IMMUNOLOGICAL ROLE OF THE MATERNAL MICROBIOME IN PREGNANCY

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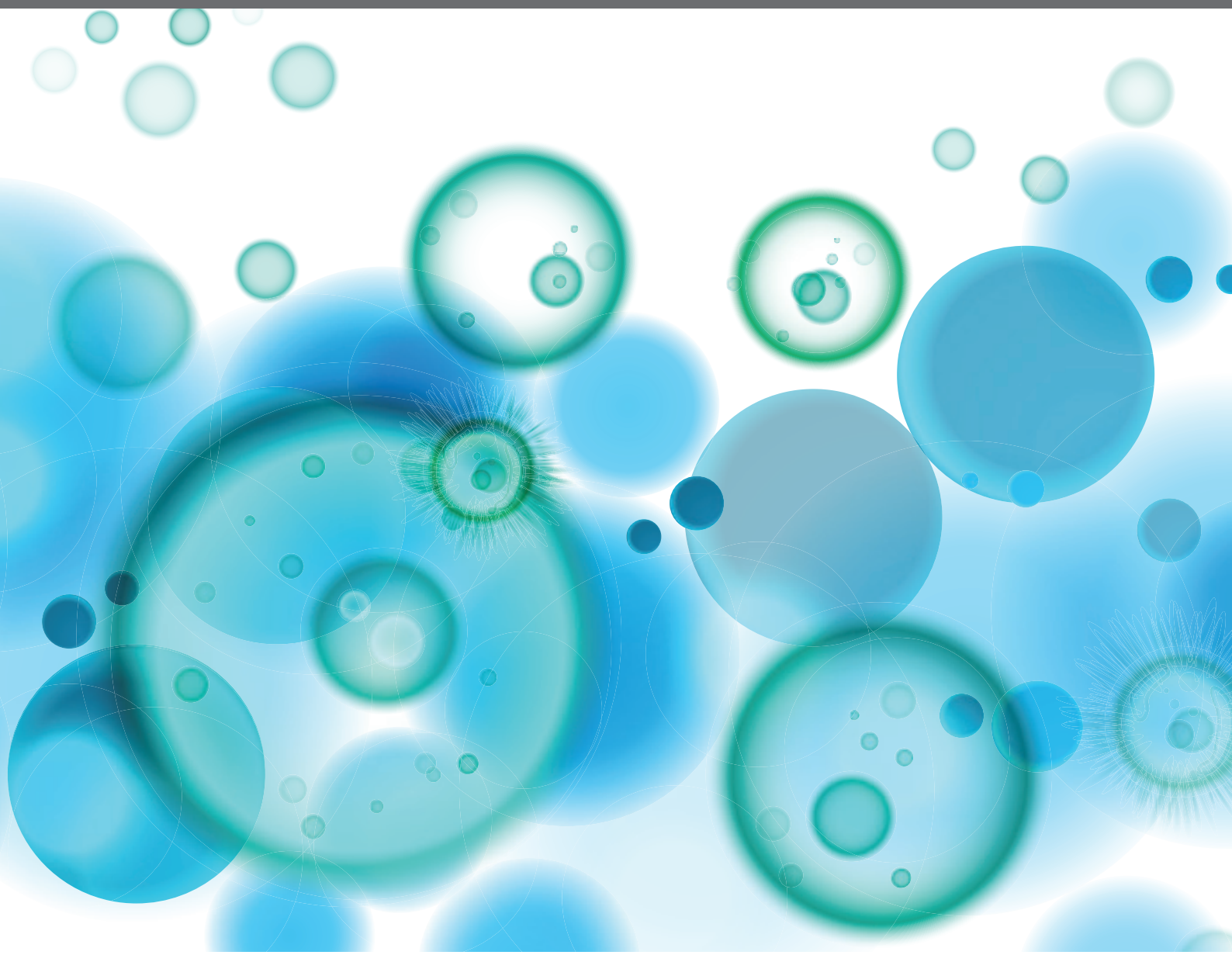

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IMMUNOLOGICAL ROLE OF THE MATERNAL MICROBIOME IN PREGNANCY

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EDITORIAL
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Editorial: Immunological Role of the Maternal Microbiome in Pregnancy

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Keywords: microbiota, immunity, pregnancy, implantation, preterm (birth), preterm/full term infants

Editorial on the Research Topic

Immunological Role of the Maternal Microbiome in Pregnancy

In this Research Topic of Frontiers in Mucosal Immunity we have focused on the immunological role of the maternal microbiome in pregnancy. We have invited leading researchers in the medical field to contribute articles that summarize the advancements in this broad and complex topic. The understanding of microbiota and its function has been steadily expanding and recent advancements in molecular biology and the Human Microbiome Project have allowed us to take a new biological look on the microbiota and the host interactions. In case of pregnancy, the host has to be separated in two compartments namely the mother and the fetus. Furthermore, pregnancy itself induces a number of changes, which include immunological, hormonal, and metabolic changes - necessary for the maternal adaptation process and fetal development. Undoubtedly, the maternal microbiome influences the course of pregnancy and fetal development beyond plain colonization. Therefore in pregnancy, we have to consider the potential implications of microbiota or it`s changes on both compartments (the maternal and fetal) and importantly visa-versa. We have therefore selected 10 review and original articles covering the important aspect of the field.

In this Research Topic we have selected two contributions reviewing the global aspects of maternal microbiota and pregnancy interaction (Di Simone et al.; Al-Nasiry et al.). The article written by Di Simone et al. summarizes the abnormal changes of gastrointestinal, vaginal, and endometrial microbiota in pregnancy and potential morbidities throughout the pregnancy. An example is the association between abnormally increased intestinal permeability and uterine innate immunity affecting obstetrical outcomes. Al-Nasiry et al. summarize the composition of reproductive tract microbiome in relation to pregnancy and focuses on host-microbiota immune interactions. Therefore, the readers will understand how microbiota changes impact clinical outcomes such as infertility, recurrent miscarriage, and placental syndromes. We have selected additional review articles addressing the implications of microbiota and pregnancy in more detail. The article written by Bardos et al. evaluates the changes specifically in early pregnancy such as the relationship between the microbiota and endometrium/embryo development. The potential implication of uterine microbiota on immunological responses and resulting placental syndromes such as preeclampsia or intrauterine growth restriction are discussed. Another example is the complex interaction between microbiota and uterine immune cells, which are the topic of the article written by Agostinis et al. The article discusses the role of microbiota in the control of uterine cells

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character and function in pregnancy and beyond. Beyond expected and classical approach to review the complex topic of maternal microbiome in pregnancy, we have selected three additional review articles to summarize evidence pregnancy related morbidities and treatment approaches. The potential of fecal microbiota transplantation is reviewed by Quaranta et al. and the implications in the postpartum period such as neonatal outcomes is reviewed by Tirone et al. Finally, the immunological role of uterine microbiota in postpartum hemorrhage is reviewed by Escobar et al. Together, we have selected seven review articles covering the topic of the maternal microbiome in pregnancy and beyond. To further dissect the complexity of maternal microbiome in pregnancy we have selected additional three original articles.

The selected original articles cover different aspects of immune responses in pregnancy. Using germfree mice, Faas et al. show different immunological adaptations to pregnancy modulated by microbiota. For example, they detected the increase in Treg and tendency to an increase in Th2 cells in conventional pregnant mice only. Another article by Cui et al. focuses on the role of $ROP16_I$, a rhoptry protein of Toxoplasma gondii, and macrophage polarization. The findings suggest that $ROP16_I$ might be a protective factor and involved in the M1–Th1 biased pathological process in the early phase of gestation. Finally, Mohr et al. provide a prospective Case-Control study investigating inflammation in pregnant women with periodontitis and preterm prelabor rupture of membranes (PPROM). They concluded that systemic inflammation, initiated and triggered by periodontal disease, might play a role in developing preterm prelabor rupture of membranes.

Together, we have selected 10 review and original articles dissecting the various aspects of maternal microbiome in pregnancy. Although the selected articles provide an overview in this complex topic, we have placed particular emphasis on the immunological changes and potential implications – having both the mother and the fetus in mind.

AUTHOR CONTRIBUTIONS

All authors contributed equally. All authors contributed to the article and approved the submitted version.

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REVIEW
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Uterine Immunity and Microbiota: A Shifting Paradigm

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The female reproductive tract harbors distinct microbial communities, as in the vagina, cervical canal, uterus, and fallopian tubes. The nature of the vaginal microbiota is well-known; in contrast, the upper reproductive tract remains largely unexplored. Alteration in the uterine microbiota, which is dependent on the nutrients and hormones available to the uterus, is likely to play an important role in uterine-related diseases such as hysteromyoma, adenomyosis, and endometriosis. Uterine mucosa is an important tissue barrier whose main function is to offer protection against pathogens and other toxic factors, while maintaining a symbiotic relationship with commensal microbes. These characteristics are shared by all the mucosal tissues; however, the uterine mucosa is unique since it changes cyclically during the menstrual cycle as well as pregnancy. The immune system, besides its role in the defense process, plays crucial roles in reproduction as it ensures local immune tolerance to fetal/paternal antigens, trophoblast invasion, and vascular remodeling. The human endometrium contains a conspicuous number of immune cells, mainly Natural Killers (NK) cells, which are phenotypically distinct from peripheral cytotoxic NK, cells and macrophages. The endometrium also contains few lymphoid aggregates comprising B cell and $CD8^+$ T cells. The number and the phenotype of these cells change during the menstrual cycle. It has become evident in recent years that the immune cell phenotype and function can be influenced by microbiota. Immune cells can sense the presence of microbes through their pattern recognition receptors, setting up host-microbe interaction. The microbiota exerts an appropriately controlled defense mechanism by competing for nutrients and mucosal space with pathogens. It has recently been considered that uterus is a non-sterile compartment since it seems to possess its own microbiota. There has been an increasing interest in characterizing the nature of microbial colonization within the uterus and its apparent impact on fertility and pregnancy. This review will examine the potential relationship between the uterine microbiota and the immune cells present in the local environment.

Keywords: uterus, pregnancy, immune cells, cellular immunity, microbiota, menstruation

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INTRODUCTION

Mucosal barriers are the first line of immune defense designed to induce protection against noxious environmental agents including pathogens, and simultaneously allow symbiosis with commensal microbes (1). The largest number of commensals are segregated along the gastrointestinal tract; this segregation is achieved by the combined action of epithelial cells, mucus, IgA, anti-microbial peptides and immune cells (2, 3). One major benefit from the homeostatic relationship between the host and the microbiota is the resistance to pathogen colonization (4).

It has been demonstrated that the microbiota is able to influence the function of the host immune system, which keeps a symbiotic relationship with the microbiota. The ability of microbes to modulate the immunological response, both locally and systemically, requires sensing of microbes, followed by complex dialogue between innate and adaptive components of the immune system (2).

The uterine mucosal immune system is quite unique compared to other mucosal surfaces since it has to adapt to menstruation in response to hormonal stimuli (1). In addition to binding with the pathogens, the uterine immunity has evolved to tolerate the semi-allogeneic fetus, thus, playing a fundamental role in implantation and pregnancy (5).

The uterine microenvironment, for a long time, has been considered to be a sterile compartment, although the likely existence of commensal colonization within the uterus was always debated. Only recently, thanks to the discovery of the 16S rRNA in the uterine compartment, it has been possible to demonstrate and characterize the presence of commensal bacteria in the uterus. This review will focus on the immune system that characterizes the endometrium, the microbiota present in the uterine microenvironment, and their mutual homeostatic relationship.

ANATOMY OF THE UTERUS

The female reproductive tract comprises the fallopian tubes, the uterus, and the vagina.

The uterus is the organ involved in the gestation; it has the function of accepting the fertilized egg and allowing its development. The uterus is an unequal organ, which at the top receives the outlet of the uterine tubes and at the bottom opens into the vaginal cavity (6–8).

The uterus, a pear-shaped viscus, can be divided into a fundus, body, and cervix. During pregnancy, it houses and supports the developing embryo and fetus. It is composed of a thick, muscular myometrium (covered by serosa and/or adventitia) and a spongy mucosal layer, the endometrium. The cervix is the inferior portion of the uterus, which protrudes into the vagina. The lumen (canal) of the cervix is continuous with the lumen of the uterus (superiorly) and the vaginal canal (inferiorly). It is divided into two main parts: the endocervix is the inner part of the cervix lining the canal leading into the uterus, whereas the ectocervix is the outer part of the cervix. It is rounded and lip-like and sticks out into the vagina. The endocervical canal is the passage from inside the uterus to the vagina (6–8). The superior surface of the uterus is convex and directed forward. The anterior surface is flat and looks downward and forward, resting on the bladder. Its peritoneal covering is reflected at the level of isthmus to the upper aspect of the bladder, creating the vesico-uterine pouch. The posterior surface of the uterus is convex and lies in relation to the pelvic colon and rectum. The peritoneum of the posterior wall covers the body and upper cervix, and then extends over the posterior fornix of the vagina to the rectum, to form the recto-uterine pouch or cul-de-sac of Douglas. The cervix is directed downward and backward to rest again the posterior wall. Only the upper half of its posterior surface is covered by peritoneum. The external side of the cervix lies at about the level of the upper border of the symphysis pubis in the plane of ischial spine (6–8).

The uterine wall is constituted by mucous or endometrium tunic, muscular or myometrium tunic, and serous tunic, where it is referred to as perimetry. A connective tissue constitutes the perimeter that surrounds the uterus, below the peritoneum, extending into the base of the broad ligament (6–8).

The endometrium, composed of an epithelial lamina propria, with its superficial functional and deep basal layers, undergoes hormonally modulated cyclic changes during the menstrual cycle. The three cyclic stages of the endometrium are the: follicular (proliferative) phase, luteal (secretory) phase, and the menstrual phase. The basal layer, which remains intact during menstruation, is served by short straight arteries and is occupied by the base of the uterine glands. The functional layer, served by the helicine (coiled) arteries, undergoes hormonally modulated cyclic changes. Follicle-stimulating hormone (FSH) facilitates the proliferative phase (follicular phase), characterized by thickening of the endometrium and the renewal of the connective tissue, glandular structures and blood vessels (helicine arteries) subsequent to the menstrual phase. Luteinizing hormone (LH) facilitates the secretory phase (luteal phase), characterized by the further thickening of the endometrium, coiling of the endometrial glands, accumulation of glandular secretions, and further coiling and lengthening of the helicine arteries. Decreased levels of LH and progesterone are responsible for the menstrual phase, which begins with long-term, intermittent vasoconstriction of the helicine arteries, with subsequent necrosis of the vessel walls as well as of the endometrial tissue of the functional layer. It should be noted that the basal layer is unaffected because it is supplied by the straight arteries. During relaxation (between episodes of vasoconstriction), the helicine arteries rupture, and the rapid blood flow dislodges the blood-filled necrotic functional layer, which becomes sloughed as the hemorrhagic discharge, so that only the basal layer of the endometrium remains as the lining of the uterus (7, 9).

CHARACTERISTICS OF THE MUCOSA OF THE FEMALE REPRODUCTIVE TRACT

As mentioned earlier, the female reproductive tract comprises the fallopian tubes, the uterus, and the vagina. The mucosa of the female reproductive tract differs between the upper and the

lower tracts. The upper female reproductive tract that includes the uterine tubes, uterus and the endocervix, and is covered by a single-layered columnar epithelium. The lower reproductive tract includes the ectocervix and vagina, and is lined with a single layer of stratified, squamous, non-keratinized epithelium, which forms a more protective barrier than the columnar epithelium (10, 11).

Anti-microbial molecules, produced in the mucosa of the female reproductive tract, are significantly influenced by estrogen, which acts differently in the upper and lower reproductive tracts. High levels of estrogen, a characteristic of the pre-ovulatory period, increase the production of some anti-microbial peptides, such as secretory leukocyte peptidase inhibitor (SLPI), β-defensin 1-2 (HBD 1-2), and elafin from the endometrial epithelium (12). On the other hand, estrogen suppresses the LPS- and poly (I:C)-induced secretion of pro-inflammatory cytokines, including TNF-α, macrophage inflammatory protein 3α (MIP3α, CCL20), IL-1β, IL-6, and IL-8 from uterine epithelial cells. IgG levels in the uterine cavity peak in the periovulatory period. In contrast, the lower reproductive tract, including the cervix and the vagina, maintains a low level of IgA, IgG, and lactoferrin and decreased secretions of β-defensin 2, elafin, SLPI, and α-defensin1-3 (HNP1-3). Furthermore, concentrations of IL-6 and IL-8 in the vagina are decreased in the period of highest estrogen concentration (12). This decreased immunity in the lower genital tract may contribute to enhanced sperm survival in the periovulation period. In addition, the estrogen rise in the periovulatory period increases the levels of anti-microbial peptides and decreases pro-inflammatory cytokines in the upper genital tract, which prevents ascending infections by vaginal pathogens and provides favorable conditions for the transit of sperm and embryos.

UTERINE MICROBIOTA

The endometrium of healthy women, for a long time, has been considered as a sterile environment. However, the use of next-generation sequencing techniques has enhanced our understanding of the complexity and number of microbiota that inhabit human mucosal surfaces. Recently, it has been demonstrated that the sites of the body that were previously thought to be devoid of bacteria, such as the lungs (13), bladder (14), placenta and endometrium (15, 16), are in fact colonized by low-abundance and unique microbiota (17), each tissue and microenvironment hosting unique microbial communities (18).

The first study that provided information about uterine colonization by bacteria used endometrium of 78 patients after hysterectomy. The authors were able to show the presence of pathogens in culture in 6% of these patients. They also demonstrated the presence of carcinoma of the cervix but no other pathologic condition may predispose to microbial invasion of the uterine cavity (19).

