recruitment) within the FRT, which can facilitate HIV-1 transmission [330, 331]. In macaques, our teams has demonstrated that repeated intravaginal inoculations of seminal fluid in animals induced a recruitment of immune cells within the cervix [332]. A PhD student, in our team, is currently studying the impact of seminal fluid on CT infection and CT/SIV coinfection *in vitro* and *in vivo*.

The variation of other immune cell populations according to menstrual cycle could also be investigated. Due to sampling limitation, we were not able to study other immune cells. NK cell and macrophage total numbers were described to be altered in the endometrium. Previous studies described that there is no variation of immune cells in the vagina [115, 162]. however, they only focused on the total number of each cell type and not on their activation status or specific marker expression. Consequently, it would be very interesting to investigate the variation of other immune cells locally, using cervicovaginal cytobrushes, but also in peripheral blood. In our study on cynomolgus macaques, the total number of neutrophils in peripheral blood was modified according to menstrual cycle. No evaluation of the variation of neutrophil functionalities according to menstrual cycle was performed in our study. Nevertheless, human studies described a modification of functional activity of peripheral blood neutrophils according to the hormonal cycle [149, 333]. Moreover, T (CD3⁺CD4⁺, CD4⁺ and CD8⁺ Treg) and NK cell proportions in peripheral blood are significantly impacted during menstrual cycle [334, 335]. These results highlight that more work is necessary to confirm that our animal model completely recapitulate what has been described in human. In addition, including the quantification of FSH and LH will be essential to study the ovulation

phase in a next study. In fact, a paper on cervicovaginal secretion highlights an increase of several biological processes implied in inflammation in luteal phase compared to ovulation [336]. Finally, Wira et al. described a “window of vulnerability”, period at the end of the luteal phase during which several immune components are repressed to prevent fetus rejection [108]. During this period, the FRT was described to be more susceptible to STI acquisition. All these modifications, according to menstrual cycle, have to be better investigated in cynomolgus macaques to determine if they can have an impact on STI susceptibility, but also on pathogen clearance or dissemination. These perspectives are summarized in figure



44.

Figure 44: Thesis main perspectives.

Created with Biorender.com.

II. Vaginal microbiota dysbiosis in Human vs naturally diverse vaginal microbiota in macaques

a. Association between local inflammation and vaginal microbiota

Higher cervicovaginal inflammation was clearly associated with a specific bacterial composition in human. *Lactobacillus spp.*, in women, are associated with a low inflammation in the FRT, in opposition to highly diverse microbiota [195]. On the contrary, vaginal microbiota of macaques are naturally diverse, thus increased presence of *Lactobacillus spp.* might be deleterious compared to Human. It might, for instance, increase the inflammation. In our second study, we have longitudinally characterized cytokine production and neutrophil phenotype in cervicovaginal fluid and blood sample, as well as the vaginal microbiota in nine female cynomolgus macaques. We have shown that cervicovaginal cytokine production is very variable according to the animal, with some animals exhibiting a higher production of cytokines compared to the others [Fig43]. We can speculate that, as observed in women, a genus or a group of bacteria are capable of reducing local immune response. Unfortunately, we were not able to directly link vaginal microbiota composition to specific increase or decrease of cytokine concentrations. Principal component analysis (PCA) was performed on cervicovaginal cytokine concentration measured throughout the whole study [data not show] to determine if animals could be split in different groups that might be associated with a specific vaginal microbiota composition. The division of the samples was time points dependent and not animal dependent. Therefore, the vaginal microbiota could be different between the two groups (ongoing analysis).

b. Impact of *L. crispatus* presence on the local environment

To investigate the effect of vaginal microbiota composition on inflammation during an STI or not, we wanted to develop a model of female macaques vaginally enriched with *L.*

crispatus, a species associated with low inflammation in women. We managed to develop a model of female cynomolgus macaques vaginally enriched with human *L. crispatus*. However, the enrichment was sustained only by repeated inoculations and was not optimal for all females. In terms of inflammation, we observed a lower vaginal cytokine expression in animals with a vaginal microbiota enriched with *L. crispatus*. However, this decrease could also be linked to a decrease in anaerobic bacteria, and not only to the presence of *L. crispatus*. Absence of repeated inoculations of *L. crispatus* and menstruation decrease the abundance of *L. crispatus* [Fig43]. Therefore, we can speculate that *L. crispatus* did not colonize the vaginal microbiota of those females. We thought that, by treating the females with Depo-provera thus stopping the menstrual bleeding, we would be able to have a better enrichment with *L. crispatus*. It was actually the opposite since we completely avoided vaginal enrichment with *L. crispatus* by treating them with Depo-provera. This result gave rise to several questions involving contraceptive used, vaginal microbiota composition and STI susceptibility. Indeed, depot medroxyprogesterone acetate (DMPA) is a progesterone based contraceptive often used in Sub-Saharan Africa [337] which have been linked to bacterial vaginosis, but also increased HIV-1 susceptibility [174, 178, 235, 338, 339].

c. Study perspectives

To improve vaginal colonization with *Lactobacillus spp.*, a natural strain obtained in cynomolgus macaques could be used. *L. johnsonii*, for instance, is detected in rhesus and cynomolgus macaques [317, 319]. Anti-inflammatory properties of *Lactobacillus spp.* were observed to be strain specific [212]. Furthermore, we have shown that *L. johnsonii* was associated with CT load (C. Adapen et al., Influence of vaginal microbiota composition on cervicovaginal and systemic inflammation induced by *Chlamydia trachomatis* infection – article III). Consequently, *Lactobacillus spp.* screening (lactic acid, low production of cytokine, adhesion to epithelial cells or pathogens and antimicrobial compounds) will be essential to select the best *Lactobacillus spp.* strain. Another possibility would be to modify completely the vaginal microbiota of female cynomolgus macaques using antibiotic treatment, followed by vaginal microbiota transplantation of a women donor [203] [Fig44].

III. Effect of vaginal microbiota composition on CT infection and inflammation

a. Antibiotic effect on CT infection and immunity

To study the effect of vaginal microbiota composition on CT infection and inflammation, two groups of females were studied. One group (n=6) was antibiotic treated only and the second (n=6) received antibiotic followed by Human *L. crispatus* inoculations. Both groups were then repeatedly inoculated with CT. A non-persisting CT infection was detected in ten animals out of twelve, demonstrated that animals cleared rapidly the infection. However, repeated infections are needed in some animals to induce the production of specific anti-CT antibodies in the serum [Fig43]. It appears that animals that produce serum specific anti-CT antibodies do not suffer from CT reinfection. However, a recent study performed in women suffering from recurrent CT infection described stable level of anti-CT MOMP IgG antibodies, suggesting that antibodies do not completely protect from reinfection [79]. Indeed, neutralizing antibodies against a VD4 region of MOMP as well as CD4⁺ T cells were demonstrated to be essential for bacterial burden clearance and reduced pathology in mice [340]. In our macaque study, specific peripheral T cell responses are currently being studied to evaluate a possible link between a better serum antibody response and T cell response. In the laboratory, a previous study conducted by M. Siguier under the supervision of D. Desjardin evaluated CT infection by intravaginal route of cynomolgus macaques. During this study, repeated inoculations of CT induced a sustained infection in two females out of two [Data described in the introduction, chapter 4]. We did not observe this sustained presence of CT, wondering if antibiotic treatment could have an impact on the susceptibility to CT infection. Metronidazole is not prescribed for CT elimination in women, but it removes anaerobic bacteria, thus inducing increased abundance of *Lactobacillus spp.* [218]. Interestingly, a recent publication demonstrated that metronidazole treatment by inducing a shift in the vaginal microbiota was able to permit CT eradication without azithromycin or doxycycline treatment [341]. This result suggests that

vaginal microbiota has a central role during CT infection. Doxycycline or azithromycin treatment is highly effective in the eradication of CT, but it was also associated with impaired specific immune response and increased risk of CT reinfection in mice and women [342–344]. Overall, metronidazole, by depleting anaerobes, might trigger a better immune response against a pathogen, thus facilitating pathogen eradication. However, doxycycline and azithromycin will directly eradicate CT, consequently impairing the development of anti-CT immunity facilitating CT reinfection.

b. Effect of *L. crispatus* enrichment on immune responses

We have demonstrated that animals treated with *L. crispatus* develop higher titers of specific anti-CT antibodies in serum, compared to *L. crispatus* untreated [Fig43]. Bacterial load, measured in cervicovaginal swabs, between treated and untreated animal, was similar. We do not know yet the mechanism involved in this phenomenon. Our hypothesis is that local production of anti-CT IgG might be stimulated by the presence of *L. crispatus* and or a specific vaginal microbiota and that local antibodies would be released in the bloodstream. We only measured specific antibody production in the serum, but quantifications in cervicovaginal fluids are planned. Indeed, it will be interesting to see if the differences can also be observed or not locally. So far, we were not able to link a vaginal microbiota composition and anti-CT IgG production, this could be due to the delay between the actual production of antibodies locally and its increase presence in the blood. Quantification of specific antibodies in cervicovaginal fluids might answer these questions. In terms of cytokine production after CT infection, an increased production was noticed in cervicovaginal fluids of *L. crispatus* treated animal, compared to untreated, whereas the opposite was seen in peripheral blood. So far, the mechanisms by which the vaginal microbiota composition might affect peripheral blood immune responses are unknown. Neutrophil phenotype was characterized in cervicovaginal cytobrushes and peripheral blood at baseline, before CT inoculation and one and four weeks after the last CT inoculation. We

did not performed cervicovaginal cytobrushes for neutrophil phenotype analysis during the CT inoculations, to avoid the induction of local inflammation due to sampling, therefore we could not detect if an accumulation of neutrophils or a change of neutrophil phenotype occurs just after infection in the FRT. Since no persisting infection was induced, the sampling at the end of the protocol only allow us to determine the long-term effect of CT infection on neutrophils. We have shown that blood neutrophils were more activated and mature after CT infection in both groups. Concerning cervicovaginal neutrophils, only a significant decrease of the expression of CD11b was noticed. CD11b associated with neutrophil priming, is usually overexpressed on neutrophils during an infection [134, 136] but, since the sampling was quite far away from the peak of infection, a lower activation state of neutrophils in the vaginal mucosa, compared to blood, might be expected. Indeed, it we can presume that resident cervicovaginal neutrophils are affected very quickly after CT infection, therefore modification of activation should be observed right after inoculation or infection. No persisting infection was noticed in the cervicovaginal fluids suggesting that neutrophil phenotype might return to a "resting" phenotype as soon as CT is eradicated. On the contrary, the systemic response takes a longer time to set up. We have observed in our study that two animals develop a strong systemic production of cytokine after CT infection therefore, we wondered if CT had disseminated in the FRT upper tract of these animals. No significant differences of cervicovaginal neutrophil phenotype was noticed in those two animals compared to others but we did not monitor neutrophil phenotype in the upper tract. We could not verify neutrophil phenotype as well as CT persistence in the upper tract since no euthanasia was performed.

c. Do human and macaque *Lactobacillus spp* have similar anti-inflammatory effect?

In the cynomolgus macaque *in vivo* model, we have shown that the antibiotic treatment increased the abundance of *L. johnsonii*. Moreover, we have associated the presence of *L. johnsonii* with CT infection in cynomolgus macaques [Fig43]. This observation was quite

surprising since many *Lactobacillus spp* in human are associated with low CT susceptibility [254, 255]. Only *L. iners*, described to have an intermediate metabolomics signature between a eubiotic and dysbiotic environment, has been associated to an increased CT susceptibility [199, 259, 260].

This discovery highlights a possible difference between *Lactobacillus spp* in macaques and in Human. In Human, most of the studies focus on genus or species for the analyses of the impact on inflammation. However, a recent article described that *Lactobacillus spp.* strains have different inflammatory properties, depending if they were isolated from a women with a *Lactobacillus spp.* dominant microbiota or with BV [212]. We can hypothesize that the strains induced in the vaginal microbiota of cynomolgus macaques might be equivalent to strains isolated from women with BV. Unfortunately, we were not able to link the abundance of *L. johnsonii* with a specific inflammatory environment, which could be associated to this increased susceptibility to CT.

Therefore, it would be interesting to investigate more deeply the mechanisms involved in the control of CT infection using *in vitro* experiments. We could, for instance, isolate *L. johnsonii* strain from the macaque vaginal microbiota and test its ability to induce cytokines, adhere to epithelial cells and produce lactic acid. Then, this strain could be tested in the *in vitro* model to study its ability to induce CT infection.

d. Study perspectives

CT persistence within the FRT is also an issue for CT clearance. It has been shown that azithromycin is not sufficient to clear CT infection in macrophages, thus allowing CT persistence within macrophages. Moreover, the authors hypothesized that early infection of macrophages could drive chronic upper tract inflammation and pathology [345]. *C. pneumoniae* can also persist within neutrophils [346]. We presume that those two cell types could be involved in CT dissemination and upper tract infection. We did not detect a variation of the phenotype of cervicovaginal neutrophils in cynomolgus macaques upon CT infection. However, we did not monitor CT infection of neutrophils, neither did we quantified

the infection in macrophages. Moreover, neutrophil functions were not tested. Further experiments, including neutrophil functions (NET production, survival), are required [Fig44].

IV. Modification of the inflammation induced by CT and its effect on neutrophil phenotype/survival and HIV-1 co-infection

In vivo in cynomolgus macaques, we have studied the impact of the vaginal environment on CT infection and inflammation in the FRT and in peripheral blood. However, we did not investigate the mechanisms involved in the regulation of the immune response by the vaginal microbiota. We wanted to set up a Human *in vitro* model to investigate more deeply the mechanisms observed in human, and determine if we can recapitulate what we observed *in vivo*. In the *in vitro* model that we have set up, we sought to investigate the effect of two bacteria, *L. crispatus* and *G. vaginalis*, on CT induced inflammation. We determined if this modified environment had: (1) an impact on neutrophil phenotype and survival and (2) an impact on HIV-1 susceptibility.

a. Inflammation: cytokine production and neutrophil activation and survival

We demonstrated that cytokines induced during CT infection in presence of *G. vaginalis* or *L. crispatus* are similar to a simple interaction between *G. vaginalis* or *L. crispatus* with epithelial cells. *G. vaginalis*, a bacteria often present during bacterial vaginosis in women, has been associated with higher inflammation [246]. In the *in vitro* model, *G. vaginalis* was able to increase the production of IFN α , IL1-RA and CXCL10 mainly. C.R. Eade et al., observed an increase of different cytokines upon *G. vaginalis* interaction with epithelial cell lines [249]. An important difference between both studies is that C.R. Eade performed the experiments using transwells and the epithelial cells were polarized. *G. vaginalis* is known to have a central role in BV by being a part of a complex environment, including *Prevotella*,

Atopium, *Sneathia* and BV associated bacteria for instance. Indeed, association of several anaerobic bacteria is necessary to induce BV, thus a pro-inflammatory environment [220].

In the *in vitro* model, we demonstrated that peripheral blood neutrophils are more activated by an environment composed of bacteria from the microbiota than CT infection environment. *In vivo*, we did not see a higher activation of cervicovaginal neutrophils in cynomolgus macaques, according to microbiota composition or CT infection. These apparent opposite results could be explained by the use of human neutrophils that are isolated from the blood. Indeed, we have demonstrated different phenotype of neutrophils according to compartment (C. Adapen et al., Local immune innate markers and vaginal microbiota composition are influenced by hormonal cycle phases – submitted manuscript). We can presume that blood neutrophils are more prone to activation via the microbiota, compared to tissue specific neutrophils.

b. Impact of CT on HIV-1 susceptibility

We have shown an inhibition of HIV-1 infection by the supernatants obtained after *G. vaginalis* interaction with CT infected or uninfected epithelial cells. This result is unexpected with regard to our initial hypothesis that *G. vaginalis* will increase the inflammation, and subsequently the susceptibility for HIV-1 infection. We detected more CCL5 production in supernatants after *G. vaginalis* stimulation, compared to *L. crispatus*. CCL5 is a chemokine with an anti-HIV-1 effect, indeed, it can bind to the co-receptor CCR5 present on HIV-1 target cells, enabling HIV-1 virions to bind and enter the cell [347]. Therefore, CCL5 concentration might be sufficient to decrease HIV-1 infection. Antibodies against CCL5 could be used to neutralize this chemokine and confirm its role on HIV-1 inhibition in this context.

c. Strength and limitation of the *in vitro* model

In vitro models are very useful to dissect a complex environment for understanding the involvement of each element. It allows in deep mechanistic studies with very straightforward results that are not biased by others factors. However, tissue specific environments are very

complex and the analysis of one factor does not completely reflect what happens in the tissue. For instance, in our study, the analysis of cytokine production of epithelial cells is relevant, since epithelium are the cells that are preferentially infected by CT and are in contact with the microbiota. Therefore, they are the first responders to the infection. Nevertheless, the FRT mucosa is also composed of immune cells that are recruited in case of an infection/inflammation, and a complex interplay takes place in the FRT, allowing either pathogen eradication or dissemination. Our current *in vitro* model was focusing on the interaction between the vaginal microbiota and epithelial cells, but does not recapitulate the complex environment present in the FRT. Other cell populations are needed to complete the model. Recently, human epithelial 2D cell culture and cervical organoid have been described and could be used to better characterize the impact of vaginal microbiota composition [348]. Nevertheless, these cultures do not include other immune cells, such as myeloid or T cells. Pyles et al., developed an *in vitro* model composed of a multilayer of vaginal epithelial cells supplemented with HIV-1 target cells. In this model, they showed that HIV-1 infection was enhanced when cocultured with cervicovaginal fluids of women suffering from BV [262]. This experimental model or human *ex vivo* explant could be adapted to study the impact of vaginal microbiota composition on CT/HIV-1 co-infection.