The presence of microbes in the uterine cavity was later demonstrated by Moller et al. (20). A prospective study involved the uteri from 99 women admitted for hysterectomy for persistent irregular vaginal bleeding and fibromyomas. The uteri were opened under sterile conditions immediately after hysterectomy and samples were obtained from the isthmus and fundus of the uterine cavity for microbiological examination. In nearly a quarter of all the patients, the uterine cavity was colonized with potentially pathogenic organisms, harboring one or more microorganisms in the uterus, most notably *Gardnerella vaginalis, Enterobacter*, and *Streptococcus agalactiae*. Mitchell et al. recently evaluated the presence of vaginal bacterial species by species-specific quantitative PCR in excised uteri obtained from 58 patients (21). They detected mostly the presence of *Liners, Prevotella* spp. *and L crispatus* with the median quantities of bacteria 2–4 $\log_{10}$ lower those that present in the vagina. Recent studies via next generation sequencing have yielded important information about the presence of many genera of bacteria in the non-pregnant human uterus.

An interesting study by Moreno et al. analyzed endometrial fluid and vaginal aspirates obtained from 13 fertile women in pre-receptive and receptive phases within the same menstrual cycle (22). They also investigated endometrial fluid collected at pre-receptive and receptive phases from 22 fertile women to study the hormonal regulation of the endometrial microbiota. Genomic DNA was sequenced for the 16S ribosomal RNA (rRNA) gene. The data demonstrated that the presence of endometrial microbiota was not hormonally regulated during the acquisition of endometrial receptivity. Furthermore, the presence of a non-lactobacillus-dominated microbiota in a receptive endometrium was associated with significant decrease in implantation and pregnancy outcome.

Chen et al. analyzed the microbiota present in the lower vagina, posterior fornix, cervical mucus, endometrium, fallopian tubes, and peritoneal fluid obtained from the pouch of Douglas in women being operated for benign and non-infectious conditions (23). A significant fraction of the non-pregnant uterine microbiota comprises not only Lactobacillus spp., which is similar to the vaginal mucosa, but also a variety of bacteria that grow in mildly alkaline conditions (23). Although characterized by a lower bacterial biomass, endometrial samples have a higher bacterial diversity compared to the vaginal microbiota (24). The metabolic profiles of the vagino-uterine microbiota change throughout the menstrual cycle. The functions of these microbes in uterine mucosal immune homeostasis in non-pregnant and pregnant states remain to be ascertained (1).

Few studies have attempted to investigate the correlation between bacterial colonization and pregnancy outcome, by culturing catheter tips used for embryo transfer during *in vitro* fertilization. The results were not conclusive, probably due to the fact that in culture, the aerobic species dominate, in addition to potential contamination through the cervicovaginal canal (25).

IMMUNE CELLS RESIDING IN THE FEMALE REPRODUCTIVE TRACT

There are conflicting reports in terms of the leukocyte population in the female reproductive tract. This discrepancy arises from the differences in the sampling phase during the menstrual cycle, sample size, analytical methods, and antibodies used to identify immune cells (10). However, it is clear that the number of

leukocytes per gram of endometrial tissue is greater than that of other reproductive tissues, including fallopian tube, endocervix, ectocervix, and vagina (10).

Immune cells residing in the reproductive tract are required to play paradoxical roles since they maintain immunity against pathogens and also establish immune tolerance for sperm and embryo/fetus in the upper tract (10). Natural killer (NK) cells and regulatory T (Treg) cells are extremely important in decidual angiogenesis, trophoblast migration, and immune tolerance during pregnancy (10). Dysregulation of endometrial/decidual immune cells is strongly associated with infertility, miscarriage, and other obstetric complications (10).

The leukocytes in the female reproductive tract are distributed in either an aggregated or a dispersed form in the epithelial layer, lamina propria, and stroma (10). Although differentially distributed in each organ of the female reproductive tract, the predominant immune cells are T cells, macrophages/dendritic cells, NK cells, neutrophils, and mast cells (10). B cells are rare in the female reproductive tract (10) (**Figures 1**, **2**).

Sex steroid hormones significantly influence these cells although they lack receptors for estrogen and progesterone (12, 26). As in the peripheral blood, leukocytes in the female reproductive tract also fluctuate in number cyclically through the menstrual cycle, probably as an indirect response to estrogenic and progesterone (12). The importance of these uterine immune cells is being discussed below.

Innate Lymphoid Cells (ILCs)

Innate lymphoid cells (ILCs) are divided into three primary groups based on their phenotype and functions: type 1 (ILC1), type 2 (ILC2), and type 3 (ILC3) (27). Recently, the classification of ILCs has been be expanded to five subsets to reflect their distinct developmental pathways: NK cells, ILC1, ILC2, ILC3, and lymphoid tissue inducer (LTi) cells (28). It has been shown that only ILC3s are present in human endometrium and decidua, whereas all three groups of ILCs are present in the mouse uterus (29). The differences in the distribution of ILCs between human and mouse may reflect the dramatic changes that the mucosa undergoes in the human menstrual cycle with cyclical degeneration and renewal (29).

ILC1 include the prototypical NK cells and non-cytotoxic IFN-γ producing ILC1, characterized by expression of the transcription factor T-bet (27). ILC1s (Lin-CD56$^+$CD127$^-$CD117$^-$RORγt$^-$cells) are found in human non-pregnant endometrium, are further distinguished on the basis of their expression of NKp44 and CD103. CD103$^+$NKp44$^-$ cells are the principal source of IFN-γ (30), whereas, as previously described for tonsillar ILC1s (31), CD103$^+$ expression facilitate the molecular communication between lymphocytes and epithelial cells (32), suggesting an epithelial association of such cells in the uterus.

ILC2 function through the release of helper T cell type 2 (Th2) cytokines such as IL-5 and IL-13 (33) and participate in immune responses, for example, against parasitic infection (34) and allergy (35); they also serve as systemic regulators of homeostasis (36). Most of the information about these cells are based on mouse models. For instance, murine uterine ILC2 are able to express the estrogen receptor α (37) and their percentage is increased in response to *in vitro* stimulation with 17β-estradiol.

ILC3 are divided into two main groups: LTi cells and non-LTi ILC3, referred to hereafter as ILC3 (27). For the first time, ILC3 have been described in the human non-pregnant endometrium.

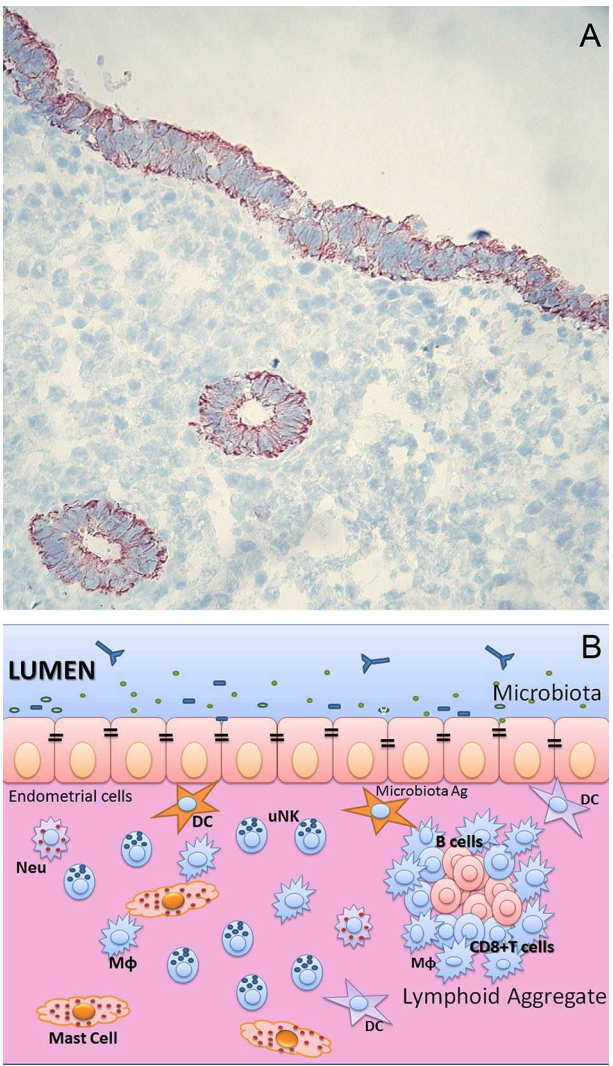

FIGURE 1 | (A) Endometrium of human uterus. Immunohistochemical analysis of human endometrium on frozen section stained with CK7 monoclonal antibody (Dako) of uterus in proliferative phase. Bound antibody was revealed using the LSAB+ HRP kit and 3,3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen. The sections were counterstained with hematoxylin. Monostratified columnar epithelium and the basal layer, which remains intact during menstruation, is occupied by the base of the uterine glands. Original magnifications 200×. **(B)** Immunological components of uterine mucosa. The endometrium is populated by a range of immune cells, such as mast cells, Macrophages (MΦ), Neutrophils (Neu), Dendritic cells (DC), T and B cells. The presence of lymphoid aggregates in the endometrial tissue suggests that this is an active site for cell-mediated immunity. Lymphoid aggregates found beneath the endometrium are composed of B cells in the inner core, surrounded by CD8$^+$ CD4$^-$ T cells and an outer layer of macrophages. Scattered CD56$^+$ natural killer (NK) cells and CD4$^+$ T cells can also be found in between lymphoid aggregates.

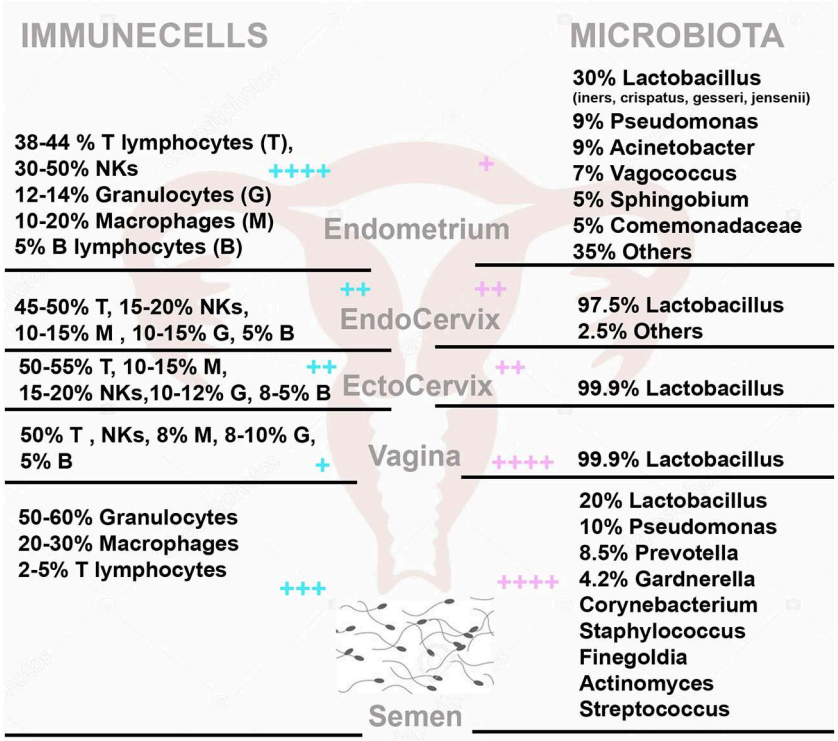

FIGURE 2 | The immune cells **(Left)** and microbiota **(Right)** within the female genital tract and semen. On the left side are the percentage with (respect to total CD45 positive cells) of T Lymphocytes (T), NK cells (NKs), Granulocytes (G), Macrophages (M), and B Lymphocytes (B) in Endometrium, Endocervix, Ectocervix, Vagina and semen. On the right side, is shown the median relative abundance of microbial genera. + + ++/+ represent the relative abundance amount of microorganisms and immune cells in different areas.

They represent a distinct subset of NK precursor-like cells that present ILC-associated markers such as CD127 and CD161 (38). The expression of the RORC and IL-22 genes that characterize an ILC3 phenotype, was also demonstrated (38). Subsequently, other studies confirmed the presence of ILC3 in the human endometrium (37, 38). These cells are divided into two main subsets: NCR^- (human $NKp44^-$; mouse $NKp46^-$) and NCR^+ (human $NKp44^+$; mouse $NKp46^+$) ILC3s (7), with the NCR^- ILC3 being the dominant population in mice and the NCR^+ ILC3 in humans. Apart from the NK cells, the potential role of other ILCs in the uterus is not fully known.

Uterine NK Cells

Uterine NK cells have also been called as Large Granular Lymphocytes (LGL), endometrial granulocytes, K cells, endometrial Granulated Lymphocytes (eGL), and decidual Granulated Lymphocytes (dGL) (39). The major phenotype of endometrial NK cells is $CD3^-$ $CD56^{bright}$ $CD16^-$, which distinguishes this cell subset from $CD3^-$ $CD56^{dim}$ $CD16^+$ NK cells in the peripheral blood (10, 39). It is possible that peripheral blood $CD56^{bright}$ NK cells home in to the uterus where they undergo tissue-specific differentiation. NK cells in the endometrium significantly expand in the late secretory phase and further increase their number during early pregnancy (10, 26).

In the proliferative phase, only a few NK cells are scattered throughout the stroma of the functional layer. However, they show a dramatic increase in their number after ovulation and continue to increase until a few days prior to menstruation. In the late secretory phase, the NK cells number surges up to 40% of the total cells in the stromal compartment and ∼70% of endometrial leukocytes (10, 39). NK cells, particularly, surround both the arteries and glands of the endometrium. Since there are few NK cells in the endometrium in the menstrual and proliferative phases, an association between falling progesterone and apoptosis of uterine NK cells has been suggested. Considering that the NK cells and other leukocytes in the uterus do not express progesterone receptors, progesterone might exert its effects indirectly via cytokines or other soluble factors produced by stromal cells. Stromal cells strongly express both estrogen receptors (ERs) and progesterone receptors (PRs) (39). Carlino et al. demonstrated that uterine stromal cells from fertile or menopausal women were able to release chemerin, and exposure of uterine stromal cells to progesterone and 17β-estradiol resulted in enhanced chemerin release, supporting peripheral blood NK cell migration through uterine stromal cells (40).

Macrophages

$CD68^+$ macrophages are detected in all phases of the menstrual cycle (41). These cells also express specific markers such as CD71, CD69, and CD54 (42). These macrophages are scattered throughout the endometrium and are found especially around the glands (43). Tissue macrophages acquire distinct functional

phenotypes, ranging between pro-inflammatory (M1) and anti-inflammatory pro-wound healing (M2) phenotype, in response to environmental cues (44). Studies using human tissues have revealed a progressive increase in the number of macrophages during the secretory phase of the cycle that peaks during menstruation, representing up to ∼15% of the leukocyte population (45). $CD68^+$ macrophages are abundant in the human endometrium during tissue breakdown and repair, and probably have a role in tissue clearance and tissue remodeling associated with menstruation (46). Macrophages within the endometrium have been suggested to have an important role in fertility and induction of a pro-inflammatory cytokine production (42). There is a significant increase in the macrophage number in the secretory phase, especially prior to menses, and this increase is particularly notable at the implantation site (39, 47).

Neutrophils

Neutrophils, the most abundant leukocytes in the human immune system, comprise about ∼10% of $CD45^+$ cells in the cervix, with a greater presence in the ectocervix (11). Neutrophils have been identified in endometrial tissue by their morphology, by immunolocalization of the neutrophil-specific protease, elastase; in terms of surface markers, they have been defined as CD11b bright, $CD66b^+$, and $CD16^+$ (48). During most of the cycle, neutrophils are barely detectable in normal endometrium, but the numbers rise dramatically perimenstrually, making upto 6–15% of the total cell number in the tissue (41).

IFN-γ has also been detected in intra-epithelial neutrophils in human endometrium (48). In fact, neutrophils isolated from normal donors produce IFN-γ in response to stimulation with LPS, IL-12, and TNF-α. IFN-γ has a role in macrophage activation (49), suggesting at least one potential interaction between adjacent leukocytes during menstruation.

Mast Cells

Mast cells are present in the endometrium throughout the menstrual cycle. They are detected by immunostaining for the two-mast cell-specific serine proteinases, tryptase, and chymase, and the extracellular relocation of the enzymes is indicative of their activation state (41).

Endometrial mast cell activation corresponds closely to the phases of tissue edema and is most marked prior to menstruation (50). As mentioned above, mast cells fall into two well-known phenotypic subtypes. Mucosal mast cells are positive for tryptase but not chymase, whereas connective tissue mast cells contain both enzymes. Both of these subtypes have been detected in endometrium, with regional differences. Those in the basalis region express both enzymes whereas those in the functionalis are positive only for tryptase (50). While it is the functionalis that is shed during menstruation, there is considerable tissue destruction at the basalis functionalis interface, and it is likely that both enzymes contribute to menstruation. Tryptase, and to a lesser extent, chymase, can play an important role in establishing a cascade of matrix metalloproteinase (MMP) activation and this could represent a critical function for these cells in the menstruation. Mast cells also produce histamine, heparin, arachidonate products and a variety of pleiotropic cytokines and growth factors, all of which have marked effects on endothelial cell function and local induction of edema (51).

Dendritic Cells

Dendritic cells (DCs) are a heterogeneous and dynamic population of leukocytes; they are the most potent antigen capturing (immature DCs) and antigen presenting (mature DCs) cells. They are highly involved in the stimulation and modulation of the immune response within mucosal surfaces (52). In the basal layer of the endometrium, the density of endometrial $CD1a^+$ immature DCs is significantly higher than that of $CD83^+$ mature DCs throughout the menstrual cycle, whereas the number of $CD83^+$ mature DCs remain relatively constant (39, 53). The number of $CD1a^+$ immature DCs is likely to be indirectly regulated by steroid hormones (53). The density of $CD83^+$ DCs was significantly greater in the basal layer compared with the functional layer during both the proliferative and secretory phases, whereas for $CD1a^+$ DCs, the greater density in the basal layer was only observed in the secretory phase (53). Cyclical changes in DC populations during the normal menstrual cycle may be important for local regulatory mechanisms relevant to menstruation and implantation. Alterations in this normal profile may contribute to menstrual disturbances and fertility.

Lymphocytes

$CD3^+$ T cells are a minor population in the endometrium, comprising only ∼1–2% of the total lymphomyeloid cells (45). These T cells are distributed at three different sites of the endometrium: aggregated in the basal lymphoid, and scattered in the stroma, and in the epithelial sites (41). In contrast to $CD3^+$ T cells in peripheral blood, endometrial $CD3^+$ T cells consist of a larger proportion of $CD8^+$ cells (66%) and smaller proportion of $CD4^+$ cells (33%) (41). Cytolytic activity of endometrial $CD8^+$ T cells is maintained during the proliferative phase, but this activity weakens in the secretory phase without any drop in the $CD8^+$ cell number (12, 41). This suppression of $CD8^+$ cytolytic activity has been observed only in the fallopian tubes and endometrium, but not in the cervix and vagina (12). $CD45RA^+$ T cells were found throughout the cycle, but their numbers are very low (41).

Hormonal changes may be drivers for Treg changes. In particular, estrogen has been shown to induce expansion of $Foxp3^+$ Treg cells (54, 55), including in the pregnant uterus (56), which is essential for promoting immune tolerance toward the fetus. Activation of Treg is needed for a successful pregnancy, while suppression of Treg was associated with pregnancy failure (56).

Gamma/delta (γδ) T Cells

Two types of T cells, αβ T cells and γδ T cells are present in vertebrates and are defined by surface expression of either the αβ or the γδ TCR complex. Thymus is the origin tissue of both these subsets but there are several key differences between αβ and γδ T cells: (i) αβ T cells are primarily localized in secondary lymphoid organs, whereas γδ T cells are predominant at epithelial surfaces; (ii) αβ T cells recognize peptide ligands in the context of MHC class I and class II molecules, whereas γδ T cells do not recognize peptides in an MHC-restricted manner but recognize and respond to a broad range of antigens, including heat shock

proteins, and lipids; and (iii) the role of αβ T cells in immune response is well-defined whereas that of γδ T cell are not yet fully characterized (57).

Haller et al. analyzed the presence of γδ-positive cells in non-pregnant endometrium, and in first trimester and third trimester basal decidua. They did not observe any substantial differences in the number of γδ T cells in first and third trimester basal decidua compared with the non-pregnant endometrium. γδ T cells were occasionally found scattered throughout the endometrial/decidual stroma and showed no clear distribution pattern, nor was there any obvious relation to the pregnancy in the first or third trimester (58). Although Heyborn et al. showed that γδ T cells were significantly enriched at the feto-maternal interface, compared to the periphery or the non-pregnant uterus (59), the role of these lymphocytes in pregnancy is not yet fully understood. Hill et al. observed an increased number of TcRγδ-positive cells in spontaneous miscarriages endometrium compared to normal pregnancies (60). Vassiliadou and Bulmer showed that γδ-T cells were present in both normal pregnancy and spontaneous pregnancy loss, often located adjacent to endometrial glands (61). These cells formed a very small proportion of T cells in both groups and there was no difference in their numbers and proportions between normal and pathological tissues.

ENDOMETRIAL LYMPHOID AGGREGATES

Endometrial lymphoid aggregates comprise a $CD19^+$ B cell core, surrounding T cells, and an outer halo of macrophages in the stratum basalis (12, 39). These lymphocyte aggregates vary in size with the phase of the menstrual cycle, becoming larger in the secretory phase (3,000∼4,000 cells) than the proliferative phase (300∼400 cells) (12). To form lymphoid aggregates, immune cell trafficking to the nucleation sites is likely to be the principle mechanism, rather than *in situ* proliferation of precursor cells (62). Recruited immune cells from the circulation are likely to undergo tissue-specific differentiation in the local microenvironment, which confers new characteristics on these tissue-specific cells that are distinct from their original properties (39, 63).

CYCLIC FLUCTUATIONS OF ENDOMETRIAL LEUKOCYTES

Endometrial leukocytes exhibit profound cyclic fluctuations between the follicular and luteal phases of the menstrual cycle (10). The mean number of $CD45^+$ cells in the endometrium remains low from the early follicular phase to the early secretory phase, but increases considerably (about 5-fold) during the secretory phase (64). The major population of $CD45^+$ leukocytes in the late secretory phase is NK cells comprising ∼80% of $CD45^+$ cells, while $CD3^+$ T cells decrease to <10% (64). Even though the percentage of $CD3^+$ T cells is lower in the late secretory phase, the absolute number of T cells remained unchanged during the menstrual cycle. Comparing the late proliferative phase with the late secretory phase, the proportion of $CD3^+$ $CD8^+$ T cells decreases significantly from 63 to 54% (64) (**Figures 2**, **3**).

The number of $CD3^-$ $CD56^+$ NK cells significantly increases in the late secretory phase compared with other phases (64). The cytotoxicity of endometrial NK cells is high, comparable to peripheral NK cells in the late proliferative phase (65). Similarly, the expression of the activation markers, CD69 and HLA-DR, is increased on NK cells in the proliferative phase (66). These characteristics of NK cells are likely to contribute to protective immunity against microbial infections.

During the menstrual cycle, neutrophils and eosinophils remain at a very low number in the endometrium, but their numbers profoundly increase in the premenstrual period, up to 15% and 5% of endometrial cells, respectively (41, 45).

$CD68^+$ macrophages were found throughout the cycle and significantly increased from the proliferative phase to the secretory phase (67). The density of endometrial $CD1a^+$ DCs, but not $CD83^+$ DCs, in the basal layer gradually increased through the menstrual cycle, showing a nadir at the proliferative phase and reaching its peak in the menstrual phase (53). Only in the secretory phase was the density of $CD1a^+$ DCs greater in the basal layer than in the functional layer. Endometrial $CD83^+$ DCs showed greater density in the basal layer than in the functional layer throughout the menstrual cycle (53).

ROLE OF CERVICAL MUCUS IN BACTERIAL UTERINE COLONIZATION

The portal between the uterus and the vagina is represented by the cervix, which functions as an entrance to the endometrial cavities. Cervix contains several hundred crypts (glands) lined by cells which, under hormonal and neural influence, produce mucus, composed mostly of water (95–99%) and a complex mixture of organic components, inorganic ions, enzymes, mucins, and a high concentration of cytokines, anti-microbial peptides, immunoglobulins, and MMPs to protect the uterus against bacterial colonization (68, 69). Mucus released into the cervical canal moves into the vagina, where it acts as pathogen traps. It is an important protective barrier, preventing the ascendance of microorganisms into the uterus cavity; on the other hand, it is essential for the migration of spermatozoa. It has been shown that the hydration of the mucus and its glycosylation state play a pivotal role in both these processes (70, 71). Mucins in the cervix are known to change conformation during the menstrual cycle due to pH; these variations possibly allow passage of bacteria from vagina (72). However, bacteria from the lower female reproductive tract are able to cross. Hansen et al. have shown that the cervical mucus plug only inhibits (and does not completely block) the passage of *Ureaplasma parvum* during its ascendance from the vagina through the cervical canal (17, 73).

ROLE OF SEMEN IN BACTERIAL UTERINE COLONIZATION

Besides all the putative bacterial transmission routes between uterine microbiota and distal sites such as hematogenous spread

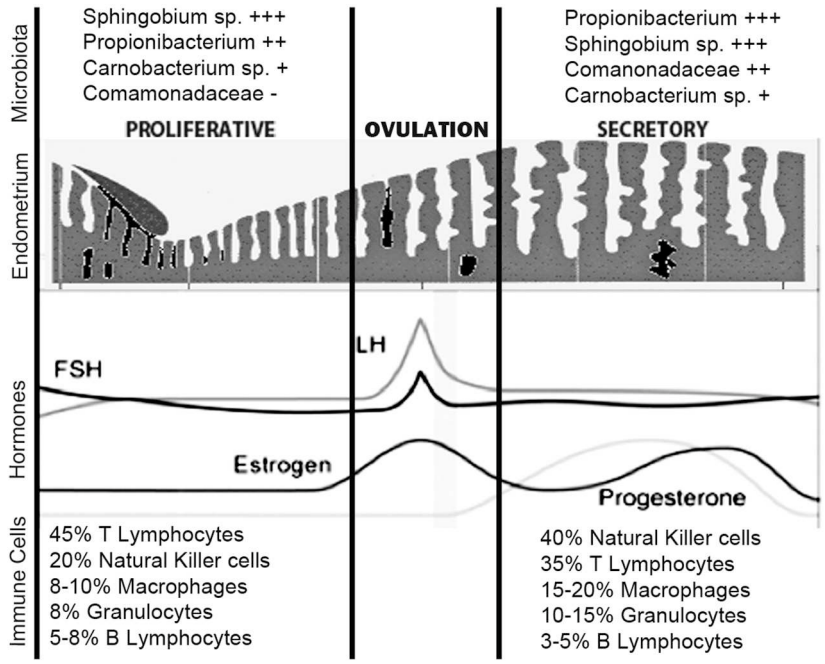

FIGURE 3 | Uterine immune stages during menstruation cycle. During the normal menstrual cycle, the human endometrium is exposed to cyclical fluctuations of sex hormones. The repetitive cycles of proliferation, differentiation, decidualization, and shedding of this tissue during mensuration and the steroid hormones *per se* cause profound changes in the immune cells' population. The estrogen-dominant proliferative phase is characterized by the regeneration of the functional layer of the endometrium. In the progesterone driven secretory phase, the endometrium undergoes a number of changes in preparation for implantation of the embryo. The immune cells that undergo greater number variations in the secretory phase are the NKs and macrophages. The microbiota, on the other hand, does not undergo large variations during different phases of the menstrual cycle. *Sphingobium* sp., *Propionibacterium* acnes, and *Pseudomonas* sp. are differentially enriched during the proliferative and secretory phases; *P. acnes* is more abundant in the secretory phase and has previously been identified in the placenta and cultured from follicular fluid. Functionally, the proliferative phase, compared to the secretory phase, appears associated with increased bacterial proliferation. + + ++/+ represent the relative abundance amount of microorganisms.

of bacteria through either oral or gut route, migration of bacteria through the cervix, and retrograde spread through fallopian tubes, another possible transmission route that influences uterine microbiota is the semen (24, 69). It is essentially composed of the spermatozoa cells and seminal plasma, which contains many components contributing to the immune tolerance at foeto-maternal interface (74). Semen, despite being synthesized in a tissue that is not typically colonized by a microbiota, is very far from being sterile; even in normal individuals, usually the microbial count is 10^3 organisms/mL semen (75). Weng et al. showed that the most abundant genera present in seminal fluid are *Lactobacillus* (19.9%), *Pseudomonas* (9.85%), *Prevotella* (8.51%), and *Gardnerella* (4.21%) (**Figure 2**). Unsupervised clustering analysis revealed that the seminal bacterial communities were clustered into three main groups: *Lactobacillus*, *Pseudomonas*, and *Prevotella* predominant group (76). For this reason, the seminal fluid is a microorganism source for female genital tract. Indeed, it has been shown that bacteria are shared among partners influencing the species composition of each other's reproductive tract microbiota (24). Semen, being slightly basic and enriched with carbohydrates, represents an ideal habitat for microorganisms so that it may function as a perfect medium for the transmission of microorganisms, which finally may reach the uterus and become resident.

PERSPECTIVES

Mucosal barriers are the first line of immune defense against the external environment and one major benefit resulting from the homeostatic relationship between the host with the commensal microbiota is the resistance to pathogen colonization (4). Although the uterine microenvironment has been considered a sterile compartment in the past, it is now clear that some commensal bacteria are present in the uterus. Therefore, it is possible to hypothesize a cross-regulation of the uterine microbiota and the local immune system. The uterine microbiota and the uterine immune system have to adapt to the functional changes of the uterus: first of all, they have to adapt to the cyclic hormonal stimulation during the menstrual cycle implantation and pregnancy. The uterine microbiota and the uterine immune cells must together tolerate the paternal antigens in response to the immunological stimulation by the semen, the embryo and the fetus.

We have provided an overview of the immunological order in an healthy endometrium since the recognition of the changes in the local microbial communities may point toward reproductive failure at different levels (from implantation failure in the uterus to the pregnancy complications), as well as other gynecological diseases (10, 11, 77). Furthermore, alteration of the uterine

microbiota can also be a potential trigger for tumorigenesis. It is well-known that the etiology and progression of cancer may be influenced by microbial infections, as is *Helicobacter pylori* for gastric cancer, and *Fusobacteria* and *Porphyromonas* for colorectal cancer. It has been suggested that the presence of *Atopobium vaginae* and *Porphyromonas* sp. in the gynecologic tract is statistically associated with endometrial cancer (78).

It is an emerging notion that the microbiota is able to influence the function of the host immune system, which keeps a symbiotic relationship with the microbiota. Normally in other mucosa, such as the intestinal mucosa, bacteria are confined to the luminal side of the epithelial mucosa but occasionally they breach the physical barrier. These commensal microbes that penetrate the lamina propria are phagocytosed by resident macrophages and engulfed and carried by DCs to the local lymphoid aggregates and consequently modulate the adaptive immune system (79). The nature of interplay between uterine microbiota and the immune cells is not known. One can only speculate that a similar mechanism may work in the case of uterine compartment involving local lymphoid aggregates. The symbiotic relationship between the uterine microbiota and the innate and adaptive immune system probably has a fundamental role in maintaining a balanced inflammatory milieu since this mild bacterial stimulation could induce a potentially favorable microenvironment for embryo implantation. On the other hand, regulated stimulation of the immune system by microbiota could facilitate induction of tolerance to the non-sterile semen traversing through the uterine cavity.

In conclusion, we have discussed the potential role of microbiota in the control of uterine cells present in the microenvironment. We deliberately avoided reviewing and summarizing the literature on the uterine microbiome in animal models because our aim was to focus on human milieu. This can be a limitation since important information on local mechanisms can be obtained only using animal models. For example, it is possible to compare the reproductive performances of germ free and conventional animals and to study the rates of "immune abortion" in germ free or in various conventional breeding conditions. Animal models could also be used for analyzing the potential synergistic effect of different immunological trigger in the presence of bacterial products: this point has been indicated as "ecology of danger" as suggested by DA Clark (80). These studies are however not feasible to carry out in human subjects.

AUTHOR CONTRIBUTIONS

CA, AM, FB, GR, UK, and RB reviewed the literature and wrote sections of the review article. CA and RB created figures. All authors contributed to manuscript revision and approved the submitted version.

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REFERENCES

1. Zhou JZ, Way SS, Chen K. Immunology of uterine and vaginal mucosae. *Trends Immunol.* (2018) 39:302–14. doi: 10.1016/j.it.2018.01.007
2. McGuckin MA, Linden SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. *Nat Rev Microbiol.* (2011) 9:265–78. doi: 10.1038/nrmicro2538
3. Belkaid Y, Harrison OJ. Homeostatic immunity and the microbiota. *Immunity.* (2017) 46:562–76. doi: 10.1016/j.immuni.2017.04.008
4. Schnupf P, Gaboriau-Routhiau V, Cerf-Bensussan N. Modulation of the gut microbiota to improve innate resistance. *Curr Opin Immunol.* (2018) 54:137–44. doi: 10.1016/j.coi.2018.08.003
5. 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. doi: 10.1038/nri3819
6. Netter FH. *Atlas of Human Anatomy*. 7th ed. Philadelphia, PA: Elsevier (2019).
7. Standring S. *Gray's Anatomy : The Anatomical Basis of Clinical Practice*. 41st ed. New York, NY: Elsevier Limited (2016). xviii, 1562 p.
8. Drake RL, Vogl W, Mitchell AWM. *Gray's Atlas of Anatomy*. 2nd ed. Philadelphia, PA: Churchill Livingstone/Elsevier (2015). xvi, 626 p.
9. Hall JE. *Guyton and Hall Textbook of Medical Physiology*. 13th ed. Philadelphia, PA: Elsevier (2016). xix, 1145 p.
10. Lee SK, Kim CJ, Kim DJ, Kang JH. Immune cells in the female reproductive tract. *Immune Netw.* (2015) 15:16–26. doi: 10.4110/in.2015.15.1.16
11. 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. doi: 10.1111/aji.12198
12. Wira CR, Fahey JV, Rodriguez-Garcia M, Shen Z, Patel MV. Regulation of mucosal immunity in the female reproductive tract: the role of sex hormones in immune protection against sexually transmitted pathogens. *Am J Reprod Immunol.* (2014) 72:236–58. doi: 10.1111/aji.12252
13. Dickson RP, Erb-Downward JR, Martinez FJ, Huffnagle GB. The microbiome and the respiratory tract. *Annu Rev Physiol.* (2016) 78:481–504. doi: 10.1146/annurev-physiol-021115-105238
14. Brubaker L, Wolfe AJ. The new world of the urinary microbiota in women. *Am J Obstet Gynecol.* (2015) 213:644–9. doi: 10.1016/j.ajog.2015.05.032
15. Verstraelen H, Vilchez-Vargas R, Desimpel F, Jauregui R, Vankeirsbilck N, Weyers S, et al. Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16S rRNA gene. *PeerJ.* (2016) 4:e1602. doi: 10.7717/peerj.1602
16. Franasiak JM, Werner MD, Juneau CR, Tao X, Landis J, Zhan Y, et al. Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. *J Assist Reprod Genet.* (2016) 33:129–36. doi: 10.1007/s10815-015-0614-z
17. Laniewski P, Gomez A, Hire G, So M, Herbst-Kralovetz MM. Human three-dimensional endometrial epithelial cell model to study host interactions with vaginal bacteria and *Neisseria gonorrhoeae*. *Infect Immun.* (2017) 85:e01049-16. doi: 10.1128/IAI.01049-16
18. Belkaid Y, Naik S. Compartmentalized and systemic control of tissue immunity by commensals. *Nat Immunol.* (2013) 14:646–53. doi: 10.1038/ni.2604