In addition, we focused our analysis on two bacteria that have a central role in inflammation in Human. This analysis is essential to study specifically the effect of those two bacteria on the infection and inflammation. However, two bacteria cannot recapitulate the whole microbiota. The human vaginal microbiota is composed of a wide range of bacteria, and even if it is dominated by *Lactobacillus spp.* other bacteria are present at low abundances. To better reproduce bacterial vaginosis, our model needs to be improved, first, by stimulating epithelial cells with multiple strains of bacteria associated with BV. We could also directly collect cervicovaginal fluids of women with BV and non-BV, and test their ability to induce pro-inflammatory cytokines by epithelial cells [Fig44].

Conclusion and Perspectives

Conclusion

In conclusion, my PhD work has allowed to:

- (1) Characterize the composition of the vaginal and rectal microbiota of cynomolgus macaques. The rectal microbiota is very stable according to animal and time, in contrast to the vaginal microbiota which is influenced by the menstrual cycle and varies greatly according to animal. The vaginal microbiota was observed to be similar to the one of women suffering from dysbiosis (CST IV) in terms of taxa and abundances.
- (2) Highlight the impact of menstruation on cervicovaginal inflammation translating into a surge of cytokines and accumulation of mature/activated neutrophils that originate from the blood. These results demonstrated that further studies focusing on local responses have to take into account those variations to avoid false interpretation of results.
- (3) Establish a novel model of cynomolgus macaques with a vaginal microbiota enriched with *L. crispatus*. This model will be useful in studies aiming at understanding the impact of microbiota composition and inflammation control.
- (4) Show a non-persisting CT infection in all animals (*L. crispatus* treated or untreated) exposed to repeated low doses of CT whereas higher specific antibody titers are detected in the serum of *L. crispatus* treated animals. Higher systemic inflammation was demonstrated in untreated animals upon CT infection, compared to *L. crispatus* treated animals. However, CT infection of *L. crispatus* treated animals triggered a better local innate response compared to untreated. *L. johnsonii* abundance was associated with higher CT load.

- (5) Test an *in vitro* model to study the effect of bacterial stimulation on epithelial cells and the subsequent effect on immune cell populations or STI. Differential cytokine profiles according to bacterial stimulation/infection (CT vs *L. crispatus* vs *G. vaginalis*) were characterized. In addition, modification of neutrophil phenotype and survival according to bacterial stimulation was described. HIV-1 infection of target cells was also impacted.

Perspectives

This project focused on the role and regulation of the local inflammation and its influence on STI susceptibility. It updates our knowledge on the interaction between numerous cervicovaginal environmental factors in presence or absence of a STI. It highlights that cervicovaginal environment is a complex milieu composed of a wide array of factors that interact between each other and are influenced by hormones. This is the first study describing the phenotype and accumulation of resident cervicovaginal neutrophils in presence or absence of an STI. We were able to set up an *in vivo* model that could be used to better characterize the impact of vaginal microbiota composition on immune response to STI, but also vaccines among other things. It will be important to better understand the local inflammation and how to control it to reduce STI susceptibility. More importantly, this project gives a solid basis for the development of future projects focusing on other vectors of inflammation, such as the seminal fluid, or DMPA use and their impact on STI acquisition. The impact of the seminal fluid on FRT mucosal inflammation in the context of CT/HIV-1-SIV co-infection is currently under evaluation in our team. Moreover, co-infection could also be studied in order to understand the role of the vaginal microbiota composition in the regulation of the immune response during an infection, and its ability to favour or repress another STI. In our laboratory, SIV infection of cynomolgus macaques through intravaginal route are set up. Therefore, an *in vivo* study aiming to better understand the impact of vaginal microbiota on CT/SIV coinfection could be performed. This study would allow to evaluate the nature and amplitude of the innate and specific immune responses in the mucosae and in peripheral blood.

Altogether, my PhD project was the first part of a story aiming at understanding the complex environment of the FRT mucosae, and how the factors in the FRT could favour or inhibit STI acquisition by regulating the inflammation.

Bibliography

1. Sexually transmitted infections (STIs). <https://www.who.int/westernpacific/health-topics/sexually-transmitted-infections>. Accessed 31 Aug 2021.
2. Raiford JL, DiClemente RJ, Wingood GM. Effects of Fear of Abuse and Possible STI Acquisition on the Sexual Behavior of Young African American Women. *Am J Public Health*. 2009;99:1067–71.
3. Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, et al. Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. *PLoS One*. 2015;10.
4. Infections sexuellement transmissibles. <https://www.santepubliquefrance.fr/maladies-et-traumatismes/infections-sexuellement-transmissibles>. Accessed 31 Aug 2021.
5. Jansen K, Steffen G, Potthoff A, Schuppe A-K, Beer D, Jessen H, et al. STI in times of PrEP: high prevalence of chlamydia, gonorrhea, and mycoplasma at different anatomic sites in men who have sex with men in Germany. *BMC Infect Dis*. 2020;20:110.
6. Walker J, Tabrizi SN, Fairley CK, Chen MY, Bradshaw CS, Twin J, et al. Chlamydia trachomatis Incidence and Re-Infection among Young Women – Behavioural and Microbiological Characteristics. *PLoS One*. 2012;7.
7. Chlamydia infection - Annual Epidemiological Report for 2018. European Centre for Disease Prevention and Control. 2020. <https://www.ecdc.europa.eu/en/publications-data/chlamydia-infection-annual-epidemiological-report-2018>. Accessed 31 Aug 2021.
8. Goulet V, De Barbeyrac B, Raheison S, Prudhomme M, Semaille C, Warszawski J. Prevalence of Chlamydia trachomatis: results from the first national population-based survey in France. *Sexually Transmitted Infections*. 2010;86:263.
9. Lot F, Françoise Cazein, Josiane Pillonel, Bruyand M, Ndeindo Ndeikoundam, Delphine Viriot, et al. Surveillance du VIH et des IST bactériennes.
10. Chandra NL, Broad C, Folkard K, Town K, Harding-Esch EM, Woodhall SC, et al. Detection of Chlamydia trachomatis in rectal specimens in women and its association with anal intercourse: a systematic review and meta-analysis. *Sex Transm Infect*. 2018;94:320–6.
11. Yeruva L, Spencer N, Bowlin AK, Wang Y, Rank RG. Chlamydial infection of the gastrointestinal tract: a reservoir for persistent infection. *Pathog Dis*. 2013;68:88–95.
12. Rank RG, Yeruva L. Hidden in Plain Sight: Chlamydial Gastrointestinal Infection and Its Relevance to Persistence in Human Genital Infection. *Infect Immun*. 2014;82:1362–71.
13. Howe SE, Shillova N, Konjufca V. Dissemination of Chlamydia from the reproductive tract to the gastrointestinal tract occurs in stages and relies on Chlamydia transport by host cells. *PLOS Pathogens*. 2019;15:e1008207.
14. Ming Tan, Patrik Bavoil. Intracellular Pathogens I: Chlamydiales. 1: Departments of Microbiology & Molecular Genetics, and Medicine, University of California, Irvine, CA; 2: Departments of Microbial Pathogenesis, and Microbiology & Immunology, University of Maryland, Baltimore, MD: ASM Press; 2012.
15. Murthy AK, Li W, Ramsey KH. Immunopathogenesis of Chlamydial Infections. *Curr Top Microbiol Immunol*. 2018;412:183–215.
16. Ziklo N, Huston WM, Hocking JS, Timms P. Chlamydia trachomatis Genital Tract Infections: When Host Immune Response and the Microbiome Collide. *Trends Microbiol*. 2016;24:750–65.
17. Hafner LM. Pathogenesis of fallopian tube damage caused by Chlamydia trachomatis infections. *Contraception*. 2015;92:108–15.
18. Weström L, Joesoef R, Reynolds G, Hagdu A, Thompson SE. Pelvic Inflammatory Disease and Fertility: A Cohort Study of 1,844 Women with Laparoscopically Verified Disease and 657 Control Women with Normal Laparoscopic Results. *Sexually Transmitted Diseases*. 1992;19:185–192.
19. Chlamydiae. <https://www.santepubliquefrance.fr/maladies-et-traumatismes/infections-sexuellement-transmissibles/chlamydiae>. Accessed 31 Aug 2021.

20. Kong FYS, Tabrizi SN, Law M, Vodstrcil LA, Chen M, Fairley CK, et al. Azithromycin Versus Doxycycline for the Treatment of Genital Chlamydia Infection: A Meta-analysis of Randomized Controlled Trials. *Clin Infect Dis*. 2014;59:193–205.
21. Caldwell HD, Wood H, Crane D, Bailey R, Jones RB, Mabey D, et al. Polymorphisms in *Chlamydia trachomatis* tryptophan synthase genes differentiate between genital and ocular isolates. *J Clin Invest*. 2003;111:1757–69.
22. An BB, Adamis AP. Chlamydial ocular diseases. *Int Ophthalmol Clin*. 1998;38:221–30.
23. Hybiske K, Stephens RS. Mechanisms of *Chlamydia trachomatis* Entry into Nonphagocytic Cells. *Infect Immun*. 2007;75:3925–34.
24. Nans A, Ford C, Hayward RD. Host-pathogen reorganisation during host cell entry by *Chlamydia trachomatis*. *Microbes Infect*. 2015;17:727–31.
25. Ford C, Nans A, Boucrot E, Hayward RD. *Chlamydia* exploits filopodial capture and a macropinocytosis-like pathway for host cell entry. *PLoS Pathog*. 2018;14:e1007051.
26. Keb G, Ferrell J, Scanlon KR, Jewett TJ, Fields KA. *Chlamydia trachomatis* TmeA Directly Activates N-WASP To Promote Actin Polymerization and Functions Synergistically with TarP during Invasion. *mBio*. 2021;12:e02861-20.
27. Subbarayal P, Karunakaran K, Winkler A-C, Rother M, Gonzalez E, Meyer TF, et al. EphrinA2 Receptor (EphA2) Is an Invasion and Intracellular Signaling Receptor for *Chlamydia trachomatis*. *PLOS Pathogens*. 2015;11:e1004846.
28. Cocchiari JL, Valdivia RH. New insights into *Chlamydia* intracellular survival mechanisms. *Cellular Microbiology*. 2009;11:1571–8.
29. Kurihara Y, Itoh R, Shimizu A, Walenna NF, Chou B, Ishii K, et al. *Chlamydia trachomatis* targets mitochondrial dynamics to promote intracellular survival and proliferation. *Cellular Microbiology*. 2019;21:e12962.
30. Ibana JA, Sherchand SP, Fontanilla FL, Nagamatsu T, Schust DJ, Quayle AJ, et al. *Chlamydia trachomatis*-infected cells and uninfected-bystander cells exhibit diametrically opposed responses to interferon gamma. *Sci Rep*. 2018;8.
31. Ziklo N, Huston WM, Taing K, Katouli M, Timms P. In vitro rescue of genital strains of *Chlamydia trachomatis* from interferon- γ and tryptophan depletion with indole-positive, but not indole-negative *Prevotella* spp. *BMC Microbiol*. 2016;16.
32. Luján A, Fili S, Damiani M. Female Infertility Associated to *Chlamydia trachomatis* Infection. 2016.
33. Rasmussen SJ, Eckmann L, Quayle AJ, Shen L, Zhang YX, Anderson DJ, et al. Secretion of proinflammatory cytokines by epithelial cells in response to *Chlamydia* infection suggests a central role for epithelial cells in chlamydial pathogenesis. *J Clin Invest*. 1997;99:77–87.
34. Buckner LR, Lewis ME, Greene SJ, Foster TP, Quayle AJ. *Chlamydia trachomatis* infection results in a modest pro-inflammatory cytokine response and a decrease in T cell chemokine secretion in human polarized endocervical epithelial cells. *Cytokine*. 2013;63:151–65.
35. Lu H, Shen C, Brunham RC. *Chlamydia trachomatis* Infection of Epithelial Cells Induces the Activation of Caspase-1 and Release of Mature IL-18. *The Journal of Immunology*. 2000;165:1463–9.
36. Xu JZ, Kumar R, Gong H, Liu L, Ramos-Solis N, Li Y, et al. Toll-Like Receptor 3 Deficiency Leads to Altered Immune Responses to *Chlamydia trachomatis* Infection in Human Oviduct Epithelial Cells. *Infect Immun*. 2019;87:e00483-19.
37. Hvid M, Baczynska A, Deleuran B, Fedder J, Knudsen HJ, Christiansen G, et al. Interleukin-1 is the initiator of Fallopian tube destruction during *Chlamydia trachomatis* infection. *Cellular Microbiology*. 2007;9:2795–803.
38. Spear GT, Kendrick SR, Chen HY, Thomas TT, Bahk M, Balderas R, et al. Multiplex Immunoassay of Lower Genital Tract Mucosal Fluid from Women Attending an Urban STD Clinic Shows Broadly Increased IL1 β and Lactoferrin. *PLOS ONE*. 2011;6:e19560.
39. Poston TB, Lee DE, Darville T, Zhong W, Dong L, O'Connell CM, et al. Cervical Cytokines Associated With *Chlamydia trachomatis* Susceptibility and Protection. *J Infect Dis*. 2019;220:330–9.
40. Agrawal T, Vats V, Salhan S, Mittal A. The mucosal immune response to *Chlamydia trachomatis* infection of the reproductive tract in women. *Journal of Reproductive Immunology*. 2009;83:173–8.
41. Arno JN, Ricker VA, Batteiger BE, Katz BR, Caine VA, Jones R. Interferon- γ in Endocervical Secretions of Women Infected with. :5.
42. Wyrick PB. *Chlamydia trachomatis* Persistence in Vitro – An Overview. *J Infect Dis*. 2010;201 Suppl 2:S88–95.

43. Shemer-Avni Y, Wallach D, Sarov I. Inhibition of *Chlamydia trachomatis* growth by recombinant tumor necrosis factor. *Infect Immun*. 1988;56:2503–6.
44. Radomski N, Karger A, Franzke K, Liebler-Tenorio E, Jahnke R, Matthiesen S, et al. *Chlamydia psittaci*-Infected Dendritic Cells Communicate with NK Cells via Exosomes To Activate Antibacterial Immunity. *Infection and Immunity*. 2019;88.
45. Gyorke CE, Kollipara A, Allen J, Zhang Y, Ezzell JA, Darville T, et al. IL-1 α Is Essential for Oviduct Pathology during Genital Chlamydial Infection in Mice. *The Journal of Immunology*. 2020;205:3037–49.
46. Lijek RS, Helble JD, Olive AJ, Seiger KW, Starnbach MN. Pathology after *Chlamydia trachomatis* infection is driven by nonprotective immune cells that are distinct from protective populations. *Proc Natl Acad Sci U S A*. 2018;115:2216–21.
47. Lehr S, Vier J, Häcker G, Kirschnek S. Activation of neutrophils by *Chlamydia trachomatis*-infected epithelial cells is modulated by the chlamydial plasmid. *Microbes and Infection*. 2018;20:284–92.
48. Rajeeve K, Das S, Prusty BK, Rudel T. *Chlamydia trachomatis* paralyzes neutrophils to evade the host innate immune response. *Nature Microbiology*. 2018;3:824.
49. Naglak EK, Morrison SG, Morrison RP. Neutrophils Are Central to Antibody-Mediated Protection against Genital Chlamydia. *Infect Immun*. 2017;85.
50. Lausen M, Christiansen G, Bouet Guldbæk Poulsen T, Birkelund S. Immunobiology of monocytes and macrophages during *Chlamydia trachomatis* infection. *Microbes and Infection*. 2019;21:73–84.
51. Stephens RS. The cellular paradigm of chlamydial pathogenesis. *Trends in Microbiology*. 2003;11:44–51.
52. Prantner D, Darville T, Sikes JD, Andrews CW, Brade H, Rank RG, et al. Critical Role for Interleukin-1 β (IL-1 β) during *Chlamydia muridarum* Genital Infection and Bacterial Replication-Independent Secretion of IL-1 β in Mouse Macrophages. *Infect Immun*. 2009;77:5334–46.
53. Sims JE, Smith DE. The IL-1 family: regulators of immunity. *Nat Rev Immunol*. 2010;10:89–102.
54. Vignali DAA, Kuchroo VK. IL-12 Family Cytokines: Immunological Playmakers. *Nat Immunol*. 2012;13:722–8.
55. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol*. 2014;6:a016295.
56. Gonzalez-Aparicio M, Alfaro C. Influence of Interleukin-8 and Neutrophil Extracellular Trap (NET) Formation in the Tumor Microenvironment: Is There a Pathogenic Role? *J Immunol Res*. 2019;2019.
57. Balkwill F. TNF- α in promotion and progression of cancer. *Cancer Metastasis Rev*. 2006;25:409–16.
58. Dorhoi A, Kaufmann SHE. Tumor necrosis factor α in mycobacterial infection. *Seminars in Immunology*. 2014;26:203–9.
59. Idriss HT, Naismith JH. TNF α and the TNF Receptor Superfamily: Structure-Function Relationship(s). :12.
60. Trinchieri G. Type I interferon: friend or foe? *J Exp Med*. 2010;207:2053–63.
61. Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunological Reviews*. 2005;206:306–35.
62. Rutz S, Ouyang W. Regulation of Interleukin-10 Expression. In: Ma X, editor. *Regulation of Cytokine Gene Expression in Immunity and Diseases*. Dordrecht: Springer Netherlands; 2016. p. 89–116.
63. Yang L, Pang Y, Moses HL. TGF- β and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends Immunol*. 2010;31:220–7.
64. Bhattacharya P, Budnick I, Singh M, Thiruppathi M, Alharshawy K, Elshabrawy H, et al. Dual Role of GM-CSF as a Pro-Inflammatory and a Regulatory Cytokine: Implications for Immune Therapy. *J Interferon Cytokine Res*. 2015;35:585–99.
65. Apte RS, Chen DS, Ferrara N. VEGF in Signaling and Disease: Beyond Discovery and Development. *Cell*. 2019;176:1248–64.
66. Aliper AM, Frieden-Korovkina VP, Buzdin A, Roumiantsev SA, Zhavoronkov A. A role for G-CSF and GM-CSF in nonmyeloid cancers. *Cancer Med*. 2014;3:737–46.
67. Kunkel SL. Through the looking glass: the diverse in vivo activities of chemokines. *J Clin Invest*. 1999;104:1333–4.
68. Gschwandtner M, Derler R, Midwood KS. More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis. *Front Immunol*. 2019;10:2759.
69. Chatterjee A, Rathore A, Vidyant S, Kakkar K, Dhole TN. Chemokines and Chemokine Receptors in Susceptibility to HIV-1 Infection and Progression to AIDS. *Dis Markers*. 2012;32:143–51.

70. Maurer M, von Stebut E. Macrophage inflammatory protein-1. *The International Journal of Biochemistry & Cell Biology*. 2004;36:1882–6.
71. Marques RE, Guabiraba R, Russo RC, Teixeira MM. Targeting CCL5 in inflammation. *Expert Opin Ther Targets*. 2013;17:1439–60.
72. Ahmadi Z, Arababadi MK, Hassanshahi G. CXCL10 Activities, Biological Structure, and Source Along with Its Significant Role Played in Pathophysiology of Type I Diabetes Mellitus. *Inflammation*. 2013;36:364–71.
73. Menon S, Timms P, Allan JA, Alexander K, Rombauts L, Horner P, et al. Human and Pathogen Factors Associated with Chlamydia trachomatis-Related Infertility in Women. *Clin Microbiol Rev*. 2015;28:969–85.
74. O'Meara CP, Armitage CW, Harvie MC, Andrew DW, Timms P, Lycke NY, et al. Immunity against a Chlamydia infection and disease may be determined by a balance of IL-17 signaling. *Immunology & Cell Biology*. 2014;92:287–97.
75. Østergaard O, Follmann F, Olsen AW, Heegaard NH, Andersen P, Rosenkrands I. Quantitative Protein Profiling of Chlamydia trachomatis Growth Forms Reveals Defense Strategies Against Tryptophan Starvation. *Mol Cell Proteomics*. 2016;15:3540–50.
76. Belland RJ, Nelson DE, Virok D, Crane DD, Hogan D, Sturdevant D, et al. Transcriptome analysis of chlamydial growth during IFN- γ -mediated persistence and reactivation. *Proc Natl Acad Sci U S A*. 2003;100:15971–6.
77. Ziklo N, Huston WM, Taing K, Timms P. High expression of IDO1 and TGF- β 1 during recurrence and post infection clearance with Chlamydia trachomatis, are independent of host IFN- γ response. *BMC Infectious Diseases*. 2019;19:218.
78. Russell AN, Zheng X, O'Connell CM, Taylor BD, Wiesenfeld HC, Hillier SL, et al. Analysis of Factors Driving Incident and Ascending Infection and the Role of Serum Antibody in Chlamydia trachomatis Genital Tract Infection. *J Infect Dis*. 2016;213:523–31.
79. Öhman H, Rantsi T, Joki-Korpela P, Tiitinen A, Surcel H-M. Prevalence and persistence of Chlamydia trachomatis-specific antibodies after occasional and recurrent infections. *Sex Transm Infect*. 2020;96:277–82.
80. Agrawal T, Vats V, Salhan S, Mittal A. Mucosal and peripheral immune responses to chlamydial heat shock proteins in women infected with Chlamydia trachomatis. *Clinical & Experimental Immunology*. 2007;148:461–8.
81. Mascellino MT, Boccia P, Oliva A. Immunopathogenesis in Chlamydia trachomatis Infected Women. *ISRN Obstet Gynecol*. 2011;2011.
82. El Hakim EA, Gordon UD, Akande VA. The relationship between serum Chlamydia antibody levels and severity of disease in infertile women with tubal damage. *Arch Gynecol Obstet*. 2010;281:727–33.
83. Cohen CR, Koochesfahani KM, Meier AS, Shen C, Karunakaran K, Ondondo B, et al. Immunoepidemiologic Profile of Chlamydia trachomatis Infection: Importance of Heat-Shock Protein 60 and Interferon- γ . *J Infect Dis*. 2005;192:591–9.
84. Li L-X, McSorley SJ. B Cells Enhance Antigen-Specific CD4 T Cell Priming and Prevent Bacteria Dissemination following Chlamydia muridarum Genital Tract Infection. *PLoS Pathog*. 2013;9.
85. Moore-Connors JM, Fraser R, Halperin SA, Wang J. CD4+CD25+Foxp3+ Regulatory T Cells Promote Th17 Responses and Genital Tract Inflammation upon Intracellular Chlamydia muridarum Infection. *The Journal of Immunology*. 2013;191:3430–9.
86. Lu C, Zeng H, Li Z, Lei L, Yeh I-T, Wu Y, et al. Protective immunity against mouse upper genital tract pathology correlates with high IFN γ but low IL-17 T cell and anti-secretion protein antibody responses induced by replicating chlamydial organisms in the airway. *Vaccine*. 2012;30:475–85.
87. Farris CM, Morrison SG, Morrison RP. CD4+ T Cells and Antibody Are Required for Optimal Major Outer Membrane Protein Vaccine-Induced Immunity to Chlamydia muridarum Genital Infection. *Infect Immun*. 2010;78:4374–83.
88. HIV/AIDS. <https://www.who.int/news-room/fact-sheets/detail/hiv-aids>. Accessed 26 Mar 2020.
89. Fiche d'information — Dernières statistiques sur l'état de l'épidémie de sida. <https://www.unaids.org/fr/resources/fact-sheet>. Accessed 1 Sep 2021.
90. Sentís A, Martín-Sánchez M, Arando M, Vall M, Barbera MJ, Ocaña I, et al. Sexually transmitted infections in young people and factors associated with HIV coinfection: an observational study in a large city. *BMJ Open*. 2019;9.
91. Rodrigues LLS, Hardick J, Nicol AF, Morgado MG, Martinelli KG, de Paula VS, et al. Sexually transmitted infections among HIV-infected and HIV-uninfected women in the Tapajós region, Amazon, Brazil: Self-collected vs. clinician-collected samples. *PLoS One*. 2019;14.

92. Mafokwane TM, Samie A. Prevalence of chlamydia among HIV positive and HIV negative patients in the Vhembe District as detected by real time PCR from urine samples. *BMC Res Notes*. 2016;9.
93. Johnson LF, Lewis DA. The effect of genital tract infections on HIV-1 shedding in the genital tract: a systematic review and meta-analysis. *Sex Transm Dis*. 2008;35:946–59.
94. Ghys PD, Fransen K, Diallo MO, Ettiègne-Traoré V, Coulibaly IM, Yeboué KM, et al. The associations between cervicovaginal HIV shedding, sexually transmitted diseases and immunosuppression in female sex workers in Abidjan, Côte d'Ivoire. *AIDS*. 1997;11:F85–93.
95. Venkatesh KK, van der Straten A, Cheng H, Montgomery ET, Lurie MN, Chipato T, et al. The relative contribution of viral and bacterial sexually transmitted infections on HIV acquisition in southern African women in the Methods for Improving Reproductive Health in Africa study. *Int J STD AIDS*. 2011;22:218–24.
96. Peterman TA, Newman DR, Maddox L, Schmitt K, Shiver S. Risk for HIV following a diagnosis of syphilis, gonorrhoea or chlamydia: 328,456 women in Florida, 2000–2011. *Int J STD AIDS*. 2015;26:113–9.
97. Shen R, Richter HE, Smith PD. Interactions between HIV-1 and Mucosal Cells in the Female Reproductive Tract. *Am J Reprod Immunol*. 2014;71:608–17.
98. Magérus-Chatinet A, Yu H, Garcia S, Ducloux E, Terris B, Bomsel M. Galactosyl ceramide expressed on dendritic cells can mediate HIV-1 transfer from monocyte derived dendritic cells to autologous T cells. *Virology*. 2007;362:67–74.
99. Schust DJ, Ibana JA, Buckner LR, Ficarra M, Sugimoto J, Amedee AM, et al. Potential mechanisms for increased HIV-1 transmission across the endocervical epithelium during *C. trachomatis* infection. *Curr HIV Res*. 2012;10:218–27.
100. Sperling R, Kraus TA, Ding J, Veretennikova A, Lorde-Rollins E, Singh T, et al. Differential profiles of immune mediators and in vitro HIV infectivity between endocervical and vaginal secretions from women with Chlamydia trachomatis infection: A pilot study. *J Reprod Immunol*. 2013;99.
101. Buckner LR, Amedee AM, Albritton HL, Kozłowski PA, Lacour N, McGowin CL, et al. Chlamydia trachomatis Infection of Endocervical Epithelial Cells Enhances Early HIV Transmission Events. *PLoS One*. 2016;11.
102. Kelly KA, Walker JC, Jameel SH, Gray HL, Rank RG. Differential Regulation of CD4 Lymphocyte Recruitment between the Upper and Lower Regions of the Genital Tract during Chlamydia trachomatis Infection. *Infect Immun*. 2000;68:1519–28.
103. Iijima N, Thompson J, Iwasaki A. Dendritic cells and macrophages in the genitourinary tract. *Mucosal Immunol*. 2008;1:451–9.
104. Petrova MI, van den Broek M, Balzarini J, Vanderleyden J, Lebeer S. Vaginal microbiota and its role in HIV transmission and infection. *FEMS Microbiol Rev*. 2013;37:762–92.
105. Ramanathan R, Woodrow K. Engineering immunity in the mucosal niche against sexually transmitted infections. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*. 2016;8:107–22.
106. Hickey DK, Patel MV, Fahey JV, Wira CR. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *J Reprod Immunol*. 2011;88:185–94.
107. Kaushic C. HIV-1 infection in the female reproductive tract: role of interactions between HIV-1 and genital epithelial cells. *Am J Reprod Immunol*. 2011;65:253–60.
108. Wira CR, Rodriguez-Garcia M, Patel MV. The role of sex hormones in immune protection of the female reproductive tract. *Nat Rev Immunol*. 2015;15:217–30.
109. Andersch-Björkman Y, Thomsson KA, Larsson JMH, Ekerhovd E, Hansson GC. Large Scale Identification of Proteins, Mucins, and Their O-Glycosylation in the Endocervical Mucus during the Menstrual Cycle. *Molecular & Cellular Proteomics*. 2007;6:708–16.
110. Benjelloun F, Quillay H, Cannou C, Marlin R, Madec Y, Fernandez H, et al. Activation of Toll-Like Receptors Differentially Modulates Inflammation in the Human Reproductive Tract: Preliminary Findings. *Front Immunol*. 2020;11:1655.
111. Reis Machado J, da Silva MV, Cavellani CL, Antônia dos Reis M, Monteiro MLG dos R, Teixeira V de PA, et al. Mucosal Immunity in the Female Genital Tract, HIV/AIDS. *Biomed Res Int*. 2014;2014.
112. Strbo N, Alcaide ML, Romero L, Bolivar H, Jones D, Podack ER, et al. Loss of intraepithelial endocervical gamma delta (GD) 1 T cells in HIV infected women. *Am J Reprod Immunol*. 2016;75:134–45.
113. Gibbs A, Leeansyah E, Introini A, Paquin-Proulx D, Hasselrot K, Andersson E, et al. MAIT cells reside in the female genital mucosa and are biased towards IL-17 and IL-22 production in response to bacterial stimulation. *Mucosal Immunol*. 2017;10:35–45.

114. Duluc D, Banchereau R, Gannevat J, Thompson-Snipes L, Blanck J-P, Zurawski S, et al. Transcriptional fingerprints of antigen-presenting cell subsets in the human vaginal mucosa and skin reflect tissue-specific immune microenvironments. *Genome Med.* 2014;6:98.
115. Givan AL, White HD, Stern JE, Colby E, Guyre PM, Wira CR, et al. Flow Cytometric Analysis of Leukocytes in the Human Female Reproductive Tract: Comparison of Fallopian Tube, Uterus, Cervix, and Vagina. *American Journal of Reproductive Immunology.* 1997;38:350–9.
116. Starkey PM, Clover LM, Rees MCP. Variation during the menstrual cycle of immune cell populations in human endometrium. *European Journal of Obstetrics & Gynecology.* 1991;39:203–7.
117. Lahoz-Beneytez J, Elemans M, Zhang Y, Ahmed R, Salam A, Block M, et al. Human neutrophil kinetics: modeling of stable isotope labeling data supports short blood neutrophil half-lives. *Blood.* 2016;127:3431–8.
118. Jilma B, Hergovich N, Stohlawetz P, Eichler H-G, Bauer P, Wagner OF. Circadian variation of granulocyte colony stimulating factor levels in man. *British Journal of Haematology.* 1999;106:368–70.
119. Martin C, Burdon PCE, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity.* 2003;19:583–93.
120. Casanova-Acebes M, Pitaval C, Weiss LA, Nombela-Arrieta C, Chèvre R, A-González N, et al. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell.* 2013;153:1025–35.
121. Hidalgo A, Chilvers ER, Summers C, Koenderman L. The Neutrophil Life Cycle. *Trends in Immunology.* 2019;40:584–97.
122. Ley K, Smith E, Stark MA. IL-17A-producing neutrophil-regulatory Tn lymphocytes. *Immunol Res.* 2006;34:229–42.
123. Gombart AF, Kwok SH, Anderson KL, Yamaguchi Y, Torbett BE, Koeffler HP. Regulation of neutrophil and eosinophil secondary granule gene expression by transcription factors C/EBP epsilon and PU.1. *Blood.* 2003;101:3265–73.
124. Borregaard N. Neutrophils, from marrow to microbes. *Immunity.* 2010;33:657–70.
125. Kolaczowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology.* 2013;13:159–75.
126. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes and Infection.* 2003;5:1317–27.
127. Shabani F, Farasat A, Mahdavi M, Gheibi N. Calprotectin (S100A8/S100A9): a key protein between inflammation and cancer. *Inflamm Res.* 2018;67:801–12.
128. Cowland JB, Borregaard N. Granulopoiesis and granules of human neutrophils. *Immunol Rev.* 2016;273:11–28.
129. Ng LG, Ostuni R, Hidalgo A. Heterogeneity of neutrophils. *Nature Reviews Immunology.* 2019;19:255–65.
130. Evrard M, Kwok IWH, Chong SZ, Teng KWW, Becht E, Chen J, et al. Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions. *Immunity.* 2018;48:364–379.e8.
131. Zinsly Sampaio Camargo T, Marra AR, Bacal NS, Casaroto E, Pinto LM, Pasternak J, et al. Evaluation of Two Methods for Determination of CD64 as a Diagnostic Marker of Infection in Critically Ill Adults. *Biomed Res Int.* 2016;2016:6593232.
132. Bowers NL, Helton ES, Huijbregts RPH, Goepfert PA, Heath SL, Hel Z. Immune suppression by neutrophils in HIV-1 infection: role of PD-L1/PD-1 pathway. *PLoS Pathog.* 2014;10:e1003993.
133. Luo Q, Huang Z, Ye J, Deng Y, Fang L, Li X, et al. PD-L1-expressing neutrophils as a novel indicator to assess disease activity and severity of systemic lupus erythematosus. *Arthritis Res Ther.* 2016;18.
134. Lemaitre J, Cosma A, Desjardins D, Lambotte O, Le Grand R. Mass Cytometry Reveals the Immaturity of Circulating Neutrophils during SIV Infection. *J Innate Immun.* 2020;12:170–81.
135. Miralda I, Uriarte SM, McLeish KR. Multiple Phenotypic Changes Define Neutrophil Priming. *Front Cell Infect Microbiol.* 2017;7:217.
136. Condliffe AM, Chilvers ER, Haslett C, Dransfield I. Priming differentially regulates neutrophil adhesion molecule expression/function. *Immunology.* 1996;89:105–11.
137. Ivetic A. A head-to-tail view of L-selectin and its impact on neutrophil behaviour. *Cell Tissue Res.* 2018;371:437–53.
138. Kruger P, Saffarzadeh M, Weber ANR, Rieber N, Radsak M, von Bernuth H, et al. Neutrophils: Between host defence, immune modulation, and tissue injury. *PLoS Pathog.* 2015;11:e1004651.