19. Ansbacher R, Boyson WA, Morris JA. Sterility of the uterine cavity. *Am J Obstet Gynecol.* (1967) 99:394–6. doi: 10.1016/S0002-9378(16)34549-5
20. Moller BR, Kristiansen FV, Thorsen P, Frost L, Mogensen SC. Sterility of the uterine cavity. *Acta Obstet Gynecol Scand.* (1995) 74:216–9. doi: 10.3109/00016349509008942
21. Mitchell CM, Haick A, Nkwopara E, Garcia R, Rendi M, Agnew K, et al. Colonization of the upper genital tract by vaginal bacterial species in nonpregnant women. *Am J Obstet Gynecol.* (2015) 212:611 e1–9. doi: 10.1016/j.ajog.2014.11.043
22. Moreno I, Codoner FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazan J, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am J Obstet Gynecol.* (2016) 215:684–703. doi: 10.1016/j.ajog.2016.09.075
23. Chen C, Song X, Wei W, Zhong H, Dai J, Lan Z, et al. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat Commun.* (2017) 8:875. doi: 10.1038/s41467-017-00901-0
24. Altmae S. Commentary: uterine microbiota: residents, tourists, or invaders? *Front Immunol.* (2018) 9:1874. doi: 10.3389/fimmu.2018.01874
25. Benner M, Ferwerda G, Joosten I, van der Molen RG. How uterine microbiota might be responsible for a receptive, fertile endometrium. *Hum Reprod Update.* (2018) 24:393–415. doi: 10.1093/humupd/dmy012
26. 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. *J Immunol.* (2010) 185:756–62. doi: 10.4049/jimmunol.0904192
27. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells–a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–9. doi: 10.1038/nri3365
28. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell.* (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
29. Doisne JM, Balmas E, Boulenouar S, Gaynor LM, Kieckbusch J, Gardner L, et al. Composition, development, and function of uterine innate lymphoid cells. *J Immunol.* (2015) 195:3937–45. doi: 10.4049/jimmunol.1500689
30. Montaldo E, Vacca P, Chiossone L, Croxatto D, Loiacono F, Martini S, et al. Unique Eomes(+) NK cell subsets are present in uterus and decidua during early pregnancy. *Front Immunol.* (2015) 6:646. doi: 10.3389/fimmu.2015.00646
31. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. *Immunity.* (2013) 38:769–81. doi: 10.1016/j.immuni.2013.02.010
32. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, et al. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature.* (1994) 372:190–3. doi: 10.1038/372190a0
33. Miller D, Motomura K, Garcia-Flores V, Romero R, Gomez-Lopez N. Innate lymphoid cells in the maternal and fetal compartments. *Front Immunol.* (2018) 9:2396. doi: 10.3389/fimmu.2018.02396
34. Fallon PG, Ballantyne SJ, Mangan NE, Barlow JL, Dasvarma A, Hewett DR, et al. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med.* (2006) 203:1105–16. doi: 10.1084/jem.20051615
35. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity.* (2012) 36:451–63. doi: 10.1016/j.immuni.2011.12.020
36. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol.* (2011) 12:1045–54. doi: 10.1038/ni.2131
37. Bartemes K, Chen CC, Iijima K, Drake L, Kita H. IL-33-responsive group 2 innate lymphoid cells are regulated by female sex hormones in the uterus. *J Immunol.* (2018) 200:229–36. doi: 10.4049/jimmunol.1602085
38. Male V, Hughes T, McClory S, Colucci F, Caligiuri MA, Moffett A. Immature NK cells, capable of producing IL-22, are present in human uterine mucosa. *J Immunol.* (2010) 185:3913–8. doi: 10.4049/jimmunol.1001637
39. King A. Uterine leukocytes and decidualization. *Hum Reprod Update.* (2000) 6:28–36. doi: 10.1093/humupd/6.1.28
40. Carlino C, Trotta E, Stabile H, Morrone S, Bulla R, Soriani A, et al. Chemerin regulates NK cell accumulation and endothelial cell morphogenesis in the decidua during early pregnancy. *J Clin Endocrinol Metab.* (2012) 97:3603–12. doi: 10.1210/jc.2012-1102
41. Salamonsen LA, Lathbury LJ. Endometrial leukocytes and menstruation. *Hum Reprod Update.* (2000) 6:16–27. doi: 10.1093/humupd/6.1.16
42. Thiruchelvam U, Dransfield I, Saunders PT, Critchley HO. The importance of the macrophage within the human endometrium. *J Leukoc Biol.* (2013) 93:217–25. doi: 10.1189/jlb.0712327
43. Song JY, Fraser IS. Effects of progestogens on human endometrium. *Obstet Gynecol Surv.* (1995) 50:385–94. doi: 10.1097/00006254-199505000-00026
44. Rami D, La Bianca M, Agostinis C, Zauli G, Radillo O, Bulla R. The first trimester gravid serum regulates procalcitonin expression in human macrophages skewing their phenotype *in vitro*. *Mediators Inflamm.* (2014) 2014:248963. doi: 10.1155/2014/248963
45. Salamonsen LA, Woolley DE. Menstruation: induction by matrix metalloproteinases and inflammatory cells. *J Reprod Immunol.* (1999) 44:1–27. doi: 10.1016/S0165-0378(99)00002-9
46. Cousins FL, Kirkwood PM, Saunders PT, Gibson DA. Evidence for a dynamic role for mononuclear phagocytes during endometrial repair and remodelling. *Sci Rep.* (2016) 6:36748. doi: 10.1038/srep36748
47. Salamonsen LA, Zhang J, Brasted M. Leukocyte networks and human endometrial remodelling. *J Reprod Immunol.* (2002) 57:95–108. doi: 10.1016/S0165-0378(02)00011-6
48. Yeaman GR, Collins JE, Currie JK, Guyre PM, Wira CR, Fanger MW. IFN-gamma is produced by polymorphonuclear neutrophils in human uterine endometrium and by cultured peripheral blood polymorphonuclear neutrophils. *J Immunol.* (1998) 160:5145–53.
49. Haddad EK, Duclos AJ, Antecka E, Lapp WS, Baines MG. Role of interferon-gamma in the priming of decidual macrophages for nitric oxide production and early pregnancy loss. *Cell Immunol.* (1997) 181:68–75. doi: 10.1006/cimm.1997.1199
50. Jeziorska M, Salamonsen LA, Woolley DE. Mast cell and eosinophil distribution and activation in human endometrium throughout the menstrual cycle. *Biol Reprod.* (1995) 53:312–20. doi: 10.1095/biolreprod53.2.312
51. Ribatti D, Vacca A, Nico B, Crivellato E, Roncali L, Dammacco F. The role of mast cells in tumour angiogenesis. *Br J Haematol.* (2001) 115:514–21. doi: 10.1046/j.1365-2141.2001.03202.x
52. Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, et al. Dendritic cell subsets in health and disease. *Immunol Rev.* (2007) 219:118–42. doi: 10.1111/j.1600-065X.2007.00551.x
53. Schulke L, Manconi F, Markham R, Fraser IS. Endometrial dendritic cell populations during the normal menstrual cycle. *Hum Reprod.* (2008) 23:1574–80. doi: 10.1093/humrep/den030
54. Prieto GA, Rosenstein Y. Oestradiol potentiates the suppressive function of human CD4 CD25 regulatory T cells by promoting their proliferation. *Immunology.* (2006) 118:58–65. doi: 10.1111/j.1365-2567.2006.02339.x
55. Polanczyk MJ, Hopke C, Vandenbark AA, Offner H. Estrogen-mediated immunomodulation involves reduced activation of effector T cells, potentiation of Treg cells, and enhanced expression of the PD-1 costimulatory pathway. *J Neurosci Res.* (2006) 84:370–8. doi: 10.1002/jnr.20881
56. Tai P, Wang J, Jin H, Song X, Yan J, Kang Y, et al. Induction of regulatory T cells by physiological level estrogen. *J Cell Physiol.* (2008) 214:456–64. doi: 10.1002/jcp.21221
57. Fahl SP, Coffey F, Wiest DL. Origins of gammadelta T cell effector subsets: a riddle wrapped in an enigma. *J Immunol.* (2014) 193:4289–94. doi: 10.4049/jimmunol.1401813
58. Haller H, Radillo O, Rukavina D, Tedesco F, Candussi G, Petrovic O, et al. An immunohistochemical study of leucocytes in human endometrium, first and third trimester basal decidua. *J Reprod Immunol.* (1993) 23:41–9. doi: 10.1016/0165-0378(93)90025-D
59. Heyborne KD, Cranfill RL, Carding SR, Born WK, O'Brien RL. Characterization of gamma delta T lymphocytes at the maternal-fetal interface. *J Immunol.* (1992) 149:2872–8.
60. Hill JA, Polgar K, Harlow BL, Anderson DJ. Evidence of embryo- and trophoblast-toxic cellular immune response(s) in women with recurrent

spontaneous abortion. *Am J Obstet Gynecol.* (1992) 166:1044–52. doi: 10.1016/S0002-9378(11)90589-4
61. Vassiliadou N, Bulmer JN. Characterization of endometrial T lymphocyte subpopulations in spontaneous early pregnancy loss. *Hum Reprod.* (1998) 13:44–7. doi: 10.1093/humrep/13.1.44
62. Yeaman GR, Collins JE, Fanger MW, Wira CR, Lydyard PM. CD8+ T cells in human uterine endometrial lymphoid aggregates: evidence for accumulation of cells by trafficking. *Immunology.* (2001) 102:434–40. doi: 10.1046/j.1365-2567.2001.01199.x
63. Keskin DB, Allan DS, Rybalov B, Andzelm MM, Stern JN, Kopcow HD, et al. TGFbeta promotes conversion of CD16+ peripheral blood NK cells into CD16- NK cells with similarities to decidual NK cells. *Proc Natl Acad Sci USA.* (2007) 104:3378–83. doi: 10.1073/pnas.0611098104
64. Flynn L, Byrne B, Carton J, Kelehan P, O'Herlihy C, O'Farrelly C. Menstrual cycle dependent fluctuations in NK and T-lymphocyte subsets from non-pregnant human endometrium. *Am J Reprod Immunol.* (2000) 43:209–17. doi: 10.1111/j.8755-8920.2000.430405.x
65. Jones RK, Bulmer JN, Searle RF. Phenotypic and functional studies of leukocytes in human endometrium and endometriosis. *Hum Reprod Update.* (1998) 4:702–9. doi: 10.1093/humupd/4.5.702
66. Kodama T, Hara T, Okamoto E, Kusunoki Y, Ohama K. Characteristic changes of large granular lymphocytes that strongly express CD56 in endometrium during the menstrual cycle and early pregnancy. *Hum Reprod.* (1998) 13:1036–43. doi: 10.1093/humrep/13.4.1036
67. Bonatz G, Hansmann ML, Buchholz F, Mettler L, Radzun HJ, Semm K. Macrophage- and lymphocyte-subtypes in the endometrium during different phases of the ovarian cycle. *Int J Gynaecol Obstet.* (1992) 37:29–36. doi: 10.1016/0020-7292(92)90974-N
68. Taherali F, Varum F, Basit AW. A slippery slope: on the origin, role and physiology of mucus. *Adv Drug Deliv Rev.* (2018) 124:16–33. doi: 10.1016/j.addr.2017.10.014
69. Baker JM, Chase DM, Herbst-Kralovetz MM. Uterine microbiota: residents, tourists, or invaders? *Front Immunol.* (2018) 9:208. doi: 10.3389/fimmu.2018.00208
70. Wallach EE, Zacur HA. *Reproductive Medicine and Surgery*. 1st ed. St. Louis, MO: Mosby (1994). xvi, 1182 p.
71. Moghissi KS. The function of the cervix in fertility. *Fertil Steril.* (1972) 23:295–306. doi: 10.1016/S0015-0282(16)38890-2
72. Brunelli R, Papi M, Arcovito G, Bompiani A, Castagnola M, Parasassi T, et al. Globular structure of human ovulatory cervical mucus. *FASEB J.* (2007) 21:3872–6. doi: 10.1096/fj.07-8189com
73. Hansen LK, Becher N, Bastholm S, Glavind J, Ramsing M, Kim CJ, et al. The cervical mucus plug inhibits, but does not block, the passage of ascending bacteria from the vagina during pregnancy. *Acta Obstet Gynecol Scand.* (2014) 93:102–8. doi: 10.1111/aogs.12296
74. Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res.* (2005) 322:43–52. doi: 10.1007/s00441-005-1127-3
75. Keck C, Gerber-Schafer C, Clad A, Wilhelm C, Breckwoldt M. Seminal tract infections: impact on male fertility and treatment options. *Hum Reprod Update.* (1998) 4:891–903. doi: 10.1093/humupd/4.6.891
76. Weng SL, Chiu CM, Lin FM, Huang WC, Liang C, Yang T, et al. Bacterial communities in semen from men of infertile couples: metagenomic sequencing reveals relationships of seminal microbiota to semen quality. *PLoS ONE.* (2014) 9:e110152. doi: 10.1371/journal.pone.0110152
77. Moreno I, Simon C. Relevance of assessing the uterine microbiota in infertility. *Fertil Steril.* (2018) 110:337–43. doi: 10.1016/j.fertnstert.2018.04.041
78. Walther-Antonio MR, Chen J, Multinu F, Hokenstad A, Distad TJ, Cheek EH, et al. Potential contribution of the uterine microbiome in the development of endometrial cancer. *Genome Med.* (2016) 8:122. doi: 10.1186/s13073-016-0368-y
79. McCoy KD, Burkhard R, Geuking MB. The microbiome and immune memory formation. *Immunol Cell Biol.* (2019) 97:625–35. doi: 10.1111/imcb.12273
80. Clark DA, Manuel J, Lee L, Chaouat G, Gorczynski RM, Levy GA. Ecology of danger-dependent cytokine-boosted spontaneous abortion in the CBA x DBA/2 mouse model. I. Synergistic effect of LPS and (TNF-alpha + IFN-gamma) on pregnancy loss. *Am J Reprod Immunol.* (2004) 52:370–8. doi: 10.1111/j.1600-0897.2004.00237.x

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Systemic Inflammation in Pregnant Women With Periodontitis and Preterm Prelabor Rupture of Membranes: A Prospective Case-Control Study

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Aims: Periodontal disease is associated with adverse pregnancy outcome, but the underlying pathophysiologic mechanism is still unknown. In this prospective, longitudinal, non-interventional case-control study, 45 women with preterm premature rupture of membranes and 26 controls with uncomplicated pregnancies were examined at three time-points (T1: 20–34 weeks of gestations; T2: within 48 h after delivery; T3: 4–6 weeks post partum). Examinations included subgingival, blood, vaginal, and placenta sampling for microbiologic, cytokine, and histology assessment. Objective of this study was to test the hypothesis that systemic inflammatory changes and not specific bacteria are predominantly involved in the association between periodontal disease and adverse pregnancy outcome.

Results: Demographic data and gestational age at T1 were comparable between groups. While there was no correlation between vaginal and gingival fluid microbiome, cytokine levels in the assessed compartments differed between cases, and controls. Vaginal smears did not show a higher rate of abnormal flora in the cases at the onset of preterm premature rupture of membranes. Number and variety of bacteria in the case group placental membranes and vagina were higher, but these bacteria were not found in membranes at birth.

Conclusions: On the basis of our results we speculate that an inflammatory pathway sequentially involving periodontal tissue, maternal serum, and finally vaginal compartment contributes to the underlying pathomechanism involved in preterm premature rupture of membranes associated with periodontitis.

Keywords: adverse pregnancy outcome, cytokines, inflammatory mediators, periodontal inflammation, periodontopathogenic bacteria, preterm birth, PPROM, preterm premature rupture of membranes

INTRODUCTION

Preterm birth (PTB) is the leading cause of perinatal morbidity and mortality in developed countries (1). About 10% of all live births worldwide are preterm and account for 28–75% of all perinatal deaths and over 50% of all severe developmental disorders in children worldwide (1). Prevention of PTB would have an extensive impact on public health and understanding its pathogenesis would pave the way for prevention strategies.

PTB is associated with preterm labor in 40–45%, with preterm prelabor rupture of membranes (PPROM) in 25–30%, and with maternal or fetal indications in 30–35% (1, 2). PPROM occurs in 3% of pregnancies (2).

Ascending (asymptomatic) intrauterine infections cause an inflammatory decidual activation resulting in preterm labor or PPROM (3–5). Albeit this is believed to be the primary cause of PPROM (1, 6, 7), to what extent remote infections contribute to PTB and specifically PPROM is not known (1, 8, 9). Many of the risk factors for PTB culminate in increased systemic inflammation which might as well result in decidual activation (1).

Periodontal disease is known to be related to systemic inflammation in other diseases (10). Periodontitis is an inflammatory disease destroying tooth-supporting connective tissue and bone. It has been linked to adverse pregnancy outcome (APO) like PTB, low birth weight and preeclampsia (8, 11–17), but this association is inconsistently reported to exist or not to exist (18, 19).

The pathophysiologic mechanism of a potential association between APO and periodontal disease remains to be elucidated (8, 20). Possibly a common pathway of increased systemic inflammation is responsible (8, 21), since intrauterine infection with oral flora in PTB patients is rare (22) and bacteria are found in the majority of elective cesarean membranes at term, hence the presence of bacteria may not be sufficient to cause PTB (1, 23).

Recent findings challenge the view that ascending infections are the primary cause for PTB because the placental microbiome profile is much more akin to the oral microbiome than to the lower genital tract (24). Furthermore, bacteria detected in the PTB placenta are not typically found in the lower genitourinary tract (24). Intrauterine infections causing PTB may originate from the mother's mouth rather than the vagina and the oral cavity is a major reservoir for microbial infections (25, 26). Nevertheless, it is unclear whether the organisms need to enter fetal tissue or whether inflammatory substances are sufficient to potentiate PTB (27).

Microorganisms activate the immune system to release inflammatory markers and prostaglandins stimulating uterine contractility and matrix-degrading enzymes which lead to PPROM (1, 7). Increased levels of inflammatory mediators in gingival crevicular fluid (GCF) have been found in women with APO and pro-inflammatory cytokines might be able to precipitate labor (28–31).

The study of clinical parameters, bacteria and cytokines in three different body compartments (mouth, vagina, blood) might help to understand the relation between remote infections and PTB. Therefore, the aim of the present study was to investigate inflammatory markers in different body compartments in women with PPROM compared with healthy pregnant women to test the hypothesis that systemic inflammatory changes and not specific bacteria are predominantly involved in the association between periodontal disease and APO.

MATERIALS AND METHODS

Study Design and Patient Selection

This longitudinal, non-interventional, prospective case-control study was designed and conducted as a collaboration of the Department of Obstetrics and Gynecology and the Department of Periodontology (University of Bern). The study protocol was approved by the Ethics Committee of the Canton of Bern (Nr. 091/10), and all participating women gave written informed consent.

Participants were enrolled in the Department of Obstetrics and Gynecology from November 2011 to August 2013 when either presenting with PPROM or during regular pregnancy visits. Cases were defined as women with PPROM between 20 0/7 and 34 0/7 weeks of gestation, and controls had uneventful pregnancies at the time of inclusion and were recruited in the same time frame (timepoint T1).

Cases and controls were examined at three distinct time points T1, T2, T3 (T1: after inclusion; T2: within 48 h after delivery; T3: 4–6 weeks post partum). Women missing two out of three examinations were defined as dropouts. Each exam included

1. Oral investigation: Periodontal Screening Index (PSI), collection of gingival crevicular fluid [GCF; CRP, cytokines (IL-1b, IL-6, IL-8, IL-10)], subgingival bacterial sampling, Multiplex-PCR
2. Blood samples: CRP, blood count, cytokines (IL-1b, IL-6, IL-8, IL-10)
3. Vaginal exam: vaginal and cervical swabs [gram stain (Nugent-Score), microbiologic culture and PCR], CRP, cytokines (IL-1b, IL-6, IL-8, IL-10), Multiplex-PCR
4. Additionally, at T2 placental membranes were examined microbiologically [gram stain (Nugent-Score), microbiologic culture and PCR] and histopathologically.

Women with PPROM received antibiotic treatment (clindamycin or amoxicillin/clavulanic acid if group B streptococcal status was positive or unknown) for 10 days. Tocolysis was administered. Antenatal glucocorticoids (betamethasone 12 mg i.m., repeated once after 24 h) were given to promote fetal lung maturation. Samples were taken before antibiotic, tocolytic, and glucocorticoid treatment on initial exam after admission. Labor was induced or cesarean section done at 34 0/7 weeks of gestation if women did not develop spontaneous labor, persistent vaginal bleeding, signs of amnion infection, or non-reassuring fetal status.

Statistical power analysis based on data including 185 patients at 6 months post-partum (32). A 50% difference in bacterial load between women delivering preterm and controls was anticipated. Based on a significance level of $p < 0.01$ and $1\text{-}\beta = 0.85$ we assumed to need 40 women in each group to declare a difference.

Oral Investigation

For microbiological sampling subgingival plaque was collected from each first molar in all quadrants with endodontic paper points (ISO 055, Dentsply Maillefer, Montigny Le Bretonneux, France, www.dentsply.fr) inserted into the gingival crevice for 15 s. Paper points pooled and stored at −20°C until assayed for presence of periodontopathogens. The PSI (Periodontal Screening Index) was recorded. GCF (Gingival crevicular fluid) samples were taken by sterile paper strips (Periopaper, Oraflow Inc., Smithtown, NY, USA, www.oraflow.com) and stored at −80°C until assayed. The oral investigations' results have been published elsewhere (12).

Vaginal and Peripheral Blood Analysis, Histopathology

Swabs were taken from the vagina and cervix, respectively, during speculum exam for routine microbiological culture, PCR, and gram stain and stored in the appropriate media. Blood samples were examined in the central laboratory and histopathologic assessment was provided from the Department of Pathology (University Hospital Bern).

Microbiologic Analysis

Multiplex-PCR: DNA was extracted by using the Chelex method (12). For detection of periodontopathogens the microIDent®plus11 test (Hain Lifescience, Nehren, Germany, www.hain-lifescience.de) was used according to the manufacturer's description. The test is able to identify 11 periodontopathogenic bacterial species after two PCR runs and a subsequent reverse hybridization (12).

Vaginal samples were assessed via conventional microbiological analysis (cultures including aerobes and anaerobes, *Listeria* and *Candida* sp.) and PCR (Chlamydia, Mycoplasma, Ureaplasma, and Treponema pallidum). Bacterial vaginosis (BV) was diagnosed in gram stain samples according to Nugent criteria and smears were categorized as abnormal according to Donders et al. (33).

Analysis of Inflammatory Mediators

Before analyzation, samples (GCF, blood serum, vaginal fluid) were eluted at 4°C overnight into 750 µl phosphate-buffered saline containing proteinase inhibitors (Sigma-Aldrich, St. Louis, MO, USA, www.sigmaaldrich.com). From the eluates, IL-1b, IL-6, IL-8, IL-10, and CRP were determined by using commercially

TABLE 1 | Demographic data, preexisting conditions, previous and current pregnancies.

		Cases ($n = 45$)	Controls ($n = 26$)	$p=$	
Age	(years; Median, Range)	35.0 (24–48)	35.0 (25–42)	0.608*	n.s.
Race	(% caucasian)	91	81	0.272°	n.s.
BMI	(kg/m^2; Median, Range)	22.2 (16.1–44.3)	22.2 (18–40.4)	0.989**	n.s.
Primipara	(%)	58	58	1.000°	n.s.
Preexisting conditions		(n =)			
- Systemic diseases		5	4		
- Hypertensive disease		1	0		
- Obesity		3	3		
- HIV-positive		1	0		
Previous pregnancies					
- Miscarriage		15	8		
- Induced abortion		3	2		
- Preterm delivery		1	1		
- Intrauterine fetal death		1	1		
- Chorioamnionitis		1	0		
- Preeclampsia		1	1		
- PPROM		1	2		
- GTD		0	1		
Current pregnancy					
- Premature labor		26	0		
- Amnion infection syndrome		9	0		
- Vaginal bleeding		4	2		
- Polyhydramnios		1	0		
- Pregnancy-induced hypertension		0	2		
- Gestational diabetes		0	1		
- Preeclampsia		0	1		

*t-test; **Mann-Whitney-U-Test; °Fisher's Exact test; n.s., not statistically significant.

available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Europe Ltd., Abingdon, UK, www.rndsystems.com). The detection levels of the kits were 2 pg (CRP: 10 pg).

Data Analysis

Demographic data, obstetric outcomes and laboratory results were compared with the student's *t*-test and the Mann-Whitney-*U*-Test, respectively. The Mann-Whitney-*U* Rank Sum Test was used comparing medians if the normality test (Kolmogorov-Smirnov) failed or if the normality test passed but the equal variance test (Levene Median test) failed. If both tests passed the student's *t*-test was used for comparing means. Qualitative data were analyzed with Fisher's Exact Test for independent groups. The statistical tests used are specified in the respective tables. Statistical analysis was performed by using Graph Pad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). The level of significance was set at $p = 0.05$.

RESULTS

Seventy-one women were included in the study analysis of whom 45 were PPROM-cases and 26 controls. In the test group four women dropped out because they did not prove to have ruptured membranes ($n = 2$), did not deliver in our facility or were not followed-up according to the protocol. In the control group 11 women dropped out as they delivered in other hospitals ($n = 3$), developed chorioamnionitis ($n = 2$), had a severe pre-eclampsia, intrauterine death, preterm contractions, or refused further participation in the study ($n = 3$).

Study population and controls were comparable as they did not differ in regard with age, race, BMI, parity, and preexisting conditions (**Table 1**).

Characteristics of previous and current pregnancies were similar in both groups, too, except from premature labor, and amnion infection syndrome which were understandably more frequent in the test group (**Table 1**).

As expected, pregnancies lasted significantly shorter and birth weights were significantly lower in the case group. There was only a tendency toward a higher rate of cesarean deliveries in the case group which was not statistically significant (**Table 2**).

The neonates' gender did not differ in both groups. Interestingly, no difference in the rate of newborns with a 5-min-Apgar lower than 7 was found in both groups and an arterial umbilical cord pH lower than 7.15 was even more frequent in the control group. Expectedly, referral to the Neonatology department occurred more frequently in the case group (**Table 2**). Likewise respiratory distress syndrome, neonatal infection and sepsis rates were higher in the case group newborns.

In vaginal smears, gram stain showed no statistically significant difference in the rate of abnormal flora/bacterial vaginosis (BV) (34) between the groups at T1 (onset of PPROM). At T2 (delivery) the abnormal flora/BV rate was significantly higher in the case group most likely due to ascending bacteria because of the state of ruptured membranes. At T3 no difference in the rate of abnormal flora/BV was found for both groups. The variety and number of bacteria found in cultures were increased in the case group at T1 and (possibly affected by the state of ruptured membranes) remained increased at T2 and even T3 (**Table 3**).

Smears from the membranes showed again a larger number and variety of bacteria in the case group membranes (**Table 3**). Remarkably, in only one patient the same bacterium (Haemophilus influenzae) was found in the vagina at T1 and in the placenta/membranes at delivery (T2). Chorioamnionitis was diagnosed histopathologically significantly more often in the case placentae (**Table 3**).

TABLE 2 | Current pregnancy.

		Cases ($n = 45$)	Controls ($n = 26$)	$p =$	
Gestational weeks at PPROM/study inclusion	Median	31 + 0	28 + 1	0.033**	
	Range	22 + 5–35 + 1	21 + 1–35 + 1		
Gestational weeks at delivery	Median	32 + 2	39 + 1	<0.001**	
	Range	25 + 2–35 + 1	35 + 2–41 + 3		
Complications at delivery	*n* =				
- Retained placenta		3	3		
- Obstructed labor		1	2		
- Post partum hemorrhage		1	0		
- Prolapsed umbilical cord		1	0		
Cesarean section rate	%	47	35	0.455°	n.s.
Female neonates	%	42	46	0.813°	n.s.
Birth weight	Grams	1,666	3,348	<0.001*	
APGAR at 5′ < 7	%	21	12	0.367°	n.s.
pH art. <7.15	%	2	15	0.038°	
Transfer to NICU	%	94	8	<0.001°	

**t-test; **Man-Whitney-U-Test; °Fisher's Exact test; n.s., not statistically significant.*

TABLE 3 | Microbiota and histopathology.

	Source	Legend	Timepoint 1 cases	Controls	*p* =	Timepoint 2 cases	Controls	*p* =	Timepoint 3 cases	Controls	*p* =
Gram stain	Vagina	% normal/ abnormal/BV	51/33/16	74/9/17	n.s.	19/68/13	65/29/6	0.001	31/50/19	54/33/13	n.s.
Culture spec.	Vagina		5× *E.coli* 1× *Hämoph.inf*. 1× Gardnerella 1× Staphyloc. 1× *Strept.milleri* 6× *Mix.flora*	2× Gardner. 2× Mix.flora		5× *E.coli* 1× Enterococ. 1× Peptostrept. 1× Klebsiella 1× Enterobact. 1× Fungus 8× *Mix.flora*	5× Mix.flora		3× *E.coli* 2× *Strept.mill*. 2× Gardner. 1× Klebsiella 1× *Staph.aur*. 11× *Mix.flora*	1× *E.coli* 8× *Mix.flora*	
Candida	Vagina	*n* =	3	0	n.s.	0	1	n.s.	0	0	n.s.
GBS	Vagina	*n* =	5	1	n.s.	0	0	n.s.	3	2	n.s.
Mycoplasma	Vagina	*n* =	1	0	n.s.	1	0	n.s.	0	0	n.s.
Ureaplasma	Vagina	*n* =	11	8	n.s.	2	5	0.026	0	4	n.s.
Culture growth	Membranes	*n* =				12	7	n.s.			
Culture spec.	Membranes					4× *E.coli* 2× Enterococ. 3× Ureaplas. 1× *Hämoph.inf* 1× *Strept.vir.* 1× Peptostrept. 1× Enterobact. 1× *Bact.fragil*. 3× *Mix.flora*	1× *E.coli* 1× Serratia 2× Ureaplas. 1× *Bact.fragil*.				
Histology	Placenta	% Chorioamnionitis				40	0	0.001			

All p-values: Fisher's exact test. n.s., not statistically significant.

TABLE 4 | Cytokines: comparison of case vs. control group at the 3 timepoints.

Cytokine	Source	Cases vs. Controls		
		T1	T2	T3
IL1b	Blood	n.s.	n.s.	n.s.
	Gingiva	n.s.	↓ (12 vs. 36)	n.s.
	Vagina	n.s.	n.s.	n.s.
IL6	Blood	n.s.	n.s.	n.s.
	Gingiva	n.s.	n.s.	n.s.
	Vagina	↑ (281 vs. 32)	n.s.	n.s.
IL8	Blood	↑ (126 vs. 24)	n.s.	n.s.
	Gingiva	↓ (105 vs. 280)	n.s.	n.s.
	Vagina	n.s.	n.s.	n.s.
IL10	Blood	n.s.	n.s.	n.s.
	Gingiva	↑ (30 vs. 10)	n.s.	n.s.
	Vagina	↑ (33 vs. 9)	↑ (50 vs. 22)	n.s.
CRP	Blood	n.s.	n.s.	n.s.
	Gingiva	↓ (105 vs 294)	n.s.	n.s.
	Vagina	n.s.	↓ (3,088 vs. 4,817)	↓ (1,442 vs. 2,070)

↑: significantly higher levels; ↓: significantly lower levels; n.s., not significant.
Brackets: Means in pg/ml. All p-values: Mann-Whitney-U-test.

Neither Chlamydia nor Gonococcus or Listeria were detected in all groups at all timepoints. Candida, group B streptococci, mycoplasma and ureaplasma colonization were not statistically different between both groups at all timepoints, except ureaplasma at T2 which was even higher in controls than in women with PPROM (**Table 3**).

Leucocyte count was significantly higher in the test group at T1 and T2, while 6 weeks after delivery at T3 there was no significant difference. Leucocytes increased from T1 to T2 in the test group, but not in the control group.

The comparison of cytokine levels in the three body compartments at the three timepoints showed (**Table 4**, **Figures 1 – 3**) at

- T1: The case patients had higher levels of pro-inflammatory IL-6 in the vagina and pro-inflammatory IL-8 in the blood, lower levels of IL-8 and CRP in the gingiva, and higher levels of anti-inflammatory IL-10 in gingiva and vagina
- T2: The case patients had increased levels of pro-inflammatory IL-1b in the gingiva, increased levels of IL-10 and a decreased CRP in the vagina
- T3: The case patients only showed lower CRP levels in the vaginal.

All other parameters in blood, vaginal and gingival fluid were not significantly different at the three timepoints (**Table 4**, **Figures 1**–**3**). However, from T1 to T2 IL-1b and IL-8 decreased in gingiva while IL-6 increased in the blood and CRP increased in the vagina. These interleukin patterns might be in line with the cascade theory: Inflammatory cytokines already start decreasing in the mouth while they increase in blood and vagina reflecting a chronological process starting in the oral cavity.

DISCUSSION

Our study focuses on the relationship between periodontitis and PPROM. Three different body compartments were therefore studied at three time points. The main finding is the difference in cytokine levels in different compartments in the case group at the time of PPROM as compared to controls. This means that an inflammatory process is in progress, and cytokine patterns suggest this to be initiated in the periodontal compartment, consecutively encroaching on further body compartments. However, this does not occur via bacterial spread since nucleic acid based methods and microbiologic cultures did neither reveal an association of oral and vaginal nor of vaginal and placental bacterial colonization.

Predicting and identifying women at risk for delivering preterm is challenging. A relation between periodontitis and APO has been suggested (11, 15), but the pathophysiologic link is not established. Oral inflammatory mediator changes have been reported in APO (8, 28, 35–37), but none of these studies focused on women with PPROM or cytokine levels in different body parts. A systematic approach examining systemic and local compartments might reveal pathophysiologic links between remote infection and PPROM.

The comparison of cytokine levels in the three body compartments at three timepoints showed significant differences between PPROM patients and controls. At the onset of PPROM the periodontal inflammation seems to already decline since pro-inflammatory IL-8 and CRP are decreased compared to controls and an immune response is already initiated as anti-inflammatory IL-10 (38, 39) reactively increases in the gingiva. At the same time pro-inflammatory IL-8 and IL-6, respectively, are increased in blood and vagina. Both cytokines are early markers of a beginning inflammation (40). This suggests that inflammation starts in the periodontal compartment and already begins to resolve, while in peripheral blood and vagina it is still ongoing in terms of an inflammatory cascade. From T1 to T2 the same situation is found: while inflammatory markers (IL-1b and IL-8) further decrease in the gingiva, IL-6 increases in the blood and both CRP and IL-10 increase in the vagina. The informative value of the differences in cytokine levels at T1 is emphasized by the condition that at 6 weeks post partum (T3) interleukin levels in both groups did not differ significantly. The inflammatory response seems to regress which is consistent with the systemic inflammatory hypothesis. The finding of an increased anti-inflammatory cytokine IL-10 in the vagina at T1 and T2 might be explained by the fact that rising IL-10 levels in the vaginal fluid reflect the initiation of the birth process (41, 42), besides being associated with intraamniotic infection (43).