139. de Chaisemartin L, Hayemb G, Chollet-Martin S. When neutrophils cast their nets. *Joint Bone Spine*. 2013;80:124–6.
140. Granger V, Peyneau M, Chollet-Martin S, de Chaisemartin L. Neutrophil Extracellular Traps in Autoimmunity and Allergy: Immune Complexes at Work. *Front Immunol*. 2019;10:2824.
141. Burn GL, Foti A, Marsman G, Patel DF, Zychlinsky A. The Neutrophil. *Immunity*. 2021;54:1377–91.
142. Marini O, Costa S, Bevilacqua D, Calzetti F, Tamassia N, Spina C, et al. Mature CD10+ and immature CD10– neutrophils present in G-CSF–treated donors display opposite effects on T cells. *Blood*. 2017;129:1343–56.
143. Langereis JD, Pickkers P, Kleijn S de, Gerretsen J, Jonge MI de, Kox M. Spleen-derived IFN- γ induces generation of PD-L1+–suppressive neutrophils during endotoxemia. *Journal of Leukocyte Biology*. 2017;102:1401–9.
144. Huard B, McKee T, Bosshard C, Durual S, Matthes T, Myit S, et al. APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. *J Clin Invest*. 2008;118:2887–95.
145. Scapini P, Nardelli B, Nadali G, Calzetti F, Pizzolo G, Montecucco C, et al. G-CSF–stimulated Neutrophils Are a Prominent Source of Functional BlyS. *J Exp Med*. 2003;197:297–302.
146. Parsa R, Lund H, Georgoudaki A-M, Zhang X-M, Ortlieb Guerreiro-Cacais A, Grommisch D, et al. BAFF-secreting neutrophils drive plasma cell responses during emergency granulopoiesis. *J Exp Med*. 2016;213:1537–53.
147. Tamassia N, Bianchetto-Aguilera F, Arruda-Silva F, Gardiman E, Gasperini S, Calzetti F, et al. Cytokine production by human neutrophils: Revisiting the “dark side of the moon.” *European Journal of Clinical Investigation*. 2018;48:e12952.
148. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol*. 2011;11:519–31.
149. Smith JM, Wira CR, Fanger MW, Shen L. Human Fallopian Tube Neutrophils – A Distinct Phenotype from Blood Neutrophils. *American Journal of Reproductive Immunology*. 2006;56:218–29.
150. Robertson SA. Seminal fluid signaling in the female reproductive tract: lessons from rodents and pigs. *J Anim Sci*. 2007;85 13 Suppl:E36–44.
151. Sasaki S, Nagata K, Kobayashi Y. Regulation of the estrous cycle by neutrophil infiltration into the vagina. *Biochemical and Biophysical Research Communications*. 2009;382:35–40.
152. Salinas-Muñoz L, Campos-Fernández R, Mercader E, Olivera-Valle I, Fernández-Pacheco C, Matilla L, et al. Estrogen Receptor-Alpha (ESR1) Governs the Lower Female Reproductive Tract Vulnerability to *Candida albicans*. *Frontiers in Immunology*. 2018;9:1033.
153. Stevens JS, Criss AK. Pathogenesis of *Neisseria gonorrhoeae* in the female reproductive tract: neutrophilic host response, sustained infection, and clinical sequelae. *Curr Opin Hematol*. 2018;25:13–21.
154. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood*. 2010;115:335–43.
155. Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, et al. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS ONE*. 2012;7:e32366.
156. Fidel PL, Barousse M, Espinosa T, Ficarra M, Sturtevant J, Martin DH, et al. An Intravaginal Live *Candida* Challenge in Humans Leads to New Hypotheses for the Immunopathogenesis of Vulvovaginal Candidiasis. *Infect Immun*. 2004;72:2939–46.
157. Richardson JP, Willems HME, Moyes DL, Shoaie S, Barker KS, Tan SL, et al. Candidalysin Drives Epithelial Signaling, Neutrophil Recruitment, and Immunopathology at the Vaginal Mucosa. *Infect Immun*. 2018;86.
158. Barr FD, Ochsenbauer C, Wira CR, Rodriguez-Garcia M. Neutrophil extracellular traps prevent HIV infection in the female genital tract. *Mucosal Immunol*. 2018;11:1420–8.
159. Gardner JK, Swaims-Kohlmeier A, Herbst-Kralovetz MM. IL-36 γ Is a Key Regulator of Neutrophil Infiltration in the Vaginal Microenvironment and Limits Neuroinvasion in Genital HSV-2 Infection. *The Journal of Immunology*. 2019;203:2655–64.
160. Parkin J, Cohen B. An overview of the immune system. *The Lancet*. 2001;357:1777–89.
161. Trifonova RT, Lieberman J, van Baarle D. Distribution of immune cells in the human cervix and implications for HIV transmission. *Am J Reprod Immunol*. 2014;71:252–64.
162. Pudney J, Quayle AJ, Anderson DJ. Immunological Microenvironments in the Human Vagina and Cervix: Mediators of Cellular Immunity Are Concentrated in the Cervical Transformation Zone. *Biol Reprod*. 2005;73:1253–63.

163. Rodriguez-Garcia M, Barr FD, Crist SG, Fahey JV, Wira CR. Phenotype and susceptibility to HIV infection of CD4+ Th17 cells in the human female reproductive tract. *Mucosal Immunol*. 2014;7:1375–85.
164. Yeaman GR, Guyre PM, Fanger MW, Collins JE, White HD, Rathbun W, et al. Unique CD8+ T cell-rich lymphoid aggregates in human uterine endometrium. *Journal of Leukocyte Biology*. 1997;61:427–35.
165. Sullivan DA, Richardson GS, MacLaughlin DT, Wira CR. Variations in the levels of secretory component in human uterine fluid during the menstrual cycle. *J Steroid Biochem*. 1984;20:509–13.
166. Oh JE, Iijima N, Song E, Lu P, Klein J, Jiang R, et al. Migrant memory B cells secrete luminal antibody in the vagina. *Nature*. 2019;571:122–6.
167. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol*. 2003;3:710–20.
168. Yarbrough VL, Winkle S, Herbst-Kralovetz MM. Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications. *Hum Reprod Update*. 2015;21:353–77.
169. Keller MJ, Guzman E, Hazrati E, Kasowitz A, Cheshenko N, Wallenstein S, et al. PRO 2000 elicits a decline in genital tract immune mediators without compromising intrinsic antimicrobial activity: AIDS. 2007;21:467–76.
170. Boomsma C, Kavelaars A, Eijkemans M, Amarouchi K, Teklenburg G, Gutknecht D, et al. Cytokine profiling in endometrial secretions: a non-invasive window on endometrial receptivity. *Reproductive BioMedicine Online*. 2009;18:85–94.
171. Lieberman JA, Moscicki A-B, Sumerel JL, Ma Y, Scott ME. Determination of cytokine protein levels in cervical mucus samples from young women by a multiplex immunoassay method and assessment of correlates. *Clin Vaccine Immunol*. 2008;15:49–54.
172. Kutteh WH, Prince SJ, Hammond KR, Kutteh CC, Mestecky J. Variations in immunoglobulins and IgA subclasses of human uterine cervical secretions around the time of ovulation. *Clin Exp Immunol*. 1996;104:538–42.
173. Al-Harthi L, Wright DJ, Anderson D, Cohen M, Matityahu D, Cohn J, et al. The Impact of the Ovulatory Cycle on Cytokine Production: Evaluation of Systemic, Cervicovaginal, and Salivary Compartments. *Journal of Interferon & Cytokine Research*. 2000;20:719–24.
174. Francis SC, Hou Y, Baisley K, Wijgert J van de, Watson-Jones D, Ao TT, et al. Immune Activation in the Female Genital Tract: Expression Profiles of Soluble Proteins in Women at High Risk for HIV Infection. *PLOS ONE*. 2016;11:e0143109.
175. Wira CR, Fahey JV, Ghosh M, Patel MV, Hickey DK, Ochiel DO. Sex Hormone Regulation of Innate Immunity in the Female Reproductive Tract: The Role of Epithelial Cells in Balancing Reproductive Potential with Protection against Sexually Transmitted Pathogens. *Am J Reprod Immunol*. 2010;63.
176. Zhu L, Pollard JW. Estradiol-17beta regulates mouse uterine epithelial cell proliferation through insulin-like growth factor 1 signaling. *Proc Natl Acad Sci USA*. 2007;104:15847–51.
177. Marx PA, Spira AI, Gettie A, Dailey PJ, Veazey RS, Lackner AA, et al. Progesterone implants enhance SIV vaginal transmission and early virus load. *Nat Med*. 1996;2:1084–9.
178. Zalenskaya IA, Chandra N, Yousefieh N, Fang X, Adedipe OE, Jackson SS, et al. Use of contraceptive depot medroxyprogesterone acetate is associated with impaired cervicovaginal mucosal integrity. *J Clin Invest*. 2018. <https://doi.org/10.1172/JCI120583>.
179. Polikarpova AV, Levina IS, Sigai NV, Zavarzin IV, Morozov IA, Rubtsov PM, et al. Immunomodulatory effects of progesterone and selective ligands of membrane progesterone receptors. *Steroids*. 2019;145:5–18.
180. Kersh EN, Henning T, Vishwanathan SA, Morris M, Butler K, Adams DR, et al. SHIV susceptibility changes during the menstrual cycle of pigtail macaques. *J Med Primatol*. 2014;43:310–6.
181. Calenda G, Villegas G, Reis A, Millen L, Barnable P, Mamkina L, et al. Mucosal Susceptibility to Human Immunodeficiency Virus Infection in the Proliferative and Secretory Phases of the Menstrual Cycle. *AIDS Res Hum Retroviruses*. 2019;35:335–47.
182. Lorenzen E, Follmann F, Secher JO, Goericke-Pesch S, Hansen MS, Zakariassen H, et al. Intrauterine inoculation of minipigs with *Chlamydia trachomatis* during diestrus establishes a longer lasting infection compared to vaginal inoculation during estrus. *Microbes and Infection*. 2017;19:334–42.
183. Savage DC. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol*. 1977;31:107–33.
184. Structure, function and diversity of the healthy human microbiome. *Nature*. 2012;486:207–14.
185. Grice EA, Segre JA. The Human Microbiome: Our Second Genome. *Annu Rev Genomics Hum Genet*. 2012;13:151–70.
186. Sonnenburg JL, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. *Nature*. 2016;535:56–64.

187. Belkaid Y, Hand T. Role of the Microbiota in Immunity and inflammation. *Cell*. 2014;157:121–41.
188. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science*. 2018;359:97–103.
189. Hagan T, Cortese M, Rouphael N, Boudreau C, Linde C, Maddur MS, et al. Antibiotics-Driven Gut Microbiome Perturbation Alters Immunity to Vaccines in Humans. *Cell*. 2019;178:1313–1328.e13.
190. Klatt NR, Cheu R, Birse K, Zevin AS, Perner M, Noël-Romas L, et al. Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science*. 2017;356:938–45.
191. Gosmann C, Anahtar MN, Handley SA, Farcasanu M, Abu-Ali G, Bowman BA, et al. Lactobacillus-Deficient Cervicovaginal Bacterial Communities are Associated with Increased HIV Acquisition in Young South African Women. *Immunity*. 2017;46:29–37.
192. Balle C, Lennard K, Dabee S, Barnabas SL, Jaumdally SZ, Gasper MA, et al. Endocervical and vaginal microbiota in South African adolescents with asymptomatic Chlamydia trachomatis infection. *Sci Rep*. 2018;8.
193. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, et al. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA*. 2011;108 Suppl 1:4680–7.
194. Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UME, Zhong X, et al. Temporal dynamics of the human vaginal microbiota. *Sci Transl Med*. 2012;4:132ra52.
195. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, et al. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity*. 2015;42:965–76.
196. Fredricks DN, Fiedler TL, Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. *N Engl J Med*. 2005;353:1899–911.
197. Amsel R, Totten PA, Spiegel CA, Chen KC, Eschenbach D, Holmes KK. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *Am J Med*. 1983;74:14–22.
198. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol*. 1991;29:297–301.
199. Srinivasan S, Morgan MT, Fiedler TL, Djukovic D, Hoffman NG, Raftery D, et al. Metabolic Signatures of Bacterial Vaginosis. *mBio*. 2015;6.
200. Fredricks DN, Fiedler TL, Thomas KK, Mitchell CM, Marrazzo JM. Changes in Vaginal Bacterial Concentrations with Intravaginal Metronidazole Therapy for Bacterial Vaginosis as Assessed by Quantitative PCR. *Journal of Clinical Microbiology*. 2009;47:721–6.
201. Balkus JE, Richardson BA, Mandalika K, Kiarie J, Jaoko W, Ndinya-Achola JO, et al. Establishing and Sustaining a Healthy Vaginal Environment: Analysis of Data From a Randomized Trial of Periodic Presumptive Treatment for Vaginal Infections. *The Journal of Infectious Diseases*. 2011;204:323–6.
202. Sobel JD, Ferris D, Schwebke J, Nyirjesy P, Wiesenfeld HC, Peipert J, et al. Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis. *American Journal of Obstetrics and Gynecology*. 2006;194:1283–9.
203. Lev-Sagie A, Goldman-Wohl D, Cohen Y, Dori-Bachash M, Leshem A, Mor U, et al. Vaginal microbiome transplantation in women with intractable bacterial vaginosis. *Nature Medicine*. 2019;25:1500–4.
204. Anahtar MN, Gootenberg DB, Mitchell CM, Kwon DS. Cervicovaginal Microbiota and Reproductive Health: The Virtue of Simplicity. *Cell Host & Microbe*. 2018;23:159–68.
205. Martín R, Soberón N, Vaneechoutte M, Flórez AB, Vázquez F, Suárez JE. Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. *Int Microbiol*. 2008;11:261–6.
206. Martín R, Sánchez B, Suárez JE, Urdaci MC. Characterization of the adherence properties of human Lactobacilli strains to be used as vaginal probiotics. *FEMS Microbiol Lett*. 2012;328:166–73.
207. Witkin SS, Mendes-Soares H, Linhares IM, Jayaram A, Ledger WJ, Forney LJ. Influence of Vaginal Bacteria and d- and l-Lactic Acid Isomers on Vaginal Extracellular Matrix Metalloproteinase Inducer: Implications for Protection against Upper Genital Tract Infections. *mBio*. 2013;4:e00460-13.
208. Mirmonsef P, Hotton AL, Gilbert D, Burgad D, Landay A, Weber KM, et al. Free glycogen in vaginal fluids is associated with Lactobacillus colonization and low vaginal pH. *PLoS ONE*. 2014;9:e102467.
209. Boskey ER, Telsch KM, Whaley KJ, Moench TR, Cone RA. Acid Production by Vaginal Flora In Vitro Is Consistent with the Rate and Extent of Vaginal Acidification. *Infection and Immunity*. 1999;67:5170–5.
210. Ocaña VS, Pesce de Ruiz Holgado AA, Nader-Macías ME. Selection of vaginal H₂O₂-generating Lactobacillus species for probiotic use. *Curr Microbiol*. 1999;38:279–84.
211. O'Hanlon DE, Lanier BR, Moench TR, Cone RA. Cervicovaginal fluid and semen block the microbicidal activity of hydrogen peroxide produced by vaginal lactobacilli. *BMC Infect Dis*. 2010;10:120.