The oral investigations' results showed an association between periodontal status and PPROM and oral periodontopathogens were more prevalent in the PPROM women (12). Importantly, although periodontopathogenic bacteria were detected in vaginal samples, in none of the women these bacteria were found in both the vagina and the mouth concomitantly (12), corroborating the hypothesis that not bacterial spread but systemic inflammation might be a contributing factor in developing PPROM. This is further supported by the finding that abnormal flora and bacterial

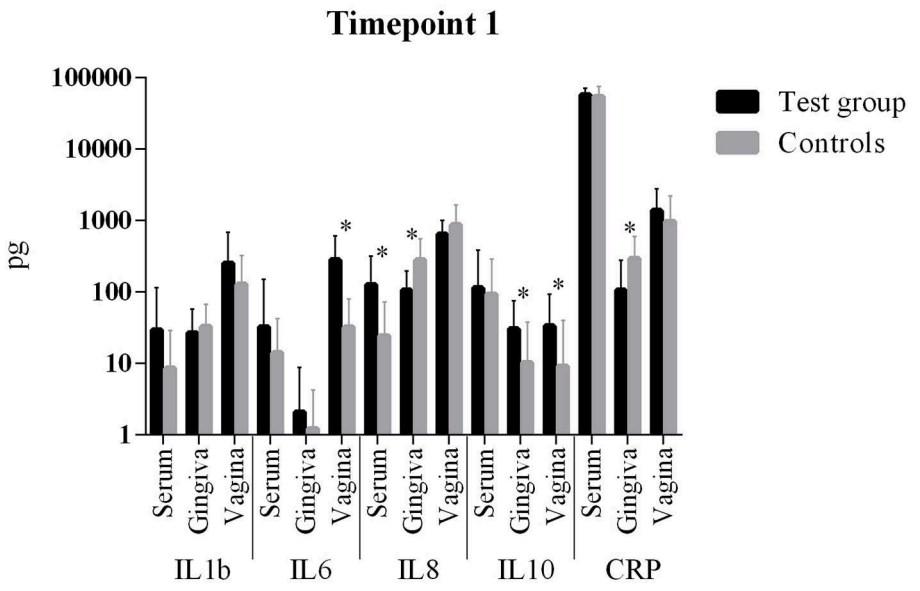

FIGURE 1 | Comparison of cytokine levels in PPROM women (cases) vs. controls at timepoint 1. *statistically significant.

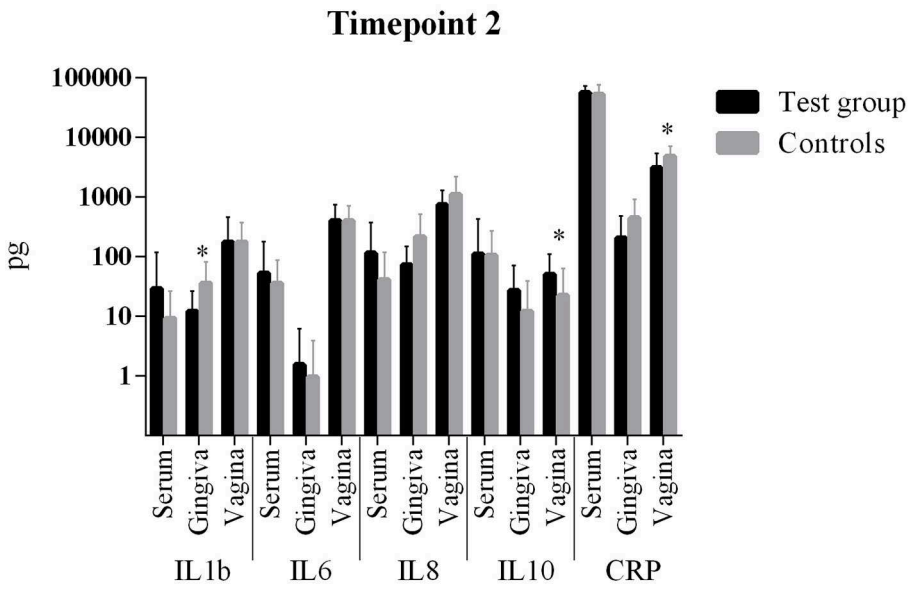

FIGURE 2 | Comparison of cytokine levels in PPROM women (cases) vs. controls at timepoint 2. *statistically significant.

vaginosis were equally frequent in both the cases and the controls, respectively, at the occurrence of PPROM (timepoint 1). Vaginal bacterial dysbalance seems not to be the exclusive cause for PPROM. The variety and number of bacteria found in cultures was increased in the case group at T1, but remarkably in only one patient the same bacterium has been affirmed in both the vagina at T1 and the placenta smears at T2 (delivery), which again is in conflict with the hypothesis that ascending infection is the primary mechanism for PPROM.

Recent findings showing that the placental microbiome profile is much more akin to the oral microbiome than to the lower genital tract (24) do not support the theory that ascending infection is the primary cause of PPROM. Likewise, microbiome profile at PPROM does not correlate with latency duration supporting our notion that systemic inflammation may rather play a role in the onset of PPROM than microbiota dysbalance (44). Furthermore, direct spread of oral bacteria to the reproductive tract is unlikely since PTB is rarely associated

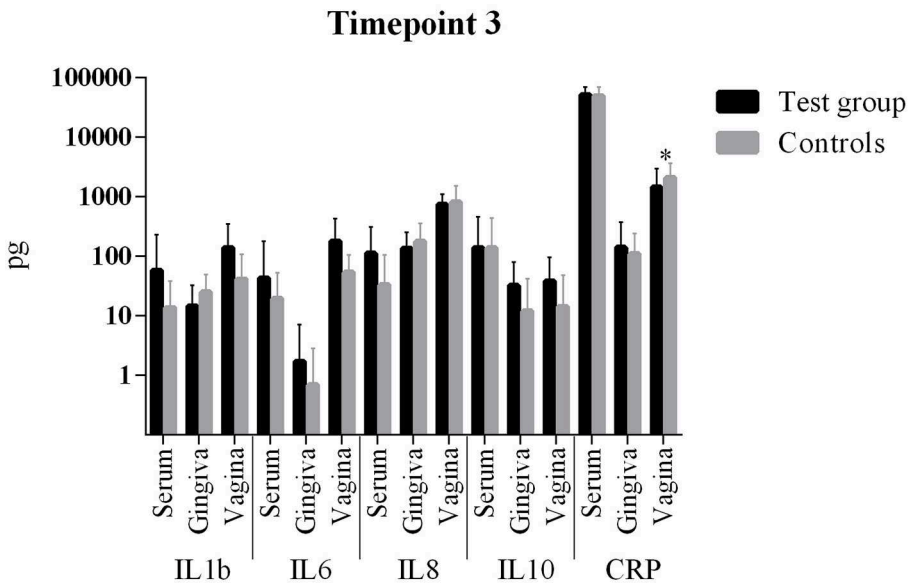

FIGURE 3 | Comparison of cytokine levels in PPROM women (cases) vs. controls at timepoint 3. *statistically significant.

with intrauterine infection with oral flora (22), and bacteria are mostly found in term membranes without causing PTB (1, 23). In our patients, periodontopathogens found in the gingiva have not been verified in the placenta/membrane swabs (12) and no difference was found in the rate of abnormal flora and bacterial vaginosis between cases and controls at the time of PPROM. Moreover, in only one patient one of the bacteria found in the vagina at T1 was verified in the placenta/membranes at T2. All these findings argue against the theory of direct spread of bacteria as the causative mechanism of PPROM. Accordingly, recent studies have shown the gut microbiome being linked to several diseases and although the exact mechanism is not fully understood a systemic cytokine release has been demonstrated and made accountable for disease occurrence (45, 46). A more extensive investigation of the microbiome-inflammation-interaction in different body compartments is desirable.

A strength of our study is the unique study design with a comprehensive approach including simultaneous examination of different body compartments, microbiological and nucleic acid based assessment and comparison of inflammation markers, in parallel with clinical periodontological assessment at different timepoints. The use of the multiplex method is considered particularly convenient (47).

A weakness is that we have no data of women at "T0," i.e., before PPROM becomes clinically evident. In particular, an early timepoint for analysis in the first trimester or even before onset of pregnancy would be valuable in order to have the whole picture and understand the mechanism how periodontitis may cause PPROM and APO. Unfortunately, due to the low incidence of PPROM and the high expenditure such a prospective study would be difficult to realize. The lack of published data and the complexity of recruiting women for examination at three time points impeded a sound power analysis and inclusion of a large number of patients. Thus, the limited number of women in our study might explain why differences have not been observed among some of the measured parameters, and maternal plasma cytokine concentrations are difficult to interpret in APO patients (48). Moreover, paper points were used for samplings of subgingival plaque. Since they do not allow to collect the biofilm but only planktonic bacteria, use of a courette might have been the better choice for sample collection.

In summary, the results of our study can be interpreted in line with our initial hypothesis that systemic inflammation—initiated and triggered by periodontal disease—might play a role in developing PPROM. Bacterial spread or ascension seem not to represent the exclusive pathophysiologic cause for PPROM. There is a systemic inflammation in progress at the time of PPROM and we speculate that the inflammatory pathway rather than ascending infection alone contributes to PPROM and—consecutively—PTB.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Canton of Bern (Nr. 091/10). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SM: project development, data management, data analysis, manuscript writing, and approval of the final manuscript. SA-M: data collection, data interpretation, and approval of the final manuscript. PS: data collection, data analysis, and approval of the final manuscript. AS and RP: project development and approval of the final manuscript. SE: data management and approval of the final manuscript. DS: project development, manuscript editing, and approval of the final manuscript.

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REFERENCES

1. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet.* (2008) 371:75–84. doi: 10.1016/S0140-6736(08)60074-4
2. Mercer BM. Preterm premature rupture of the membranes. *Obstet Gynecol.* (2003) 101:178–93. doi: 10.1016/S0029-7844(02)02366-9
3. Surbek DV, Hoesli IM, Holzgreve W. Morphology assessed by transvaginal ultrasonography differs in patients in preterm labor with vs. without bacterial vaginosis. *Ultrasound Obstet Gynecol.* (2000) 15:242–5. doi: 10.1046/j.1469-0705.2000.00102.x
4. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. *Science.* (2014) 345:760–5. doi: 10.1126/science.1251816
5. Lamont RF. Advances in the prevention of infection-related preterm birth. *Front Immunol.* (2015) 6:566. doi: 10.3389/fimmu.2015.00566
6. Witkin SS. The vaginal microbiome, vaginal anti-microbial defence mechanisms and the clinical challenge of reducing infection-related preterm birth. *BJOG.* (2015) 122:213–8. doi: 10.1111/1471-0528.13115
7. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss JF III, Petraglia F. Inflammation and pregnancy. *Reprod Sci.* (2009) 16:206–15. doi: 10.1177/1933719108329095
8. Cetin I, Pileri P, Villa A, Calabrese S, Ottolenghi L, Abati S. Pathogenic mechanisms linking periodontal diseases with adverse pregnancy outcomes. *Reprod Sci.* (2012) 19:633–41. doi: 10.1177/1933719111432871
9. Menon R, Fortunato SJ. Infection and the role of inflammation in preterm premature rupture of the membranes. *Best Pract Res Clin Obstet Gynaecol.* (2007) 21:467–78. doi: 10.1016/j.bpobgyn.2007.01.008
10. Patini R, Gallenzi P, Spagnuolo G, Cordaro M, Cantiani M, Amalfitano A, et al. Correlation between metabolic syndrome, periodontitis and reactive oxygen species production. A Pilot Study. *Open Dent J.* (2017) 11:621–7. doi: 10.2174/1874210601711010621
11. Jarjoura K, Devine PC, Perez-Delboy A, Herrera-Abreu M, D'Alton M, Papapanou PN. Markers of periodontal infection and preterm birth. *Am J Obstet Gynecol.* (2005) 192:513–9. doi: 10.1016/j.ajog.2004.07.018
12. Stadelmann PF, Eick S, Salvi GE, Surbek D, Mohr S, Burgin W, et al. Increased periodontal inflammation in women with preterm premature rupture of membranes. *Clin Oral Investig.* (2014) 16:1537–46. doi: 10.1007/s00784-014-1371-6
13. Kumar A, Basra M, Begum N, Rani V, Prasad S, Lamba AK, et al. Association of maternal periodontal health with adverse pregnancy outcome. *J Obstet Gynaecol Res.* (2013) 39:40–5. doi: 10.1111/j.1447-0756.2012.01957.x
14. Sgolastra F, Petrucci A, Severino M, Gatto R, Monaco A. Relationship between periodontitis and pre-eclampsia: a meta-analysis. *PLoS ONE.* (2013) 8:e71387. doi: 10.1371/journal.pone.0071387
15. Chambrone L, Guglielmetti MR, Pannuti CM, Chambrone LA. Evidence grade associating periodontitis to preterm birth and/or low birth weight: I. A systematic review of prospective cohort studies. *J Clin Periodontol.* (2011) 38:795–808. doi: 10.1111/j.1600-051X.2011.01755.x
16. Ide M, Papapanou PN. Epidemiology of association between maternal periodontal disease and adverse pregnancy outcomes–systematic review. *J Periodontol.* (2013) 84:S181–194. doi: 10.1902/jop.2013.134009
17. Shetty M, Shetty PK, Ramesh A, Thomas B, Prabhu S, Rao A. Periodontal disease in pregnancy is a risk factor for preeclampsia. *Acta Obstet Gynecol Scand.* (2010) 89:718–21. doi: 10.3109/00016341003623738
18. Lin D, Moss K, Beck JD, Hefti A, Offenbacher S. Persistently high levels of periodontal pathogens associated with preterm pregnancy outcome. *J Periodontol.* (2007) 78:833–41. doi: 10.1902/jop.2007.060201
19. Africa C, Kayitenkore J, Bayingana C. Examination of maternal gingival crevicular fluid for the presence of selected periodontopathogens implicated in the pre-term delivery of low birthweight infants. *Virulence.* (2010) 1:254–9. doi: 10.4161/viru.1.4.12004
20. Pretorius C, Jagatt A, Lamont RF. The relationship between periodontal disease, bacterial vaginosis, and preterm birth. *J Perinat Med.* (2007) 35:93–9. doi: 10.1515/JPM.2007.039
21. Goldenberg RL, Culhane JF. Preterm birth and periodontal disease. *N Engl J Med.* (2006) 355:1925–7. doi: 10.1056/NEJMe068210
22. Goepfert AR, Jeffcoat MK, Andrews WW, Faye-Petersen O, Cliver SP, Goldenberg RL, et al. Periodontal disease and upper genital tract inflammation in early spontaneous preterm birth. *Obstet Gynecol.* (2004) 104:777–83. doi: 10.1097/01.AOG.0000139836.47777.6d
23. Steel JH, Malatos S, Kennea N, Edwards AD, Miles L, Duggan P, et al. Bacteria and inflammatory cells in fetal membranes do not always cause preterm labor. *Pediatr Res.* (2005) 57:404–11. doi: 10.1203/01.PDR.0000153869.96337.90
24. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Sci Transl Med.* (2014) 6:237ra65. doi: 10.1126/scitranslmed.3008599
25. Han YW, Fardini Y, Chen C, Iacampo KG, Peraino VA, Shamonki JM, et al. Term stillbirth caused by oral Fusobacterium nucleatum. *Obstet Gynecol.* (2010) 115:442–5. doi: 10.1097/AOG.0b013e3181cb9955
26. Keelan JA, Wong PM, Bird PS, Mitchell MD. Innate inflammatory responses of human decidual cells to periodontopathic bacteria. *Am J Obstet Gynecol.* (2010) 202:471–11. doi: 10.1016/j.ajog.2010.02.031
27. Onderdonk AB, Delaney ML, DuBois AM, Allred EN, Leviton A. Detection of bacteria in placental tissues obtained from extremely low gestational age neonates. *Am J Obstet Gynecol.* (2008) 198:110–7. doi: 10.1016/j.ajog.2007.05.044
28. Carta G, Persia G, Falciglia K, Iovenitti P. Periodontal disease and poor obstetrical outcome. *Clin Exp Obstet Gynecol.* (2004) 31:47–9.
29. Stadelmann P, Alessandri R, Eick S, Salvi GE, Surbek D, Sculean A. The potential association between gingival crevicular fluid inflammatory mediators and adverse pregnancy outcomes: a systematic review. *Clin Oral Investig.* (2013) 17:1453–63. doi: 10.1007/s00784-013-0952-0
30. Velez DR, Fortunato SJ, Williams SM, Menon R. Interleukin-6. (IL-6) and receptor. (IL6-R) gene haplotypes associate with amniotic fluid protein concentrations in preterm birth. *Hum Mol Genet.* (2008) 17:1619–30. doi: 10.1093/hmg/ddn049
31. Ryu A, Park KH, Oh KJ, Lee SY, Jeong EH, Park JW. Predictive value of combined cervicovaginal cytokines and gestational age at sampling for intra-amniotic infection in preterm premature rupture of membranes. *Acta Obstet Gynecol Scand.* (2013) 92:517–24. doi: 10.1111/aogs.12073
32. Persson GR, Hitti J, Paul K, Hirschi R, Weibel M, Rothen M, et al. Tannerella forsythia and Pseudomonas aeruginosa in subgingival bacterial samples from parous women. *J Periodontol.* (2008) 79:508–16. doi: 10.1902/jop.2008.070350
33. Donders GG, Van CK, Bellen G, Reybrouck R, Van den Bosch T, Riphagen I, et al. Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy. *BJOG.* (2009) 116:1315–24. doi: 10.1111/j.1471-0528.2009.02237.x

34. 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.
35. Konopka T, Rutkowska M, Hirnle L, Kopec W, Karolewska E. The secretion of prostaglandin E2 and interleukin 1-beta in women with periodontal diseases and preterm low-birth-weight. *Bull Group Int Rech Sci Stomatol Odontol.* (2003) 45:18–28.
36. Chaparro A, Sanz A, Quintero A, Inostroza C, Ramirez V, Carrion F, et al. Increased inflammatory biomarkers in early pregnancy is associated with the development of pre-eclampsia in patients with periodontitis: a case control study. *J Periodontal Res.* (2013) 48:302–7. doi: 10.1111/jre.12008
37. Oettinger-Barak O, Barak S, Ohel G, Oettinger M, Kreutzer H, Peled M, et al. Severe pregnancy complication (preeclampsia) is associated with greater periodontal destruction. *J Periodontol.* (2005) 76:134–7. doi: 10.1902/jop.2005.76.1.134
38. Sato TA, Keelan JA, Mitchell MD. Critical paracrine interactions between TNF-alpha and IL-10 regulate lipopolysaccharide-stimulated human choriodecidual cytokine and prostaglandin E2 production. *J Immunol.* (2003) 170:158–66. doi: 10.4049/jimmunol.170.1.158
39. Fortunato SJ, Menon R, Lombardi SJ. The effect of transforming growth factor and interleukin-10 on interleukin-8 release by human amniochorion may regulate histologic chorioamnionitis. *Am J Obstet Gynecol.* (1998) 179:794–9. doi: 10.1016/S0002-9378(98)70085-7
40. Berney T, Gasche Y, Robert J, Jenny A, Mensi N, Grau G, et al. Serum profiles of interleukin-6, interleukin-8, and interleukin-10 in patients with severe and mild acute pancreatitis. *Pancreas.* (1999) 18:371–7. doi: 10.1097/00006676-199905000-00007
41. Gotsch F, Romero R, Kusanovic JP, Erez O, Espinoza J, Kim CJ, et al. The anti-inflammatory limb of the immune response in preterm labor, intra-amniotic infection/inflammation, and spontaneous parturition at term: a role for interleukin-10. *J Matern Fetal Neonatal Med.* (2008) 21:529–47. doi: 10.1080/14767050802127349
42. Tsiartas P, Holst RM, Wennerholm UB, Hagberg H, Hougaard DM, Skogstrand K, et al. Prediction of spontaneous preterm delivery in women with threatened preterm labour: a prospective cohort study of multiple proteins in maternal serum. *BJOG.* (2012) 119:866–73. doi: 10.1111/j.1471-0528.2012.03328.x
43. Cobo T, Kacerovsky M, Holst RM, Hougaard DM, Skogstrand K, Wennerholm UB, et al. Intra-amniotic inflammation predicts microbial invasion of the amniotic cavity but not spontaneous preterm delivery in preterm prelabor membrane rupture. *Acta Obstet Gynecol Scand.* (2012) 91:930–5. doi: 10.1111/j.1600-0412.2012.01427.x
44. Paramel JT, Wagner EC, van SJ, Albert AY, Hill JE, Money DM. High diversity and variability in the vaginal microbiome in women following preterm premature rupture of membranes (PPROM): A Prospective Cohort Study. *PLoS ONE.* (2016) 11:e0166794. doi: 10.1371/journal.pone.0166794
45. Kho ZY, Lal SK. The human gut microbiome - a potential controller of wellness and disease. *Front Microbiol.* (2018) 9:1835. doi: 10.3389/fmicb.2018.01835
46. Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, et al. Linking the human gut microbiome to inflammatory cytokine production capacity. *Cell.* (2016) 167:1125–36. doi: 10.1016/j.cell.2016.10.020
47. Polettini J, Cobo T, Kacerovsky M, Vinturache AE, Laudanski P, Peelen MJ, et al. Biomarkers of spontaneous preterm birth: a systematic review of studies using multiplex analysis. *J Perinat Med.* (2017) 45:71–84. doi: 10.1515/jpm-2016-0097
48. Romero R, Chaemsaithong P, Docheva N, Korzeniewski SJ, Tarca AL, Bhatti G, et al. Clinical chorioamnionitis at term IV: the maternal plasma cytokine profile. *J Perinat Med.* (2016) 44:77–98. doi: 10.1515/jpm-2015-0103

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REVIEW
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Fecal Microbiota Transplantation: A Potential Tool for Treatment of Human Female Reproductive Tract Diseases

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The gastro-intestinal tract is an extensive organ involved in several activities, with a crucial role in immunity. Billions of commensal and transient microorganisms, known as the gut microbiota, and potential pathogens, which are constantly stimulating intestinal immunity, colonize the intestinal epithelial surface. The gut microbiota may be regarded as analogous to a solid organ with multiple different functions. In the last decade, many studies have demonstrated that intestinal bacteria can be a decisive factor in the health-disease balance of the intestine, and they can also be responsible for illnesses in other locations. For this reason, fecal microbiota transplantation (FMT) represents an important therapeutic option for *Clostridium difficile* infections and hold promise for different clinical conditions, such as multiple sclerosis, autism, obesity, and other systemic diseases. FMT consists of the infusion of a fecal suspension from a healthy donor to a recipient in order to restore gut flora alterations. Similar to the gut, the female reproductive tract is an example of a very complex biological ecosystem. Recent studies indicate a possible relationship between the gut and female tract microbiota, associating specific intestinal bacteria patterns with genital female diseases, such as polycystic ovary syndrome (PCOS), endometriosis and bacterial vaginosis (BV). FMT could represent a potential innovative treatment option in this field.

Keywords: cervical-vaginal microbiota, uterine microbiota, gut micobiota, fecal microbiota transplant (FMT), probiotics, immunmodulation, next generation sequencing (NGS)

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INTRODUCTION

The gut microbiota, which is composed of 10^{13}-10^{14} bacterial cells (1), has multiple different functions. In the last decade, studies have shown that intestinal bacteria can interact with other organs and be a decisive factor in health/disease balance beyond just the intestine (2). The gut and vaginal microbiota are examples of very complex biological ecosystems. The female reproductive tract has developed unique structures, such as the vagina and uterus. While the vagina hosts trillions of bacteria, the upper reproductive tract remains largely unexplored and has generally been considered sterile. The vaginal microbiota interacts with the immune system. Despite being one of the simplest commensal bacterial communities in the human body, we are only beginning to appreciate its complex dynamic nature and modulation of host immunity (3). Toll-like receptor (TLR)-mediated signaling, for example, regulates mucin secretions, contributing to local bacteria

colonization (4). The relationship between the gut and female reproductive tract microbiota has been studied in-depth (5). Driving microbial colonization in the gut could be a very interesting approach for the treatment of many diseases. Fecal microbiota transplantation (FMT) is an important therapeutic option for *Clostridium difficile* [or *Clostridioides difficile*-like, as reported by Oren et al. (6)] infections (CDI) (7). Promising findings suggest that FMT may also play a role in the management of genital female disorders associated with microbiota alterations.

HUMAN GUT MICROBIOTA

Human microbiota is composed of archea, bacteria, yeasts, fungi, viruses, and protists (8), whose composition has not been completely described also because there is a substantial inter-individual. Human gut microbiota is a complex ecosystem with several functions integrated into the host organism, interacting with metabolic, immune, and nutrient absorption activities. The gastrointestinal tract harbors $\sim 10^{13}$-10^{14} bacterial cells (1, 9), consisting of strict anaerobes that outnumber the facultative anaerobes and aerobes by a factor of two to three; these include Firmicutes, Bacteroidetes, and Proteobacteria, while Actinobacteria contribute less to the total bacterial composition. Bacteroidetes include the genera *Prevotella* and *Bacteroides*; the phylum Actinobacteria includes *Bifidobacterium*; and the phylum Firmicutes includes *Clostridium* clusters and members of *Eubacterium, Faecalibacterium, Roseburia*, and *Ruminococcus* (10). The presence of many different bacterial species is crucial for defining the role of gut microbiota in various metabolic pathways. Gut microbiota is a dynamic system that changes and evolves during our lifetime according to anatomical, dietary, environmental, pathological, and pharmacological factors (e.g., the use of antibiotics, probiotics) (11). This variability in terms of bacterial species is distributed throughout the various districts of the gastrointestinal system. Starting from the upper gastro-intestinal tract, the throat and distal esophagus, the predominant genera are *Streptococcus, Prevotella, Actinomyces, Gemella, Rothia, Granulicatella, Haemophilus,* and *Veillonella* (12). In the stomach, microbial diversity depends upon the presence and absence of *Helicobacter pylori* (13, 14). A stomach lacking *H. pylori* is mainly populated by *Streptococcus* spp., *Actinomyces* spp., *Prevotella* spp., and *Gemella* spp., which are predominantly found in the throat, indicating that they may be transient residents coming from the throat (12). The recto-sigmoid colon microbiota is more complex than the jejunum, ileum, and caecum resident microbes. *Enterococci, E. coli, Klebsiella, Lactobacilli, Staphylococci*, and *Streptococci* are present in the jejunum and ileum. Most of the microbes of the jejunum and ileum are aerobes and facultative anaerobes (15). The small intestine harbors the aerobic *Enterococcus* group, *Lactobacilli, Streptococci*, and Gammaproteobacteria, while anaerobes are predominant in the large intestine. Caecal microbiota is more complex than jejunal and ileal microbiota. The caecal bacteria are predominated by *Lactobacillus, Enterococcus*, and *Escherichia coli* (16). In the recto-sigmoidal colon, strict anaerobic bacteria belonging to *Bacteroides, Clostridium coccoides*, and *Clostridium leptum* are the predominant bacterial groups (15). Given the complexity and multifactorial in terms of the evolution of the human intestinal microbiota, it is difficult to establish the composition of an ideal and healthy microbiota. Generally, a state of eubiosis is characterized by a strong presence of Firmicutes and Bacteroidetes and by a low percentage of Proteobacteria, which, instead, increase during inflammatory states (17). Another aspect that should be underlined is crosstalk between the gut microbiota and immune system. This point is extensive and critical. It allows for the tolerance of commensal bacteria and oral food antigens and also enables the immune system to recognize and attack opportunistic bacteria in order to prevent invasion and infection. In addition, microbiota has broader effects contributing to innate and adaptive immunity at multiple levels. This concept is supported in preclinical models, as germ-free mice lacking intestinal microbiota are subject to severe immunity defects, with a marked reduction of mucous layer, altered IgA secretion and reduced size and functionality of Peyer's patches and draining mesenteric lymph nodes (1).

FECAL MICROBIOTA TRANSPLANTATION

Given the fundamental role played by the human microbiota in the health/disease balance, the integrity of this system turns out to be an important therapeutic target (18). The most innovative therapeutic approach is represented by FMT. In the last decade, FMT has been an example of a valid solution, with success of ∼90%, resulting in a more effective regimen for *Clostridium difficile* infection (CDI) than vancomycin (19). FMT consists of the infusion of a feces suspension from a healthy donor to the intestinal tract of a recipient patient in order to treat a specific disorder associated with alteration of gut microbiota (7, 20). In the European Consensus Conference (7), 28 experts from 10 countries collaborated to establish practice guidelines about FMT indications, donor selection, preparation of fecal suspension, clinical management, and basic requirements for implementing an FMT center. An aspect to highlight in FMT assessment is the healthy donor selection. First, potential donors have to undergo a medical interview to exclude risk factors. The main objective of donor selection is to reduce and prevent any adverse events related to the infused fecal material (21). Subsequently, serological and microbiological exams are performed on donor's fresh stool and blood. The aim is to avoid any possible infection. Laboratorists check for the presence of any pathogens, such as HIV, HBV, HCV, *Treponema pallidum*, Human T-lymphotropic virus I and II, *Plasmodium* spp., *Trypanosoma* spp., *Mycobacterium tuberculosis*, *Campylobacter* spp., *Escherichia coli* O157 H7, *Yersinia*, vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), Gram-negative multidrug-resistant bacteria, *Norovirus,* antigens and/or acid fast staining for *Giardia lamblia* and *Cryptosporidium parvum*, protozoa (including *Blastocystis hominis*), helminths, and fecal occult blood testing. Regarding the laboratory preparation method, it is possible to choose between two different procedures: fresh and frozen preparations (22, 23). The first procedure requires that the feces of the donor be processed within 6 h after defecation to preserve the integrity of the anaerobic bacteria (24). Any amount of feces ranging from 30 to 50 g should be used and diluted in saline solution (NaCl 0.9%). The

frozen approach consists of freezing fecal suspensions with the addition of glycerol at −80°C. Glycerol is necessary to preserve bacterial vitality during freezing (7). On the day of infusion, fecal suspension thawing should be performed at 37°C and then diluted to the desired volume by adding NaCl 0.9% (7, 25). Freezing feces is important for establishing a stool bank and the availability of stool on demand. Both methods have high efficacy (7). Promising findings suggest that FMT can also be used in the management of other extra-intestinal disorders associated with microbiota alterations. Some clinical conditions in which FMT could be a potential therapeutic method are described in **Figure 1**.

GENITAL FEMALE MICROBIOTA THROUGHOUT LIFE

The vaginal district is a complex and essential ecosystem for female health and conception achievement/success. One aspect that needs to be observed is the microbial population residing in this region (34). In the last decade, metagenomic analysis has allowed researchers to outline the vaginal microbial ecosystem. The predominant species detected in the vagina of most healthy women are *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners,* and *Lactobacillus jensenii* (35). Other important microbes found in healthy women are strictly anaerobic bacteria, such as *Atopobium*, *Gardnerella* (an opportunistic pathogen), *Megasphaera*, *Prevotella*, and *Peptoniphilus* (36). One important aspect is the dynamic shift that occurs across the female lifecycle and how it contributes to maintaining vaginal health. During perinatal development, the vaginal epithelium is thickened by residual maternal estrogen. This action allows for the deposition of glycogen in epithelial cells. Subsequently, glycogen is released by exfoliation of the epithelial cells, favoring glucose-fermenting microorganisms (37). Postnatally, when maternal estrogen is metabolized, the vaginal mucosa undergoes a thinning that leads to a reduction in glycogen and glucose-fermenting microorganisms and an ecological selection for a wide range of aerobes and facultative anaerobes (35). During childhood, microbiota is mostly populated by Gram-negative anaerobes, such as *Bacteroides, Fusobacterium, Veillonella*, and some Gram-positive anaerobes, including *Actinomyces, Bifidobacterium, Peptococcus, Peptostreptococcus*, and *Propionibacterium*.

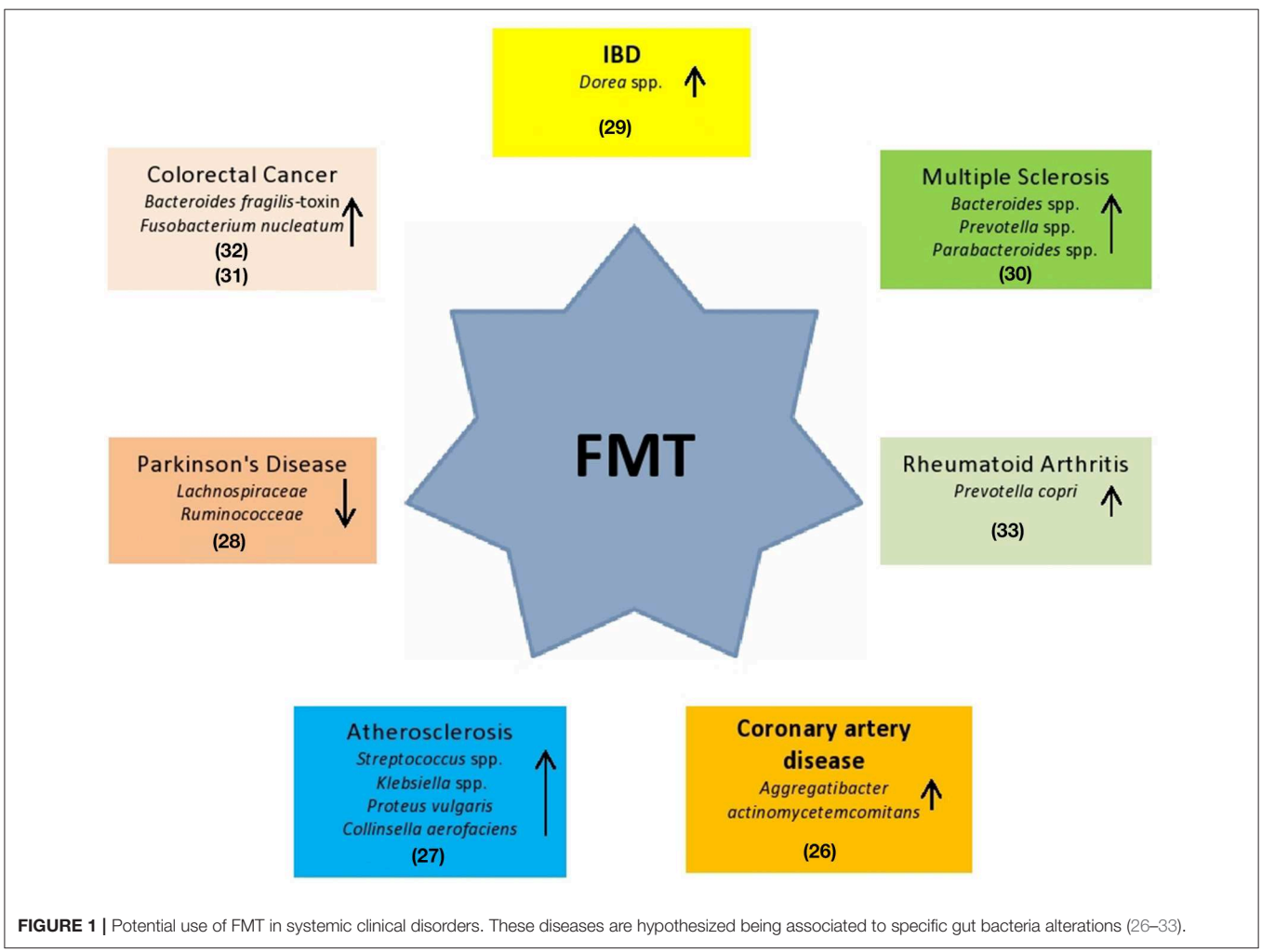

FIGURE 1 | Potential use of FMT in systemic clinical disorders. These diseases are hypothesized being associated to specific gut bacteria alterations (26–33).