212. Manhanzva MT, Abrahams AG, Gamielien H, Froissart R, Jaspan H, Jaumdally SZ, et al. Inflammatory and antimicrobial properties differ between vaginal *Lactobacillus* isolates from South African women with non-optimal versus optimal microbiota. *Sci Rep*. 2020;10.
213. Swidsinski A, Verstraelen H, Loening-Baucke V, Swidsinski S, Mendling W, Halwani Z. Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. *PLoS ONE*. 2013;8:e53997.
214. Schwebke JR, Muzny CA, Josey WE. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. *J Infect Dis*. 2014;210:338–43.
215. Jarosik GP, Land CB, Duhon P, Chandler R, Mercer T. Acquisition of Iron by *Gardnerella vaginalis*. *Infect Immun*. 1998;66:5041–7.
216. Ahmed A, Earl J, Retchless A, Hillier SL, Rabe LK, Cherpes TL, et al. Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *J Bacteriol*. 2012;194:3922–37.
217. Hilbert DW, Schuyler JA, Adelson ME, Mordechai E, Sobel JD, Gyax SE. *Gardnerella vaginalis* population dynamics in bacterial vaginosis. *Eur J Clin Microbiol Infect Dis*. 2017;36:1269–78.
218. Ahrens P, Andersen LO, Lilje B, Johannesen TB, Dahl EG, Baig S, et al. Changes in the vaginal microbiota following antibiotic treatment for *Mycoplasma genitalium*, *Chlamydia trachomatis* and bacterial vaginosis. *PLOS ONE*. 2020;15:e0236036.
219. Criswell BS, Ladwig CL, Gardner HL, Dukes CD. *Haemophilus vaginalis*: vaginitis by inoculation from culture. *Obstet Gynecol*. 1969;33:195–9.
220. Muzny CA, Łaniewski P, Schwebke JR, Herbst-Kralovetz MM. Host-Vaginal Microbiota Interactions in the Pathogenesis of Bacterial Vaginosis. *Curr Opin Infect Dis*. 2020;33:59–65.
221. Balle C, Esra R, Havyarimana E, Jaumdally SZ, Lennard K, Konstantinus IN, et al. Relationship between the Oral and Vaginal Microbiota of South African Adolescents with High Prevalence of Bacterial Vaginosis. *Microorganisms*. 2020;8.
222. Song SD, Acharya KD, Zhu JE, Deveney CM, Walther-Antonio MRS, Tetel MJ, et al. Daily Vaginal Microbiota Fluctuations Associated with Natural Hormonal Cycle, Contraceptives, Diet, and Exercise. *mSphere*. 2020;5.
223. Brotman RM, He X, Gajer P, Fadrosch D, Sharma E, Mongodin EF, et al. Association between cigarette smoking and the vaginal microbiota: a pilot study. *BMC Infect Dis*. 2014;14:471.
224. Amabebe E, Anumba DOC. The Vaginal Microenvironment: The Physiologic Role of Lactobacilli. *Front Med (Lausanne)*. 2018;5.
225. Dei M, Di Maggio F, Di Paolo G, Bruni V. Vulvovaginitis in childhood. *Best Practice & Research Clinical Obstetrics & Gynaecology*. 2010;24:129–37.
226. Mirmonsef P, Hotton AL, Gilbert D, Gioia CJ, Maric D, Hope TJ, et al. Glycogen Levels in Undiluted Genital Fluid and Their Relationship to Vaginal pH, Estrogen, and Progesterone. *PLoS ONE*. 2016;11:e0153553.
227. Spear GT, French AL, Gilbert D, Zariffard MR, Mirmonsef P, Sullivan TH, et al. Human α -amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by *Lactobacillus*. *J Infect Dis*. 2014;210:1019–28.
228. Minassian SS, Wu CH. Free and protein-bound progesterone during normal and luteal phase defective cycles. *International Journal of Gynecology & Obstetrics*. 1993;43:163–8.
229. Srinivasan S, Liu C, Mitchell CM, Fiedler TL, Thomas KK, Agnew KJ, et al. Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PLoS ONE*. 2010;5:e10197.
230. Hummelen R, Macklaim JM, Bisanz JE, Hammond J-A, McMillan A, Vongsa R, et al. Vaginal microbiome and epithelial gene array in post-menopausal women with moderate to severe dryness. *PLoS ONE*. 2011;6:e26602.
231. Mirmonsef P, Modur S, Burgad D, Gilbert D, Golub ET, French AL, et al. Exploratory comparison of vaginal glycogen and *Lactobacillus* levels in premenopausal and postmenopausal women. *Menopause*. 2015;22:702–9.
232. Brooks JP, Edwards DJ, Blithe DL, Fettweis JM, Serrano MG, Sheth NU, et al. Effects of Combined Oral Contraceptives, Depot Medroxyprogesterone Acetate, and the Levonorgestrel-releasing Intrauterine System on the Vaginal Microbiome. *Contraception*. 2017;95:405–13.
233. Balle C, Konstantinus IN, Jaumdally SZ, Havyarimana E, Lennard K, Esra R, et al. Hormonal contraception alters vaginal microbiota and cytokines in South African adolescents in a randomized trial. *Nat Commun*. 2020;11.

234. Achilles SL, Austin MN, Meyn LA, Mhlanga F, Chirenje ZM, Hillier SL. Impact of contraceptive initiation on vaginal microbiota. *Am J Obstet Gynecol.* 2018;218:622.e1-622.e10.
235. Ray RM, Maritz MF, Avenant C, Tomasicchio M, Dlamini S, van der Spuy Z, et al. The contraceptive medroxyprogesterone acetate, unlike norethisterone, directly increases R5 HIV-1 infection in human cervical explant tissue at physiologically relevant concentrations. *Sci Rep.* 2019;9.
236. Schwebke JR. New concepts in the etiology of bacterial vaginosis. *Curr Infect Dis Rep.* 2009;11:143–7.
237. Cottrell BH. An updated review of evidence to discourage douching. *MCN Am J Matern Child Nurs.* 2010;35:102–7; quiz 108–9.
238. Alcaide ML, Rodriguez VJ, Brown MR, Pallikkuth S, Arheart K, Martinez O, et al. High Levels of Inflammatory Cytokines in the Reproductive Tract of Women with BV and Engaging in Intravaginal Douching: A Cross-Sectional Study of Participants in the Women Interagency HIV Study. *AIDS Res Hum Retroviruses.* 2017;33:309–17.
239. Zozaya M, Ferris MJ, Siren JD, Lillis R, Myers L, Nsuami MJ, et al. Bacterial communities in penile skin, male urethra, and vaginas of heterosexual couples with and without bacterial vaginosis. *Microbiome.* 2016;4.
240. Mehta SD, Agingu W, Nordgren RK, Green SJ, Bhaumik DK, Bailey RC, et al. Characteristics of Women and Their Male Sex Partners Predict Bacterial Vaginosis Among a Prospective Cohort of Kenyan Women With Non-optimal Vaginal Microbiota. *Sex Transm Dis.* 2020;47:840–50.
241. Wiesenfeld HC, Hillier SL, Krohn MA, Landers DV, Sweet RL. Bacterial Vaginosis Is a Strong Predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* Infection. *Clinical Infectious Diseases.* 2003;36:663–8.
242. Tamarelle J, Barbeyrac B de, Hen IL, Thiébaut A, Bébéar C, Ravel J, et al. Vaginal microbiota composition and association with prevalent *Chlamydia trachomatis* infection: a cross-sectional study of young women attending a STI clinic in France. *Sex Transm Infect.* 2018;94:616–8.
243. Borgogna J-LC, Shardell MD, Yeoman CJ, Ghanem KG, Kadriu H, Ulanov AV, et al. The association of *Chlamydia trachomatis* and *Mycoplasma genitalium* infection with the vaginal metabolome. *Sci Rep.* 2020;10.
244. Abbai NS, Nyirenda M, Naidoo S, Ramjee G. Prevalent Herpes Simplex Virus-2 Increases the Risk of Incident Bacterial Vaginosis in Women from South Africa. *AIDS Behav.* 2018;22:2172–80.
245. Kroon SJ, Ravel J, Huston WM. Cervicovaginal microbiota, women's health, and reproductive outcomes. *Fertility and Sterility.* 2018;110:327–36.
246. Lennard K, Dabee S, Barnabas SL, Havyarimana E, Blakney A, Jaumdally SZ, et al. Microbial Composition Predicts Genital Tract Inflammation and Persistent Bacterial Vaginosis in South African Adolescent Females. *Infect Immun.* 2018;86.
247. Masson L, Barnabas S, Deese J, Lennard K, Dabee S, Gamielien H, et al. Inflammatory cytokine biomarkers of asymptomatic sexually transmitted infections and vaginal dysbiosis: a multicentre validation study. *Sex Transm Infect.* 2019;95:5–12.
248. Gautam R, Borgdorff H, Jespers V, Francis SC, Verhelst R, Mwaura M, et al. Correlates of the molecular vaginal microbiota composition of African women. *BMC Infect Dis.* 2015;15.
249. Eade CR, Diaz C, Wood MP, Anastos K, Patterson BK, Gupta P, et al. Identification and Characterization of Bacterial Vaginosis-Associated Pathogens Using a Comprehensive Cervical-Vaginal Epithelial Coculture Assay. *PLoS One.* 2012;7.
250. Jang S-E, Jeong J-J, Choi S-Y, Kim H, Han MJ, Kim D-H. *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* La-14 Attenuate *Gardnerella vaginalis*-Infected Bacterial Vaginosis in Mice. *Nutrients.* 2017;9.
251. Thurman AR, Kimble T, Herold B, Mesquita PMM, Fichorova RN, Dawood HY, et al. Bacterial Vaginosis and Subclinical Markers of Genital Tract Inflammation and Mucosal Immunity. *AIDS Res Hum Retroviruses.* 2015;31:1139–52.
252. Borgdorff H, Gautam R, Armstrong SD, Xia D, Ndayisaba GF, van Teijlingen NH, et al. Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier. *Mucosal Immunol.* 2016;9:621–33.
253. Delgado-Diaz DJ, Tyssen D, Hayward JA, Gugasyan R, Hearps AC, Tachedjian G. Distinct Immune Responses Elicited From Cervicovaginal Epithelial Cells by Lactic Acid and Short Chain Fatty Acids Associated With Optimal and Non-optimal Vaginal Microbiota. *Front Cell Infect Microbiol.* 2020;9.
254. Gong Z, Luna Y, Yu P, Fan H. *Lactobacilli* Inactivate *Chlamydia trachomatis* through Lactic Acid but Not H₂O₂. *PLoS One.* 2014;9.
255. Nardini P, Nahui Palomino RA, Parolin C, Laghi L, Foschi C, Cevenini R, et al. *Lactobacillus crispatus* inhibits the infectivity of *Chlamydia trachomatis* elementary bodies, in vitro study. *Sci Rep.* 2016;6.

256. Mastromarino P, Di Pietro M, Schiavoni G, Nardis C, Gentile M, Sessa R. Effects of vaginal lactobacilli in Chlamydia trachomatis infection. *International Journal of Medical Microbiology*. 2014;304:654–61.
257. Rizzo A, Florentin M, Buommino E, Donnarumma G, Losacco A, Bevilacqua N. Lactobacillus crispatus mediates anti-inflammatory cytokine interleukin-10 induction in response to Chlamydia trachomatis infection in vitro. *Int J Med Microbiol*. 2015. <https://doi.org/10.1016/j.ijmm.2015.07.005>.
258. van Houdt R, Ma B, Bruisten SM, Speksnijder AGCL, Ravel J, de Vries HJC. Lactobacillus iners-dominated vaginal microbiota is associated with increased susceptibility to Chlamydia trachomatis infection in Dutch women, a case control study. *Sex Transm Infect*. 2018;94:117–23.
259. Tamarelle J, Ma B, Gajer P, Humphrys MS, Terplan M, Mark KS, et al. Nonoptimal Vaginal Microbiota After Azithromycin Treatment for Chlamydia trachomatis Infection. *J Infect Dis*. <https://doi.org/10.1093/infdis/jiz499>.
260. Edwards VL, Smith SB, McComb EJ, Tamarelle J, Ma B, Humphrys MS, et al. The Cervicovaginal Microbiota-Host Interaction Modulates Chlamydia trachomatis Infection. *mBio*. 2019;10.
261. Ziklo N, Vidgen ME, Taing K, Huston WM, Timms P. Dysbiosis of the Vaginal Microbiota and Higher Vaginal Kynurenine/Tryptophan Ratio Reveals an Association with Chlamydia trachomatis Genital Infections. *Front Cell Infect Microbiol*. 2018;8.
262. Pyles RB, Vincent KL, Baum MM, Elsom B, Miller AL, Maxwell C, et al. Cultivated Vaginal Microbiomes Alter HIV-1 Infection and Antiretroviral Efficacy in Colonized Epithelial Multilayer Cultures. *PLoS One*. 2014;9.
263. Tyssen D, Wang Y-Y, Hayward JA, Agius PA, DeLong K, Aldunate M, et al. Anti-HIV-1 Activity of Lactic Acid in Human Cervicovaginal Fluid. *mSphere*. 2018;3.
264. Nunn KL, Wang Y-Y, Harit D, Humphrys MS, Ma B, Cone R, et al. Enhanced Trapping of HIV-1 by Human Cervicovaginal Mucus Is Associated with Lactobacillus crispatus-Dominant Microbiota. *MBio*. 2015;6:e01084-01015.
265. Hoang T, Toler E, DeLong K, Mafunda NA, Bloom SM, Zierden HC, et al. The cervicovaginal mucus barrier to HIV-1 is diminished in bacterial vaginosis. *PLoS Pathog*. 2020;16.
266. Ñahui Palomino RA, Zicari S, Vanpouille C, Vitali B, Margolis L. Vaginal Lactobacillus Inhibits HIV-1 Replication in Human Tissues Ex Vivo. *Front Microbiol*. 2017;8:906.
267. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat Rev Microbiol*. 2015;13:620–30.
268. Ñahui Palomino RA, Vanpouille C, Laghi L, Parolin C, Melikov K, Backlund P, et al. Extracellular vesicles from symbiotic vaginal lactobacilli inhibit HIV-1 infection of human tissues. *Nat Commun*. 2019;10:5656.
269. Cheu RK, Gustin AT, Lee C, Schifanella L, Miller CJ, Ha A, et al. Impact of vaginal microbiome communities on HIV antiretroviral-based pre-exposure prophylaxis (PrEP) drug metabolism. *PLoS Pathog*. 2020;16.
270. Taneva E, Sinclair S, Mesquita PM, Weinrick B, Cameron SA, Cheshenko N, et al. Vaginal microbiome modulates topical antiretroviral drug pharmacokinetics. *JCI Insight*. 2018;3.
271. Heffron R, McClelland RS, Balkus JE, Celum C, Cohen CR, Mugo N, et al. Efficacy of oral pre-exposure prophylaxis (PrEP) for HIV among women with abnormal vaginal microbiota: a post-hoc analysis of the randomised, placebo-controlled Partners PrEP Study. *Lancet HIV*. 2017;4:e449–56.
272. Zhang D, Frenette PS. Cross talk between neutrophils and the microbiota. *Blood*. 2019;133:2168–77.
273. Schluter J, Peled JU, Taylor BP, Markey KA, Smith M, Taur Y, et al. The gut microbiota is associated with immune cell dynamics in humans. *Nature*. 2020;588:303–7.
274. Deshmukh HS, Liu Y, Menkiti OR, Mei J, Dai N, O'Leary CE, et al. The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. *Nat Med*. 2014;20:524–30.
275. Fagundes CT, Amaral FA, Vieira AT, Soares AC, Pinho V, Nicoli JR, et al. Transient TLR activation restores inflammatory response and ability to control pulmonary bacterial infection in germfree mice. *J Immunol*. 2012;188:1411–20.
276. Zhang D, Chen G, Manwani D, Mortha A, Xu C, Faith JJ, et al. Neutrophil ageing is regulated by the microbiome. *Nature*. 2015;525:528–32.
277. Vinolo MAR, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *J Nutr Biochem*. 2011;22:849–55.
278. Yoshimura T, McLean MH, Dzutsev AK, Yao X, Chen K, Huang J, et al. The Antimicrobial Peptide CRAMP Is Essential for Colon Homeostasis by Maintaining Microbiota Balance. *J Immunol*. 2018;200:2174–85.
279. Vareille-Delarbre M, Miquel S, Garcin S, Bertran T, Balestrino D, Evrard B, et al. Immunomodulatory Effects of Lactobacillus plantarum on Inflammatory Response Induced by Klebsiella pneumoniae. *Infect Immun*. 2019;87.

280. Wang Y, Gu Y, Fang K, Mao K, Dou J, Fan H, et al. *Lactobacillus acidophilus* and *Clostridium butyricum* ameliorate colitis in murine by strengthening the gut barrier function and decreasing inflammatory factors. *Benef Microbes*. 2018;9:775–87.
281. Hensley-McBain T, Wu MC, Manuzak JA, Cheu RK, Gustin A, Driscoll CB, et al. Increased mucosal neutrophil survival is associated with altered microbiota in HIV infection. *PLoS Pathog*. 2019;15:e1007672.
282. Lavelle A, Hoffmann TW, Pham H-P, Langella P, Guédon E, Sokol H. Baseline microbiota composition modulates antibiotic-mediated effects on the gut microbiota and host. *Microbiome*. 2019;7:111.
283. Perelman P, Johnson WE, Roos C, Seuánez HN, Horvath JE, Moreira MAM, et al. A molecular phylogeny of living primates. *PLoS Genet*. 2011;7:e1001342.
284. Mestas J, Hughes CCW. Of mice and not men: differences between mouse and human immunology. *J Immunol*. 2004;172:2731–8.
285. Bjornson-Hooper ZB, Fragiadakis GK, Spitzer MH, Madhiredy D, McIlwain D, Nolan GP. A comprehensive atlas of immunological differences between humans, mice and non-human primates. *bioRxiv*. 2019;:574160.
286. Cunha GR, Sinclair A, Ricke WA, Robboy SJ, Cao M, Baskin LS. Reproductive tract biology: Of mice and men. *Differentiation*. 2019;110:49–63.
287. Estes JD, Wong SW, Brenchley JM. Nonhuman primate models of human viral infections. *Nat Rev Immunol*. 2018;18:390–404.
288. Bell JD, Bergin IL, Schmidt K, Zochowski MK, Aronoff DM, Patton DL. Nonhuman Primate Models Used to Study Pelvic Inflammatory Disease Caused by *Chlamydia trachomatis*. *Infect Dis Obstet Gynecol*. 2011;2011.
289. Bakaletz LO. Developing animal models for polymicrobial diseases. *Nat Rev Microbiol*. 2004;2:552–68.
290. Peña JC, Ho W-Z. Non-Human Primate Models of Tuberculosis. *Microbiology Spectrum*. 2016;4:4.4.10.
291. Gardner MB, Luciw PA. Macaque models of human infectious disease. *ILAR J*. 2008;49:220–55.
292. Sussman RW, Tattersall I. Distribution, Abundance, and Putative Ecological Strategy of *Macaca fascicularis* on the Island of Mauritius, Southwestern Indian Ocean. *FPR*. 1986;46:28–43.
293. Houghton P. Virus-free African cynomolgus macaques. *Lab animal (USA)*. 1988.
294. Trask JS, George D, Houghton P, Kanthaswamy S, Smith DG. Population and Landscape Genetics of an Introduced Species (*M. fascicularis*) on the Island of Mauritius. *PLOS ONE*. 2013;8:e53001.
295. Lawler SH, Sussman RW, Taylor LL. Mitochondrial DNA of the Mauritian macaques (*Macaca fascicularis*): an example of the founder effect. *Am J Phys Anthropol*. 1995;96:133–41.
296. Zitsman JS, Alonso-Guallart P, Ovanez C, Kato Y, Rosen JF, Weiner JI, et al. Distinctive Leukocyte Subpopulations According to Organ Type in Cynomolgus Macaques. *Comp Med*. 2016;66:308–23.
297. Messaoudi I, Estep R, Robinson B, Wong SW. Nonhuman Primate Models of Human Immunology. *Antioxid Redox Signal*. 2011;14:261–73.
298. Van Esch E, Cline JM, Buse E, Wood CE, de Rijk EPCT, Weinbauer GF. Summary Comparison of Female Reproductive System in Human and the Cynomolgus Monkey (*Macaca fascicularis*). *Toxicol Pathol*. 2008;36 7_suppl:171S–172S.
299. Weinbauer GF, Niehoff M, Niehaus M, Srivastav S, Fuchs A, Van Esch E, et al. Physiology and Endocrinology of the Ovarian Cycle in Macaques. *Toxicol Pathol*. 2008;36:7S–23S.
300. Shaikh AA, Naqvi RH, Shaikh SA. Concentrations of oestradiol-17 β and progesterone in the peripheral plasma of the cynomolgus monkey (*Macaca fascicularis*) in relation to the length of the menstrual cycle and its component phases. *J Endocrinol*. 1978;79:1–7.
301. Marlin R, Nugeyre M-T, Tchitchek N, Parenti M, Hocini H, Benjelloun F, et al. Modified Vaccinia Virus Ankara Vector Induces Specific Cellular and Humoral Responses in the Female Reproductive Tract, the Main HIV Portal of Entry. *J Immunol*. 2017;199:1923–32.
302. George T. Spear, Douglas Gilbert, Masoumeh Sikaroodi, Lara Doyle, Linda Green, Patrick M. Gillevet, et al. Identification of rhesus macaque genital microbiota by 16S pyrosequencing shows similarities to human bacterial vaginosis: implications for use as an animal model for HIV vaginal infection. *AIDS Research and Human Retroviruses*. 2010;26.
303. Spear GT, Kersh E, Guenther P, Vishwanathan SA, Gilbert D, Zariffard MR, et al. Longitudinal assessment of pigtailed macaque lower genital tract microbiota by pyrosequencing reveals dissimilarity to the genital microbiota of healthy humans. *AIDS Res Hum Retroviruses*. 2012;28:1244–9.
304. Chen Z, Yeoh YK, Hui M, Wong PY, Chan MCW, Ip M, et al. Diversity of macaque microbiota compared to the human counterparts. *Sci Rep*. 2018;8:15573.

305. Hallmaier-Wacker LK, Lüert S, Roos C, Knauf S. Lactation and menstruation shift the vaginal microbiota in captive rhesus monkeys to be more similar to the male urethral microbiota. *Sci Rep*. 2019;9:17399.
306. Mirmonsef P, Gilbert D, Veazey RS, Wang J, Kendrick SR, Spear GT. A comparison of lower genital tract glycogen and lactic acid levels in women and macaques: implications for HIV and SIV susceptibility. *AIDS Res Hum Retroviruses*. 2012;28:76–81.
307. Miller EA, Beasley DE, Dunn RR, Archie EA. Lactobacilli Dominance and Vaginal pH: Why is the Human Vaginal Microbiome Unique? *Front Microbiol*. 2016;7.
308. Desrosiers RC, Daniel MD, Li Y. HIV-Related Lentiviruses of Nonhuman Primates. *AIDS Research and Human Retroviruses*. 1989;5:465–73.
309. Antony JM, MacDonald KS. A critical analysis of the cynomolgus macaque, *Macaca fascicularis*, as a model to test HIV-1/SIV vaccine efficacy. *Vaccine*. 2015;33:3073–83.
310. Le Grand R, Nadal M, Cheret A, Roques P, Vaslin B, Matheux F, et al. Infection of macaques after vaginal exposure to a primary isolate of SIVmac251. *AIDS*. 1995;9:308–9.
311. Le Grand R, Dereuddre-Bosquet N, Dispinseri S, Gosse L, Desjardins D, Shen X, et al. Superior Efficacy of a Human Immunodeficiency Virus Vaccine Combined with Antiretroviral Prevention in Simian-Human Immunodeficiency Virus-Challenged Nonhuman Primates. *J Virol*. 2016;90:5315–28.
312. Boyd KL, Muehlenbachs A, Rendi MH, Garcia RL, Gibson-Corley KN. Female Reproductive System. In: *Comparative Anatomy and Histology*. Elsevier; 2018. p. 303–34.
313. Patton DL, Kuo CC, Wang SP, Halbert SA. Distal tubal obstruction induced by repeated *Chlamydia trachomatis* salpingeal infections in pig-tailed macaques. *J Infect Dis*. 1987;155:1292–9.
314. Qu Y, Frazer LC, O'Connell CM, Tarantal AF, Andrews CW, O'Connor SL, et al. Comparable Genital Tract Infection, Pathology, and Immunity in Rhesus Macaques Inoculated with Wild-Type or Plasmid-Deficient *Chlamydia trachomatis* Serovar D. *Infect Immun*. 2015;83:4056–67.
315. Patton DL, Sweeney YC, Baldessari AE, Cles L, Kari L, Sturdevant GL, et al. The *Chlamydia trachomatis* Plasmid and CT135 Virulence Factors Are Not Essential for Genital Tract Infection or Pathology in Female Pig-Tailed Macaques. *Infect Immun*. 2018;86.
316. Herbst-Kralovetz MM, Quayle AJ, Ficarra M, Greene S, Rose WA, Chesson R, et al. ORIGINAL ARTICLE: Quantification and Comparison of Toll-Like Receptor Expression and Responsiveness in Primary and Immortalized Human Female Lower Genital Tract Epithelia. *American Journal of Reproductive Immunology*. 2008;59:212–24.
317. Nugeyre M-T, Tchitchek N, Adapen C, Cannou C, Contreras V, Benjelloun F, et al. Dynamics of Vaginal and Rectal Microbiota Over Several Menstrual Cycles in Female *Cynomolgus* Macaques. *Front Cell Infect Microbiol*. 2019;9.
318. Armstrong GM, Maybin JA, Murray AA, Nicol M, Walker C, Saunders PTK, et al. Endometrial apoptosis and neutrophil infiltration during menstruation exhibits spatial and temporal dynamics that are recapitulated in a mouse model. *Sci Rep*. 2017;7:17416.
319. Yu RR, Cheng AT, Lagenaur LA, Huang W, Weiss DE, Treece J, et al. A Chinese rhesus macaque (*Macaca mulatta*) model for vaginal *Lactobacillus* colonization and live microbicide development. *J Med Primatol*. 2009;38:125–36.
320. Lagenaur LA, Sanders-Beer BE, Brichacek B, Pal R, Liu X, Liu Y, et al. Prevention of vaginal SHIV transmission in macaques by a live recombinant *Lactobacillus*. *Mucosal Immunol*. 2011;4:648–57.
321. Daggett GJ, Zhao C, Connor-Stroud F, Oviedo-Moreno P, Moon H, Cho MW, et al. Comparison of the vaginal environment in rhesus and cynomolgus macaques pre- and post-*Lactobacillus* colonization. *J Med Primatol*. 2017;46:232–8.
322. Hu K, Zheng J, Yu Z, Chen Z, Cheng H, Pan W, et al. Directed shift of vaginal microbiota induced by vaginal application of sucrose gel in rhesus macaques. *Int J Infect Dis*. 2015;33:32–6.
323. Bosinger SE, Tharp GK, Patel NB, Zhao C, Payne TL, Ostergaard SD, et al. Progestin-based contraception regimens modulate expression of putative HIV risk factors in the vaginal epithelium of pig-tailed Macaques. *American Journal of Reproductive Immunology*. 2018;80:e13029.
324. Miller L, Patton DL, Meier A, Thwin SS, Hooton TM, Eschenbach DA. Depomedroxyprogesterone-induced hypoestrogenism and changes in vaginal flora and epithelium. *Obstet Gynecol*. 2000;96:431–9.
325. Guterstam YC, Strunz B, Ivarsson MA, Zimmer C, Melin A-S, Jonasson AF, et al. The cytokine profile of menstrual blood. *Acta Obstetrica et Gynecologica Scandinavica*. 2021;100:339–46.

326. Kyongo JK, Jespers V, Goovaerts O, Michiels J, Menten J, Fichorova RN, et al. Searching for Lower Female Genital Tract Soluble and Cellular Biomarkers: Defining Levels and Predictors in a Cohort of Healthy Caucasian Women. *PLoS One*. 2012;7:e43951.
327. Janiak MC, Montague MJ, Villamil CI, Stock MK, Trujillo AE, DePasquale AN, et al. Age and sex-associated variation in the multi-site microbiome of an entire social group of free-ranging rhesus macaques. *Microbiome*. 2021;9:68.
328. Lorenz TK, Demas GE, Heiman JR. Interaction of menstrual cycle phase and sexual activity predicts mucosal and systemic humoral immunity in healthy women. *Physiology & Behavior*. 2015;152:92–8.
329. Lorenz TK, Heiman JR, Demas GE. Interactions Among Sexual Activity, Menstrual Cycle Phase, and Immune Function in Healthy Women. *J Sex Res*. 2018;55:1087–95.
330. Doncel GF, Anderson S, Zalenskaya I. Role of Semen in Modulating the Female Genital Tract Microenvironment – Implications for HIV Transmission. *American Journal of Reproductive Immunology*. 2014;71:564–74.
331. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal Fluid Induces Leukocyte Recruitment and Cytokine and Chemokine mRNA Expression in the Human Cervix after Coitus. *The Journal of Immunology*. 2012;188:2445–54.
332. Marlin R, Nugeyre M-T, Tchitchek N, Parenti M, Lefebvre C, Hocini H, et al. Seminal Plasma Exposures Strengthen Vaccine Responses in the Female Reproductive Tract Mucosae. *Front Immunol*. 2019;10:430.
333. Smirnova TG, Savochkina AY, Dolgushin II, Nikushkina KV, Samuseva IV. Changes in Functional Activity of Neutrophils and Monocytes Isolated from the Peripheral Blood of Women at Different Phases of the Menstrual Cycle. *Bull Exp Biol Med*. 2018;166:222–4.
334. Lee S, Kim J, Jang B, Hur S, Jung U, Kil K, et al. Fluctuation of Peripheral Blood T, B, and NK Cells during a Menstrual Cycle of Normal Healthy Women. *The Journal of Immunology*. 2010;185:756–62.
335. Sereshki N, Gharagozloo M, Ostadi V, Ghahiri A, Roghaei MA, Mehrabian F, et al. Variations in T-helper 17 and regulatory T cells during the menstrual cycle in peripheral blood of women with recurrent spontaneous abortion. *International Journal of Fertility and Sterility*. 2014;8:59–66.
336. Bradley F, Birse K, Hasselrot K, Noël-Romas L, Introini A, Wefer H, et al. The vaginal microbiome amplifies sex hormone-associated cyclic changes in cervicovaginal inflammation and epithelial barrier disruption. *American Journal of Reproductive Immunology*. 2018;80:e12863.
337. Bertrand JT, Ross J, Sullivan TM, Hardee K, Shelton JD. Contraceptive Method Mix: Updates and Implications. *Glob Health Sci Pract*. 2020;8:666–79.
338. Wessels JM, Lajoie J, Cooper MIJH, Omollo K, Felker AM, Vitali D, et al. Medroxyprogesterone acetate alters the vaginal microbiota and microenvironment in a Kenyan sex worker cohort and is also associated with increased susceptibility to HIV-1 in humanized mice. *Disease Models & Mechanisms*. 2019;dmm.039669.
339. Edfeldt G, Lajoie J, Röhl M, Oyugi J, Åhlberg A, Khalilzadeh-Binicy B, et al. Regular Use of Depot Medroxyprogesterone Acetate Causes Thinning of the Superficial Lining and Apical Distribution of Human Immunodeficiency Virus Target Cells in the Human Ectocervix. *The Journal of Infectious Diseases*. 2020. <https://doi.org/10.1093/infdis/jiaa514>.
340. Olsen AW, Follmann F, Erneholt K, Rosenkrands I, Andersen P. Protection Against Chlamydia trachomatis Infection and Upper Genital Tract Pathological Changes by Vaccine-Promoted Neutralizing Antibodies Directed to the VD4 of the Major Outer Membrane Protein. *The Journal of Infectious Diseases*. 2015;212:978–89.
341. Mott PD, Taylor CM, Lillis RA, Ardizzone CM, Albritton HL, Luo M, et al. Differences in the Genital Microbiota in Women Who Naturally Clear Chlamydia trachomatis Infection Compared to Women Who Do Not Clear; A Pilot Study. *Front Cell Infect Microbiol*. 2021;11:615770.
342. Su H, Morrison R, Messer R, Whitmire W, Hughes S, Caldwell HD. The Effect of Doxycycline Treatment on the Development of Protective Immunity in a Murine Model of Chlamydial Genital Infection. *J Infect Dis*. 1999;180:1252–8.
343. Brunham RC, Pourbohloul B, Mak S, White R, Rekart ML. The Unexpected Impact of a Chlamydia trachomatis Infection Control Program on Susceptibility to Reinfection. *J Infect Dis*. 2005;192:1836–44.
344. Geisler WM, Lensing SY, Press CG, Hook EW. Spontaneous Resolution of Genital Chlamydia trachomatis Infection in Women and Protection from Reinfection. *J Infect Dis*. 2013;207:1850–6.
345. Harvie MC, Carey AJ, Armitage CW, O'Meara CP, Peet J, Phillips ZN, et al. Chlamydia-infected macrophages are resistant to azithromycin treatment and are associated with chronic oviduct inflammation and hydrosalpinx development. *Immunology & Cell Biology*. 2019;97:865–76.

346. Rupp J, Pfeiderer L, Jugert C, Moeller S, Klinger M, Dalhoff K, et al. *Chlamydia pneumoniae* Hides inside Apoptotic Neutrophils to Silently Infect and Propagate in Macrophages. *PLOS ONE*. 2009;4:e6020.
347. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 α , and MIP-1 β as the Major HIV-Suppressive Factors Produced by CD8⁺ T Cells. *Science*. 1995;270:1811–5.
348. Chumduri C, Gurumurthy RK, Berger H, Dietrich O, Kumar N, Koster S, et al. Opposing Wnt signals regulate cervical squamocolumnar homeostasis and emergence of metaplasia. *Nat Cell Biol*. 2021;23:184–97.

Appendix

French detailed summary

I. Introduction

Les infections sexuellement transmissibles (IST), incluant les infections virales comme l'infection au virus de l'immunodéficience humaine (VIH) ou les infections bactériennes telles que *Chlamydia trachomatis* (CT) ou *Neisseria gonorrhea*, demeurent un problème de santé publique.

L'infection par CT est très fréquente dans le monde, et majoritairement observée chez les jeunes femmes de 15 à 24 ans sexuellement actives. CT est l'agent pathogène responsable d'une IST, c'est une bactérie gram négative qui ne peut survivre qu'à l'intérieur des cellules. CT se divise en 14 sérovars : les sérovars impliqués dans les infections de l'œil (trachome) et urogénitaux (A-K), ainsi que ceux impliqués dans la lymphogranulomatose vénérienne (L1-L3). Les sérovars D-K sont responsables de l'infection uro-génitale. L'infection par CT peut induire : (1) une infection persistante, (2) des infections répétées à CT même après élimination des bactéries naturelle ou par traitement aux antibiotiques, (3) des infections souvent peu symptomatiques mais qui, non traitées, favorisent l'apparition de graves séquelles, (4) une inflammation locale forte. Une infection même asymptomatique non traitée peut entraîner une dissémination des bactéries dans le tractus reproducteur haut (utérus, trompes de Fallope) et faciliter le développement de l'infection en induisant une infection/inflammation des trompes de Fallope, des douleurs chroniques pelviennes, des grossesses ectopiques ainsi qu'une infertilité. L'azitromycine et la doxycycline sont deux antibiotiques prescrits contre l'infection à CT.

CT infecte, de façon préférentielle, les cellules épithéliales du tractus reproducteur féminin (TRF). Cette infection conduit à la production de cytokines pro-inflammatoires (IL-1, IL-6, IL-8, TNF α , IL-12, IFN γ et IL-18), immuno-régulatrices (IL-10) ainsi que des facteurs de croissance (VEGF, G-CSF, GM-CSF). L'ensemble de ces facteurs solubles vont favoriser le recrutement de cellules immunitaires comme les cellules dendritiques (DC), monocytes, Natural-Killer (NK), macrophages et neutrophiles. Les neutrophiles ont un rôle controversé

vis-à-vis de l'infection à CT. De nombreuses études ont démontré que le recrutement intensif de neutrophiles dans le TRF augmente l'inflammation et le risque de séquelles dans le tractus reproducteur haut. Néanmoins, une autre étude démontre un rôle prédominant des neutrophiles dans la réponse immunitaire médiée par les anticorps, permettant la diminution de la charge bactérienne. Quant à la réponse immunitaire spécifique, la résolution de l'infection sans traitement est largement attribuée à la réponse CD4⁺ Th1 via la production d'IFN γ .

Les muqueuses sont des sites préférentiels pour les infections. Le TRF est divisé entre le tractus haut (trompes de Fallope, ovaires, utérus et endocol), et le tractus bas (exocol et vagin). Les pathogènes sont en contact direct avec l'épithélium vaginal ou du col favorisant leur implantation et dissémination. L'épithélium du tractus haut est composé de cellules épithéliales cylindriques ciliées reliées entre elles par des jonctions serrées, tandis que celui du tractus bas est pluristratifié avec des cellules épithéliales pavimenteuses stratifiées non kératinisées. L'organisation dans le tractus haut et bas en cellules immunitaires innées et spécifiques, ainsi que la présence des facteurs solubles comme les anticorps, diffèrent. Les cellules prédominantes au sein du TRF sont les cellules T, dont 40% sont des cellules CD4⁺ et 60% de CD8⁺. Il y a une distribution au sein du TRF des cellules macrophages, NK, DC et neutrophiles.

Les populations de neutrophiles n'ont pas encore été déterminées dans le TRF, néanmoins, des études ont été effectuées sur le sang périphérique et la moelle osseuse. Trois sous-populations de neutrophiles sont retrouvées dans la moelle osseuse humaine : pré-neutrophiles (CD15⁺ CD66⁺ CD49d⁺ CD101⁻), neutrophiles immatures (CD15⁺ CD66b⁺ CD49d^{mid} CD101⁺ CD10⁻ CD16^{mid}), neutrophiles matures (CD15⁺ CD66b⁺ CD49d⁻ CD101⁺ CD10⁺ CD16⁺). L'expression des marqueurs CD101 et CD10 facilite la distinction entre une population mature (CD10⁺ CD101⁺) et immature (CD10⁻ CD101⁺). L'activation des neutrophiles nécessite une stimulation de ceux-ci par des agents bactériens/viraux ou des cytokines pro-inflammatoires. Cette activation induit le « priming » des neutrophiles, qui correspond à une diminution de l'expression de CD62L et à une augmentation de l'expression de CD11b. Le CD62L est une sélectine permettant la liaison entre les

neutrophiles et la surface endothéliale vasculaire. Une fois les neutrophiles dans le tissu cible, ceux-ci vont pouvoir répondre à l'infection via plusieurs mécanismes : la libération de cytokines ou de granules spécifiques, la phagocytose, et/ou la netose qui consiste en un enchevêtrement d'ADN couplé avec des protéines antimicrobiennes, des protéases ou encore des myéloperoxydases (MPO).

Le cycle menstruel a un impact important sur le TRF. En effet, il va induire des modifications en nombre de cellules et de production de facteurs solubles (peptides antimicrobiens, cytokines/chemokines, anticorps). Le cycle menstruel inclut 4 phases : les menstruations, la phase ovulatoire, la phase proliférative (folliculaire) et la phase sécrétoire (lutéale). A la suite des menstruations, qui durent entre 3 et 7 jours, la phase proliférative débute. Cette phase est caractérisée par la production d'œstradiol par le follicule mature. Lors du pic d'œstradiol, l'ovule est libéré. L'ovule va par la suite produire de la progestérone et de l'œstradiol, ce qui correspond au début de la phase sécrétoire. Sans fécondation, l'ovule ne va plus produire d'hormones et être éliminé avec l'apparition des menstruations.

Au sein du TRF, la composition du microbiote vaginal est également soumise au cycle menstruel. Chez la femme, le microbiote vaginal eubiotique est dominé par un genre de bactérie, les *Lactobacillus spp.*. La production d'acide lactique par ces bactéries va maintenir un environnement acide (pH = 4 to 5) défavorable à la survie des pathogènes. De plus, cet environnement est associé à un environnement peu inflammatoire. Le microbiote vaginal peut être réparti en 6 groupes en se basant sur sa composition. Les groupes I, II, III et V sont dominés par une espèce de *Lactobacillus spp.* : *L. crispatus* (groupe I), *L. gasseri* (groupe II), *L. iners* (groupe III), *L. jensenii* (groupe V). Quant au groupe IV, il est composé de nombreuses bactéries anaérobies strictes ou facultatives autres que *Lactobacillus spp.*, incluant les *Prevotella*, *Dialister*, *Atopium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia* et *Mobiluncus*. Le pH vaginal des groupes I, II, III et V est compris entre 4-5 alors que celui du groupe IV est au-dessus de 5. Le groupe IV peut être divisé en deux sous-groupes : les groupes IV-A et IV-B. Le groupe IV-A possède une faible quantité de *Lactobacillus spp.* et un nombre variable de bactéries anaérobies. Quant au groupe IV-B, il est dominé par des bactéries anaérobies. L'augmentation de la diversité microbienne, des bactéries anaérobies

et la diminution des *Lactobacillus spp.* est associée à une condition clinique appelé la vaginose bactérienne. Un traitement antibiotique (métronidazole) est préconisé pour le traitement des vaginoses bactériennes. Ce traitement permet l'élimination de nombreuses bactéries anaérobies sans affecter les lactobacilles. Toutefois, ce traitement induit des modifications provisoires qui n'empêchent pas les rechutes.

Les lactobacilles sont des bactéries gram positives anaérobie facultative et de nombreuses espèces sont retrouvées dans le microbiote vaginal de la femme : *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. plantarum*, *L. casei*, *L. vaginalis*, *L. iners*. Elles sont largement utilisées comme probiotiques dans le but d'endiguer l'implantation et la croissance des pathogènes. En effet, les lactobacilles vont entrer en compétition avec les pathogènes pour la fixation sur les cellules épithéliales, elles vont également produire de l'acide lactique et des agents bactéricides/bacteriostatiques qui vont inhiber la croissance des pathogènes. De nombreux facteurs vont avoir un impact sur la composition du microbiote vaginal comme : les hormones, l'utilisation de contraceptifs, le statut ethnique, le comportement sexuel, l'hygiène intime et bien évidemment les IST.

Il a été démontré que le microbiote vaginal module la réponse immunitaire. Un microbiote vaginal dominant en lactobacilles va induire un environnement peu inflammatoire et donc une protection contre les IST. A l'inverse, un microbiote divers avec de nombreuses bactéries anaérobies favorise une inflammation locale traduite par une augmentation de la production de cytokines (IL-1, IL-12, IL-4, IL-8, IL-18, IL-10, TNF α , IFN γ , G-CSF, GM-CSF) et un recrutement de cellules Th17 CD4+. Cet état inflammatoire a été lié à une susceptibilité accrue aux IST comme CT ou le VIH-1. Les neutrophiles, qui sont des cellules essentielles dans la lutte contre les infections bactériennes, n'ont été que très peu étudiés au sein du TRF. Cependant, dans l'intestin, les neutrophiles sont très largement affectés par le microbiote intestinal. En effet, celui-ci va favoriser la génération par la moelle épinière de neutrophiles, mais également le priming des neutrophiles. De plus, la survie des neutrophiles est augmentée en cas d'altération du ratio *Lactobacillus spp/Prevotella*. On peut émettre l'hypothèse que les neutrophiles cervicovaginaux sont également affectés par le microbiote vaginal.

Les modèles animaux sont utilisés depuis des années et sont essentiels à la compréhension des infections. Le macaque cynomolgus est un des modèles d'étude parmi les plus répandus, avec le macaque rhésus et le macaque à queue-de-cochon. Le système immunitaire des macaques rhésus et cynomolgus est très similaire à celui de l'homme. De plus, la morphologie du TRF, la composition en cellules immunitaires du TRF, le système endocrinien et le cycle menstruel du macaque cynomolgus sont très similaires à celui de la femme. Le microbiote vaginal des macaques rhésus et à queue-de-cochon, ont été décrits comme très divers, composés majoritairement par des bactéries anaérobies et très peu de lactobacilles. A notre connaissance, le microbiote vaginal du macaque cynomolgus n'a pas encore été décrit. Les macaques sont susceptibles à différentes infections touchant l'homme, y compris l'infection par *Chlamydia trachomatis*. Cependant, après infection CT, les animaux ne développent de forme grave qu'après infection directe des trompes de Fallope, ce qui permet de déclencher une salpingite par exemple. Au sein du laboratoire, les infections vaginales à CT forte dose unique ou répétée et faible dose répétée ont été mises au point. Les infections répétées à faibles et fortes doses semblent induire une infection persistante, avec une production d'anticorps spécifiques dans le sérum environ 15 jours après exposition. L'exposition à des faibles doses répétées de CT induit une réponse cytokinique plus forte dans le sang et dans les fluides cervicovaginaux que l'exposition à des fortes doses répétées.

II. Hypothèses et Objectifs

Le but de ce projet de thèse était, dans un premier temps, d'étudier les interactions entre les marqueurs inflammatoires et le microbiote vaginal en fonction du cycle menstruel chez la femelle cynomolgus macaque, et dans un second temps d'établir l'influence de l'environnement muqueux, dont la composition du microbiote vaginal, sur l'infection par CT et l'inflammation dans des modèles *in vitro* et *in vivo*. Nos hypothèses étaient les suivantes : (1) l'environnement local ainsi que le niveau d'inflammation seront modifiés en fonction du cycle menstruel ; (2) l'augmentation de l'abondance de lactobacilles va modifier l'inflammation et affecter la susceptibilité à l'infection à CT et l'inflammation induite par l'infection.

Les objectifs de la thèse étaient les suivants :

- I. Caractériser le microbiote vaginal au cours du temps chez la femelle cynomolgus macaque.
- II. Etudier les interactions entre le microbiote vaginal, les facteurs inflammatoires locaux (cytokines et phénotype des neutrophiles) au cours du cycle menstruel chez la femelle cynomolgus macaque.
- III. Développer un modèle d'étude *in vivo* pour étudier l'impact de la composition du microbiote vaginal sur l'infection CT et l'inflammation. Pour cela, dans un premier temps, un modèle de femelles macaques cynomolgus avec un microbiote vaginal enrichi en lactobacilles a été développé. Dans un second temps, des animaux avec un microbiote enrichi ou non par des lactobacilles ont été infectés par CT et les réponses immunitaires ainsi que le microbiote vaginal ont été étudiés.
- IV. Mettre au point un modèle *in vitro* pour l'étude de l'effet de deux bactéries antagonistes du microbiote vaginal (*L. crispatus* vs *G. vaginalis*) sur l'inflammation induite par l'infection CT. Et enfin, évaluer l'impact de ces environnements sur le phénotype et la survie des neutrophiles ainsi que sur la susceptibilité au VIH-1.

III. Résultats

Dans une première étude, nous avons étudié chez cinq femelles macaques cynomolgus la composition du microbiote vaginal et rectal au cours de 3 cycles menstruels. Le microbiote vaginal chez cette espèce de macaque n'avait pas encore été étudié. Au vu des récentes avancées qui démontrent que le microbiote vaginal joue un rôle dans la régulation de la réponse immunitaire, il était nécessaire d'étudier cette composante chez le macaque pour mieux appréhender son effet potentiel sur les IST et l'inflammation. Les genres bactériens majoritaires dans le microbiote rectal sont les *Ruminococcaceae*, *Prevotella* et *Clostridiales*. La composition du microbiote rectal varie très peu au cours du temps à l'inverse du microbiote vaginal : celui-ci est plus variable en fonction des animaux et du temps.

Néanmoins, quatre genres sont observés chez l'ensemble des femelles : *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Peptoniphilus*. Peu de lactobacilles sont détectés chez les animaux. La composition du microbiote vaginal a été démontrée comme similaire, en terme de composition et d'abondance, au microbiote vaginal d'une femme ayant un microbiote de type IV (très divers, riche en anaérobies et pauvre en lactobacilles). Enfin, nous avons déterminé que le microbiote vaginal des femelles varie en fonction du cycle menstruel : l'abondance de plusieurs bactéries est modifiée en fonction du taux de progestérone. En conclusion, nous avons caractérisé de façon longitudinale la composition du microbiote vaginal et rectal d'animaux très souvent utilisés en recherche sur les ISTs. Ces animaux sont de bons modèles d'étude de la dysbiose vaginale. Cependant, uniquement le microbiote a été étudié, sans inclure les facteurs inflammatoires tels que les cytokines ou les neutrophiles. En effet, il a été démontré, chez la femme, que la composition du microbiote vaginal va influencer l'état inflammatoire local, et par la même occasion sur la susceptibilité aux IST.

Une seconde étude a été mise en place afin d'établir si l'environnement muqueux est affecté par le cycle hormonal, et si des interactions entre les différents facteurs inflammatoires et le microbiote vaginal peuvent être déterminées. Au cours de cette étude, neuf femelles cynomolgus macaques ont été étudiées pendant trois mois, soit au cours d'environ trois cycles menstruels. Différents prélèvements ont été effectués afin d'analyser le microbiote vaginal ainsi que l'état inflammatoire cervicovaginal et sanguin (production des cytokines et phénotype des neutrophiles). Nous avons démontré que la composition cytokinique et le phénotype des neutrophiles varient en fonction du compartiment. Dans le sang, peu de cytokines sont produites à l'état basal, quant aux neutrophiles, une population principale exprimant CD11b, CD32a, CD62L et CD101 est présente. Cette population peut être divisée en deux : une population mature (CD10⁺ CD101⁺) et une immature (CD101⁺ CD10⁻). Peu de variations sont observées au cours du temps en termes de cytokine ou neutrophiles dans le sang, seul le nombre total de neutrophiles augmente pendant les menstruations. Au niveau vaginal, l'expression des cytokines et chimiokines est bien plus forte et variable en fonction du temps. Nous avons pu déterminer que de nombreuses cytokines locales sont modulées par le cycle hormonal, contrairement aux cytokines

sanguines. En effet, la production des cytokines est augmentée pendant les menstruations au niveau cervicovaginal. Quant aux neutrophiles, trois populations majoritaires sont détectées : (1) CD11b^{high} CD101⁺ CD10⁺ CD32a⁺ ; (2) CD11b^{high} CD101⁺ CD10⁻ CD32a⁺ ; (3) CD11b^{low} CD101⁻ CD10⁻ CD32a⁻. Toutes ces populations ont une très faible expression de CD62L et peuvent être catégorisées en se basant sur les populations neutrophiles du sang. La population 1 a un phénotype de neutrophiles matures et activés, tandis que la population 2 sont des neutrophiles immatures mais activés. Enfin, la population 3 possède un phénotype de pré-neutrophiles qui ne sont habituellement retrouvés que dans la moelle osseuse. Cette classification se base entièrement sur le phénotype des neutrophiles observé dans le sang, aucune expérience testant la fonctionnalité des neutrophiles n'a été faite. Par la suite, l'évolution des différentes populations au cours du temps a été étudiée. Nous avons déterminé une modulation importante des populations de neutrophiles en fonction du cycle menstruel. Plus particulièrement la population de neutrophiles matures et activés est significativement augmentée pendant les menstruations. De plus, cette population pendant les menstruations exprime plus fortement CD62L, qui est normalement augmenté dans le sang et faiblement exprimé dans les tissus. Ceci laisse supposer que cette population serait originaire du sang. La composition du microbiote vaginal a ensuite été déterminée pour évaluer sa capacité à moduler l'inflammation chez les femmes. Le microbiote vaginal des femmes est très divers et pauvre en lactobacilles comme observé dans la précédente étude, de plus, le pH vaginal ne descend que rarement en dessous de 7. On observe trois phyla majoritaires : *Firmicutes*, *Bacteroidota*, *Actinobacteriota*. Une analyse différentielle se basant sur les variations d'abondance au cours du temps, nous a permis de déterminer que de nombreuses familles de bactéries évoluent au cours du cycle menstruel. Une analyse combinée des facteurs variant au cours du cycle menstruel (cytokine, neutrophiles, familles de bactéries) a démontré que les échantillons obtenus pendant les menstruations sont groupés ensemble. En effet, ces échantillons sont caractérisés par une production importante de cytokines et la présence de neutrophiles matures activés provenant du sang. De plus, certaines femmes sont similaires entre elles lorsque ces paramètres sont pris en considération. Néanmoins, une association entre un groupe de bactéries, un profil

inflammatoire et un phénotype ou une accumulation de neutrophiles n'a pas pu être établi au cours de cette étude. En conclusion, ces résultats démontrent que le cycle hormonal induit au niveau cervicovaginal des variations de production de cytokines, de sous-populations de neutrophiles et de la composition du microbiote vaginal chez la femelle macaque cynomolgus. Les menstruations favorisent un environnement plus inflammatoire que les autres phases hormonales. Cette étude met en évidence la nécessité d'un planning de prélèvement approprié pour l'étude de l'inflammation locale dans les programmes de recherche s'intéressant aux réponses immunitaires du tractus reproducteur féminin. Le cycle menstruel doit être pris en compte dans les prochaines études afin d'éviter un biais lié à l'échantillonnage.

Afin de mieux comprendre comment la composition du microbiote vaginal va impacter les réponses immunitaires et donc l'acquisition d'IST, nous souhaitions comparer deux groupes d'animaux, dont l'un des groupes aurait un microbiote enrichi en *Lactobacillus crispatus*. En effet, une dominance de *L. crispatus* chez la femme est associée à un environnement peu inflammatoire et à une protection contre les IST, à l'inverse d'un microbiote divers riche en bactéries anaérobies et pauvre en lactobacilles. Les objectifs consistaient à évaluer la susceptibilité et les réponses immunitaires induites par l'infection chez les deux groupes d'animaux. Dans un premier temps, nous avons développé un modèle macaque dont le microbiote vaginal est enrichi en *L. crispatus*. Différentes stratégies ont été testées, impliquant des capsules de *L. crispatus* (Physioflor®), des pré-traitements avec des antibiotiques et des lavages de la cavité vaginale avec de l'acide lactique afin de diminuer le pH vaginal des femelles et de faciliter l'implantation des *L. crispatus*. Au cours de ces essais, nous avons rencontré différents obstacles. Premièrement, l'apparition des menstruations empêche le maintien des lactobacilles exogènes. Afin de palier à ce problème, nous avons décidé de traiter les animaux avec un progestatif (Depoprovera®) empêchant l'apparition des menstruations, mais ce traitement ne favorisait pas non plus l'enrichissement en lactobacilles. Deuxièmement, le traitement antibiotique, bien que favorisant significativement l'enrichissement en lactobacilles et la diminution de la diversité, ne permet pas d'effacer les différences inter individuelles observées après traitement avec les

lactobacilles. Par conséquent, la stratégie d'enrichissement en lactobacilles retenue est la suivante : traitement répétitif local avec du métronidazole suivi par un lavage de la cavité vaginale par 0.02% d'acide lactique et d'un traitement répétitif avec un gel composé de 10^{10} *L. crispatus*.

Dans une troisième étude, l'impact de la composition du microbiote vaginal sur la susceptibilité à l'infection par CT et sur les réponses immunitaires induites par CT a été étudié. Douze femelles macaques cynomolgus ont été réparties aléatoirement en deux groupes de six animaux. Les deux groupes ont été traités par des antibiotiques (métronidazole) pour réduire la diversité microbienne, mais seulement un des deux groupes a été inoculé avec des *L. crispatus*. Par la suite, tous les animaux ont été exposés de façon répétée à CT en intravaginal. Différents prélèvements ont été effectués afin de suivre : les réponses immunitaires locales et systémiques (phénotypage des neutrophiles, production de cytokines/chimiokines), l'infection par CT (quantification du pathogène et mesure d'anticorps) ainsi que la composition du microbiote vaginal. Les traitements au métronidazole induisent une réduction des anaérobies, une augmentation des lactobacilles endogènes (*L. johnsonii*, *L. prophage*, *L. animalis*) et des bactéries opportunistes (*Staphylococcus*, *Streptococcus*). La présence de *L. crispatus* n'est observée qu'après son inoculation dans le groupe traité. Concernant l'infection à CT, 10 animaux sur 12 ont été infectés sans qu'aucun ne développe une infection persistante. Un animal dans chaque groupe n'a pas été infecté. L'infection des animaux n'est pas spécifique au groupe, donc aucune association entre l'augmentation de la proportion de *L. crispatus* et la susceptibilité à CT n'est observée. Cependant, les animaux du groupe traité par les *L. crispatus* possèdent une réponse anticorps spécifique dans le sang significativement plus forte que les animaux non traités. La comparaison de la composition du microbiote avec la charge bactérienne, montre que l'augmentation des bactéries *L. johnsonii* et *L. prophage* est associée à l'augmentation de la charge bactérienne. Il est intéressant de noter que cette association n'est observée que dans le groupe non traité. L'infection par CT induit des modifications de la composition du microbiote vaginal qui sont persistantes (à D63 soit 4 semaines après la dernière inoculation). Quant à l'inflammation, des différences entre groupes après infection

sont détectés dans les deux compartiments. En effet, les cytokines cervicovaginales sont augmentées dans le groupe traité par rapport au groupe non traité, et l'inverse est observé dans le sang. Ces résultats reflètent potentiellement une meilleure réponse locale dans le groupe traité après infection, et une inflammation systémique plus faible par rapport au groupe non traité. Le phénotype des neutrophiles cervicovaginaux chez l'ensemble des femmes est peu altéré par l'infection, à l'inverse des neutrophiles du sang. Dans le sang, les neutrophiles sont plus matures et activés après les inoculations/infections par CT. Pour l'instant, nous n'avons pas pu établir une association entre un groupe de bactéries, une susceptibilité accrue à CT et une plus forte inflammation. En conclusion, ces résultats démontrent que le microbiote vaginal joue un rôle important dans la régulation de la réponse immunitaire locale, mais aussi systémique. Par conséquent, la modulation du microbiote vaginal pourrait être un outil permettant de diminuer l'inflammation locale et donc la susceptibilité aux IST.

Enfin, une dernière étude visait à disséquer les mécanismes permettant au microbiote de moduler la réponse immunitaire lors d'une infection. Cette étude *in vitro* s'est focalisée sur deux bactéries du microbiote vaginal ayant un rôle antagoniste : *L. crispatus* (faible inflammation et protection contre IST) et *G. vaginalis* (inflammation et susceptibilité accrue aux IST). Ces deux bactéries ont été ajoutées séparément à des cellules issues d'une lignée endocervicale épithéliale humaine (A2EN) infectées ou non par CT. Les surnageants de culture ont ensuite été collectés et les cytokines/chimiokines présentes ont été quantifiées. Puis, ces surnageants ont été ajoutés à des cellules cibles exposées au VIH-1 ou à des neutrophiles du sang. Ainsi, nous avons pu évaluer l'effet des surnageants sur la susceptibilité au VIH-1 et sur le phénotype et la survie des neutrophiles. La production des cytokines induites par ces deux bactéries sans infection CT sont différentes. De plus, les profils de cytokines induits par la stimulation avec les bactéries après infection CT sont similaires à ceux observés lors d'une simple stimulation sans infection. Néanmoins, l'inflammation induite est plus faible lors de la stimulation par *L. crispatus* que par *G. vaginalis*. Concernant les neutrophiles, leur activation est stimulée en présence de l'ensemble des surnageants des cellules A2EN quel que soient les conditions expérimentales, mais la

présence des bactéries du microbiote augmente cette activation. Quant à la survie des neutrophiles, elle est augmentée par les surnageants collectés après infection CT en présence ou non des bactéries. Il est intéressant de noter que les surnageants obtenus après stimulation par *G. vaginalis* sans infection CT sont capables d'augmenter significativement la survie des neutrophiles. L'infection VIH-1 a été réduite après stimulation des cellules cibles du VIH-1 avec les surnageants CT+. Cette réduction de l'infection VIH-1 est également observée après stimulation par les surnageants *G. vaginalis* avec ou sans infection CT. Chez la femme, les résultats publiés indiquent que l'inflammation induite par l'infection CT augmente le risque d'acquisition du VIH-1. Le modèle *in vitro* ne suffit donc pas à récapituler l'environnement mucosal *in vivo*. En effet, ce modèle se focalise sur l'interaction du microbiote avec les cellules épithéliales sans prendre en compte les autres cellules de l'environnement muqueux. De plus, le microbiote est composé d'une multitude de bactéries et nous n'avons regardé que l'effet spécifique de deux organismes, sans prendre en compte de possibles interactions entre bactéries.

IV. Conclusion et Perspectives

Au cours de ma thèse, j'ai étudié la présence et les interactions entre les facteurs environnementaux du TRF au cours du cycle menstruel à l'état basal, mais aussi lors d'une IST. La question centrale de ce projet était la suivante : comment pourrait-on moduler le microbiote vaginal pour réduire la susceptibilité aux IST et l'inflammation induite par les IST ? Pour ce faire, différentes étapes ont été nécessaires. Tout d'abord, nous avons caractérisé le microbiote vaginal des femelles macaques cynomolgus, ce qui n'avait pas encore été réalisé. De plus, nous avons démontré que la composition du microbiote vaginal varie en fonction du cycle menstruel. Cette étude ne s'intéressait qu'au microbiote vaginal sans prendre en compte d'autres facteurs locaux. Nous avons donc, par la suite, développé une autre étude où nous avons démontré que les concentrations de cytokines ainsi que les populations de neutrophiles en cervicovaginal varient en fonction du cycle menstruel. En effet, l'apparition des menstruations est associée à une augmentation de l'inflammation locale (augmentation de la production de cytokines et recrutement de neutrophiles matures d'origine sanguine).

Nous avons, pour la première fois, identifié les sous-populations de neutrophiles vaginaux et leur évolution au cours du cycle. En parallèle de ces études, nous avons développé le modèle macaque avec un microbiote vaginal moins divers et enrichi en *L. crispatus*. Une fois ce modèle établi, des animaux avec un microbiote enrichi en *L. crispatus* ou non ont été infectés par des doses répétées de CT. Nous avons observé que la composition du microbiote vaginal influe sur les réponses immunitaires locales, mais aussi systémique. Cependant, nous n'avons pas pu déterminer pour l'instant une association entre un groupe de bactéries, une plus forte inflammation et une susceptibilité accrue aux IST. Enfin, une partie *in vitro* complète le projet et s'intéressait plus spécifiquement aux mécanismes impliqués dans la régulation de l'inflammation, induite par CT, en présence ou non de deux bactéries du microbiote et l'effet sur la co-infection avec le VIH-1 et le phénotype/la survie des neutrophiles.

Différents points peuvent être soulevés à la suite de ce projet. Tout d'abord, nous pouvons questionner l'utilisation du modèle macaque. Le macaque cynomolgus est un très bon modèle d'étude, en partie de par sa ressemblance à l'homme en termes d'anatomie, de réponses immunitaires et de susceptibilité aux pathogènes par exemple. Néanmoins, des différences persistent, par exemple la composition du microbiote naturel du macaque n'est pas dominant en lactobacilles. Il récapitule plutôt celui de femmes qui ont un microbiote très divers, dysbiotique. Nous pouvons également nous demander si la présence des lactobacilles humains dans le microbiote vaginal des femelles macaques ne va pas, à l'inverse de chez la femme, augmenter l'inflammation et la susceptibilité aux IST. Nous avons observé plutôt une diminution de l'inflammation chez les femelles avec un microbiote enrichi, mais l'implantation n'est pas optimale, suggérant que la diminution de l'inflammation n'est peut-être due, en majorité, qu'à la diminution des anaérobies. De plus, lors des infections à CT, nous avons associé la présence de *L. johnsonii* à une charge bactérienne plus forte. Chez la femme, on s'attendrait plutôt à l'inverse, nous pouvons donc nous questionner sur l'effet souche des lactobacilles. En effet, il a été déterminé qu'en fonction de la souche l'effet sur l'inflammation peut être différent.

Ensuite, l'infection par CT des animaux n'a pas induit d'infection persistante, à l'inverse des études précédentes du laboratoire. Nous pouvons nous demander si ces différences seraient dues aux traitements antibiotiques par exemple, sachant qu'en diminuant la proportion d'anaérobies, nous aurions pu diminuer la susceptibilité à CT chez l'ensemble des animaux. De façon intéressante, nous avons observé une différence de réponse anticorps spécifique à CT entre les deux groupes (traité *L. crispatus* vs non traité) sans différences de charge bactérienne. Il serait important de comprendre comment cette production a été stimulée. Est-elle due à une meilleure production locale via une meilleure stimulation des plasmocytes ? Nous avons aussi obtenu une faible réponse immunitaire induite post-infection, il serait également intéressant de développer un modèle d'infection persistante. Quant au modèle *in vitro*, il nécessite des améliorations. Il faudrait développer un modèle d'organoïde où l'ensemble de l'environnement muqueux pourrait être présent et exposer à la fois aux IST et à un microbiote complet isolé chez des femmes présentant des dysbioses vaginales ou non.

En conclusion, ce projet se focalisait sur la régulation de l'inflammation et son influence sur l'acquisition d'IST. Il démontre que l'environnement muqueux est très complexe et implique de nombreux facteurs qui vont interagir entre eux et être impactés par les hormones. Le développement du modèle *in vivo* et les études associées vont permettre l'établissement de nouvelles études ayant pour objectif d'analyser les vecteurs de l'inflammation locale, comme le plasma séminal ou l'utilisation de contraceptif sur l'acquisition d'IST. Ce modèle aura également un rôle central dans la mise en place d'une étude se focalisant sur l'impact de l'inflammation et du microbiote vaginal sur les co-infections CT/VIH-1.

Titre : Inflammation et réponses immunitaires au sein du tractus reproducteur féminin.

Mots clés : Inflammation, microbiote vaginal, tractus reproducteur féminin, infections sexuellement transmissibles

Résumé : Plusieurs facteurs environnementaux sont présents au sein du tractus reproducteur féminin (TRF). Ceux-ci évoluent au cours du cycle menstruel et interagissent entre eux. Ils vont moduler la susceptibilité aux infections sexuellement transmissibles (IST) en modifiant l'inflammation locale. La composition du microbiote vaginal (MV) possède un rôle clé dans la régulation de l'inflammation et des réponses immunitaires (RI) locales. Ce projet visait à : (1) étudier les interactions entre les facteurs inflammatoires, présents au sein du TRF, au cours du cycle menstruel ; (2) analyser l'impact de la composition du MV sur l'infection à *Chlamydia trachomatis* (CT) et l'inflammation induite par CT; (3) évaluer les mécanismes impliqués dans la régulation de la RI par le MV (*L. crispatus* vs *G. vaginalis*) au cours d'une infection à CT et les conséquences sur le phénotype et la fonction des neutrophiles ainsi que la co-infection VIH-1.

Nous avons démontré *in vivo* chez le primate non humain que la composition du microbiote vaginal ainsi que la production de cytokines et le phénotype des neutrophiles cervicovaginaux sont impactés par le cycle menstruel. En effet, les menstruations induisent une augmentation de l'inflammation locale. De plus, la réponse immunitaire induite par l'infection CT est impactée par la composition du microbiote vaginal. Enfin, *in vitro*, les profils de cytokines induits par la stimulation avec *L. crispatus* ou *G. vaginalis* sont différents et peu altérés par l'infection par CT. De plus, l'activation et la survie des neutrophiles, et la susceptibilité au VIH-1, varient en fonction de la stimulation par les bactéries du microbiote et de l'infection par CT. En conclusion, ce travail apporte de nouvelles connaissances quant à la complexité de l'environnement au sein du TRF et son impact sur l'inflammation et la susceptibilité aux IST.

Title: Inflammation and immune responses within the female reproductive tract.

Keywords: Inflammation, vaginal microbiota, female reproductive tract, sexually transmitted infections

Abstract: The female reproductive tract (FRT) environment is composed of many factors that are influenced by menstrual cycle. These factors can interact with each other and affect the susceptibility to sexually transmitted infection (STI) by modulating the local inflammation. The vaginal microbiota composition has a central role in the regulation of local inflammation and immune responses. This project aimed at: (1) understanding the interplay between FRT inflammatory markers during the menstrual cycle; (2) ascertain the impact of the vaginal microbiota composition on *Chlamydia trachomatis* (CT) infection and on the inflammation induced by CT; (3) evaluating the mechanisms involved in the regulation of the immune response by the vaginal microbiota (*L. crispatus* vs *G. vaginalis*) during CT infection and the effect on neutrophil phenotype and survival and HIV-1

co-infection. We have shown *in vivo* in the non-human primate that cervicovaginal cytokine production, neutrophil phenotype and vaginal microbiota are influenced by the menstrual cycle. Indeed, menstruations increase local inflammation. We have demonstrated that the vaginal microbiota composition has an impact on the inflammation induced by CT infection. Finally, we have shown, in an *in vitro* model, that cytokines induced by *L. crispatus* or *G. vaginalis* stimulation are different and weakly impacted by CT infection. Moreover, neutrophil activation and survival as well as HIV-1 susceptibility are modified by microbiota/CT exposure. In conclusion, this work gives new insight into how the complex FRT environment affects the inflammation and the susceptibility to STI acquisition.