Regarding facultative anaerobic bacteria, *Staphylococcus aureus*, *Staphylococccus epidermidis*, *Streptococcus viridans*, and *Enterococcus faecalis* are mostly found (38). In prepubertal age, *Lactobacillus*, *Gardnerella vaginalis,* and *Prevotella bivia* are in low abundance. As described above, estrogen levels play a key role in vaginal bacteria colonization. Specifically, with the beginning of puberty, estrogen increases lead to further thickening, selecting for glucose-fermenting microorganisms. During adolescence, the microbiota evolves and becomes similar to the vaginal microbiota of adult women. In this stage, the dominant species are *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners,* and *Lactobacillus jensenii* (38). The great decline of estrogen levels during menopause leads to a further switch in vaginal bacterial composition. In this stage of life, the microbiome is mainly characterized by *L. crispatus, L. iners, G. vaginalis*, and *Prevotella* and a lower abundance of *Mobiluncus, Staphylococcus, Bifidobacterium*, *Gemella,* and yeasts, such as *Candida* (39).

UTERINE AND CERVICOVAGINAL MICROBIOTA AND LOCAL FACTORS

The uterus and vagina are in close anatomical proximity. For this reason, it is necessary to consider their microbial community as in continuous cross-talk. Cervicovaginal microbiota consists of 10^8 bacteria/gram of vaginal fluid (40); it is decisive in women's health and reproductive outcomes. Despite being one of the simplest commensal bacterial communities in the human body, we are only beginning to appreciate its complex dynamic nature and important role in host immune modulation. Bacteria in the urogenital tract represent 9% of the total human microbiota, and most of them are difficult to culture. For almost a century, a common opinion stated that a healthy uterine cavity was germ-free (41). It was hypothesized that this sterility is guaranteed by the cervical plug, which constitutes an impermeable barrier to any bacteria coming from the vagina (42). In the last decade, many studies have challenged this assumption using culture-dependent methods and investigating the composition of the cervical mucous plug, which has been shown to not be impermeable to bacterial migration from the vaginal tract (35). In this review, our main question was whether to consider these microbes as "residents," "tourists," or "invaders." The uterus and vagina are in strict continuity, allowing for an inevitable flow of bacteria to the uterine cavity and a dysregulation of uterine contractions, which may also promote bacterial colonization. Lactobacilli are fundamental for female reproductive tract homeostasis. The principal feature that makes lactobacilli defenders of the cervicovaginal niche is represented by their production of lactic acid and hydrogen peroxide, which maintain an acidic environment, with a pH ∼4.0, which is inhospitable to the growth of catalase-negative bacteria. Ravel et al. (36), studied the cervicovaginal ecosystem among North American women of reproductive age using bacterial 16S rRNA gene sequencing. The results suggested the presence of five *community state types* (CSTs). CSTs I-IV are characterized by an abundance of *Lactobacillus* species (CST I, *L. crispatus*; CST II, *L. gasseri*; CST III, *L. iners*; CST V, *L. jensenii*), while CTS V consists mainly of anaerobes (36). *Lactobacillus*-dominant communities have shown important prevalence differences among healthy woman depending on their racial belonging. In fact, *Lactobacillus* spp. ranged from 90% in white women, 80% in Asian women and only 60% in Hispanic and black women. Moreover, it has been demonstrated that specific cervicovaginal bacteria are related to HIV acquisition. Specifically, in a South African study, a cohort of young women was screened in order to describe links between their vaginal microbiome and risk of acquiring HIV infection. The results showed that women with *L. crispatus*-dominant microbiota had a 4-fold lower risk of infection than women with highly diverse bacterial communities (43). Remarkably, not all *Lactobacillus* spp. are protective. For example, *Lactobacillus iners* increases pro-inflammatory cytokines, predisposing to infection. This action is implemented by a variety of other taxa, such as *Prevotella melaninogenica, Prevotella bivia, Veillonella, Mycoplasma*, and *Sneathia sanguinegens*, increasing the HIV acquisition rate (44, 45). A *Lactobacillus*-deficient cervicovaginal district is associated with higher genital pro-inflammatory cytokine levels and increased genital antigen-presenting cell activation via lipopolysaccharide (LPS)-sensing pathways and increased genital $CD4^+$ T cell counts, acting as a "trigger" of local immunity (3, 43). Conversely, a vaginal ecosystem with a dominance of lactobacilli, particularly *L. crispatus*, is characterized by a low inflammatory microenvironment. Endocervix and a thick mucous layer represent very important elements that contribute to innate immune barriers (46). In the mucous, we can find the presence of IgG, secretory IgA, lactoferrin, lysozyme, and other antibacterial compounds. Moreover, T-cells and antigen-presenting cells are also in the lower reproductive endothelium.

Several studies have reported conflicting findings on the beneficial activity by *Lactobacillus*abundance. Higher levels were found in groups of women with endometrial polyps (EP) or in women with EP associated with chronic endometriosis (EP + CE) compared with healthy controls (47).

In contrast, other studies suggested that high levels of *Lactobacillus* are significantly associated with increased reproductive success in women undergoing *in vitro* fertilization (IVF) (48).

In recent years, many studies have shown how bacterial communities can play a key role in fecundation (42, 49). These new considerations are decisive for the development of new approaches to improve fertility, conception, healthy pregnancy, and bacterial colonization of the infant and to prevent preterm birth. Considering this strong relationship between microbes and fertility, the main objective must be to intervene directly on these populations in order to increase reproductive success outcomes. An example of this strategy is represented by the application of the probiotics *Lactobacillus brevis CD2, Lactobacillus plantarum FV9*, and *Lactobacillus salivarius FV2.* These species seem to exercise a protective action on sperm motility and viability *in vitro* by preventing membrane damage induced by reactive oxygen species (35). This suggests that lactobacilli species resident in the vagina could be a protective factor for spermatozoa function during

and after intercourse. This relationship has also been observed in artificial reproduction technique (ART) outcomes. In fact, vaginal microbiota composition, on the day of embryo transfer and after IVF, influenced the success of pregnancy (50). As described before, some disorders strongly alter cervicovaginal flora. Uterine microbiota composition has been shown to be significantly different in women with endometriosis (51). Furthermore, Cicinelli et al. reported that endometriosis patients treated with antibiotics before implantation had significantly better reproductive outcomes compared with those not treated (52). Endometriosis patients are characterized by low levels of *Lactobacillus* spp. and an increase of *Streptococcus* spp., *Staphylococcus* spp., and Gram-negative bacteria belonging to the family *Enterobacteriaceae*. This relationship between bacteria, endometriosis and reproductive outcomes has been studied, demonstrating how a "*Lactobacillus* dominant (LD)" population and a "non-*Lactobacillus* dominant" (NLD) show significant differences in terms of successful outcomes. Specifically, women with an LD uterine microbiome had markedly higher rates of implantation [60.7 vs. 23.1% ($P = 0.02$)], pregnancy [70.6 vs. 33.3% ($P = 0.03$)], ongoing pregnancy [58.8 vs. 13.3% ($P = 0.02$)], and live births [58.8 vs. 6.7% ($P = 0.002$)] compared with those with an NLD uterine microbiome composition (53). Furthermore, researchers are studying the functional impact of uterine microbial population and their relationship with the microenvironment. The promotion of vaginal health is influenced by probiotics with various combinations of *L. reuteri L. acidophilus, L. gasseri*, *L. rhamnosus, L. plantarum,* and *L. crispatus*. When given as a short course (3–10 days), after 1 month, rates of vaginal colonization by probiotic strains ranged from 10% for oral *L. rhamnosus* to 53% for a vaginal product, including *L. gasseri, L. fermentum, L. rhamnosus*, and *Pediococcus acidilactici.* Treatment with a vaginal *L. crispatus* product established persistent colonization up to 1 month in 44%−59% of women (46). However, 1 week after the treatment, the rates of colonization decreased in sexually active women and in women yet colonized by endogenous lactobacilli (54). Another potential approach to modulate cervicovaginal microbiota is hormone therapy. In 2013, van de Wijert et al. (55) observed that combined oral contraceptives and progestin-only injectable contraceptives were mainly associated with a lower incidence of bacterial vaginosis (BV). In post-menopausal women, when the concentration of *Lactobacilli* was reduced, it was observed that treatment with oral estradiol hormone replacement therapy seemed to influence vaginal microbiota, increasing colonization by *Lactobacillus* spp. (56).

PROSPECTIVE FMT TREATMENT OF FEMALE GENITAL TRACT DISEASES

The role of commensal bacteria in stimulating local and systemic immunity appears to be established. Some of the new strategies for the treatment of diseases are focused on chemically synthetized molecules or recombinant proteins (57), and this type of approach requires oral or topical administration. Moreover, such compounds frequently show low levels of stability and, sometimes, in cases of recombinant proteins, contain remnants of inflammatory contaminants (58). Bacterial strains resident in the gut and vagina crosstalk, which leads to local and systemic immune regulation. In this regard, particular bacterial species can be engineered and used as oral vectors capable of triggering local and systemic immune responses in order to prevent (e.g., vaccines) or alleviate disease symptoms (59).

Induction of humoral and cellular immune responses represents a fundamental aspect involved in preventing female genital tract disorders. As other secretions, vaginal and cervical fluid contain mainly IgA and a certain proportion of IgG of plasma origin (60). The female genital tract does not contain organized lymphoepithelial structures analogous to intestinal Peyer's patches. This aspect is crucial to identify alternative routes of immunization to bypass local application of antigens. Thus, oral, rectal and nasal administrations were suggested (61). Furthermore, a central aspect is represented by the homing of activated lymphocytes, which depends on the expression of specific surface receptors that recognize elements on endothelial cell walls. An example is $\alpha 4\beta 7$ and *L-selectin* receptor, which guide immune cells to the gut mucosa and peripheral lymph nodes, respectively (62). An intriguing study was conducted in 2001 by Kutteh et al. They explored the efficacy of intestinal tract immunization in the induction of specific antibodies in human female genital tract secretions. A live attenuated strain of *Salmonella typhi* Ty 21a was used as a vector. In this study, 15 women were vaccinated orally, and 11 volunteers received the same dose rectally. To evaluate the effect on mucosal responses, seven women that had been vaccinated orally 6 months prior received a rectal boost. The data showed significant increases of IgA, predominantly, and IgG in vaginal and cervical fluids. Increased immunoglobulin levels were also observed after rectal boosting. This demonstrated that specific antibodies in the female genital tract induced by primary vaccination could be enhanced via subsequent rectal administration (60). Two aspects clearly emerged from this study: (1) the administration of live bacterial vectors induces a local and systemic immune response; (2) rectal administration may also induce stimulation at the cervicovaginal level. In this regard, FMT could be a natural alternative to recombinant bacterial vectors. In fact, with single or multiple infusions, large amounts of bacteria and metabolites could be administered, each of which could serve as an inducer of local and systemic immune responses.

Indeed, understanding the connection between intestinal and vaginal microbiota may represent a goal for new treatments of female genital tract disorders. New gene sequencing techniques have allowed characterization of the intestinal and vaginal populations and therefore led to hypotheses of interactions in states of health or disease. In 2017, Lindheim et al. investigated stool microbiome of women with polycystic ovary syndrome (PCOS) and healthy controls. PCOS is a common disorder affecting 5–10% of women in their reproductive years. As far as we know, this syndrome is characterized by the presence of three main features: hyperandrogenism, oligo/anovulation, and polycystic ovaries on pelvic ultrasound (63). The etiology and pathogenesis of PCOS remain unclear and may be multi-factorial,

involving genetic, neuroendocrine, and metabolic causes (64). Other parameters, such as gut barrier integrity, endotoxaemia and inflammation, were also evaluated. The stool microbiome of PCOS patients showed a lower diversity and an altered phylogenetic composition compared to controls. Significant differences in taxa, with a relative abundance >1%, were not observed (65). Regarding rare taxa, the relative abundance of bacteria from the phylum Tenericutes (relevant genera include *Mycoplasma* spp., *Ureaplasma* spp.) and the family S24-7 (phylum Bacteroidetes) was significantly lower. Moreover, patients did not show alterations in all markers of gut barrier function and endotoxaemia (65). These findings suggest that changes of gut microbiota also had trends similar to the variations of metabolic symptoms. Other studies observed that some Gram-negative bacteria belonging to the genera *Bacteroides* and *Escherichia/Shigella* significantly increased in the gut of PCOS women with obesity. In these conditions, LPS produced by these microorganisms was demonstrated to induce chronic inflammation, obesity, and insulin resistance in LPS-infused mice (5). In this status, spore-forming species, such as *Clostridiales*, decrease, while Proteobacteria and Bacteroidetes increase. Other evidence suggests a multifactorial relationship in determining the shape of cervicovaginal microbiota. Recently, a potential relationship between sex hormones and gut microbiota emerged. This novel concept has been defined as *"microgenderome"* (66). At the time of puberty, sex hormone levels exercise specific influences on the composition of the microbiota. Removal of gut microbiota showed an increase in testosterone concentration in female mice but a decrease in male mice. Thus, the commensal gut microbiota also had effects on production of the male sex hormone (67). It is interesting to explore the role of gut microbiota in PCOS, in which androgen levels in PCOS women are always elevated. Tremellen and Pearce suggest that dysbiosis of the gut microbiota (DOGMA) brought about by a high fat-sugar diet in PCOS patients leads to an increase in intestinal permeability. Lipopolysaccharide produced by Gram-negative bacteria traverse the gut wall to enter the circulation, leading to a chronic state of low-grade inflammation. Activation of the immune system interferes with insulin receptor, driving up insulin levels, which boost testosterone production in the ovary, leading to PCOS. DOGMA theory can account for the role of gut microbiota in the pathogenesis of PCOS (68). This evidence has been reinforced by a study on induced PCOS using a rat model (67), in which the results showed that PCOS rats displayed abnormal oestrous cycles, represented by increasing androgen biosynthesis, and exhibited multiple large cysts with diminished granulosa layers in ovarian tissues. Moreover, the composition of gut microbiota in rats treated with letrozole, a non-steroidal aromatase inhibitor, was different from controls. *Lactobacillus, Ruminococcus,* and *Clostridium* species were lower, while *Prevotella* species was higher in PCOS rats compared with control rats. The rats were treated using FMT from healthy rats. It was found that the oestrous cycles were improved in all rats in the FMT group, with decreased androgen biosynthesis and normalized ovarian morphologies. The composition of the restored gut microbiota in the FMT group was characterized by an increase in *Lactobacillus* and *Clostridium* and a decrease in *Prevotella*. This evidence indicated that the dysbiosis of gut microbiota was associated with the pathogenesis of PCOS and emphasized the hypothesis aimed at implementing the use of FMT for extra-intestinal disorders. In fact, there is strong evidence that host-microbial interactions play a key role in reproductive health outcomes. The challenge is to find tools and strategies capable of modulating microbial populations in the cervicovaginal microenvironment, using probiotics, as well (46). Other female tract disorders are associated with gut microbiota alterations, such as endometriosis and BV.

Endometriosis is a condition defined by the presence of endometriotic gland and stroma outside of the uterine cavity, ranging in severity levels from I to IV (69). Many studies have emphasized how immunologic alterations and microbiological factors, independently or in association with epigenetic changes, might play a role in the development of the disease (70). Dysbiosis influences estrogen levels in the circulation (71), and increased estrogen levels can stimulate growth of ectopic endometriotic foci and inflammatory activity. Thus, gut microbiota influencing the regulation of the estrogen cycle could be associated with endometriosis. In an interesting recent study, Ata et al. performed an in depth characterization and comparison of vaginal, cervical, and gut microbiota in women with stage III–IV endometriosis and healthy controls. The results showed overall comparable compositions of the vaginal, cervical, and gut microbiota. However, there were some differences between the bacterial groups, with some interesting decreases, absences or increases of some species. Specifically, the complete absence of *Atopobium* in the vagina and cervix, together with the increased abundance of *Gardnerella, Escherichia/Shigella,* and *Ureoplasma* in the cervical microbiome of patients with endometriosis could be a relevant finding (72). *Atopobium* spp. was recently implicated as a gynecological pathogen associated with bacterial vaginosis, obstetric bacteraemia, and, possibly, with endometrial cancer (73). These findings suggest an active role played by resident bacteria in the cervix-vaginal district and interactions with intestinal microbes. This bacterial network appears to be related to local and system female conditions.

As previously mentioned, menstruation and menopause, caused by hormonal changes, contribute to a drastic modification in the vaginal microbiota, specifically decreases in lactobacilli. In this condition, infections by *Gardnerella vaginalis* (GV) and *Candida albicans* are promoted (74). GV plays a key role in vaginal immunity. In fact, this bacteria is able to increase levels of several inflammatory factors, such as NF-kB, TNF-α, myeloperoxidase activity, COX-2, and iNOS. This pattern of molecules leads to BV (75, 76). Some studies have shown how the oral administration of probiotics can influence immunity in the vagina (77). Probiotics are a valid alternative to antibiotic treatment in order to avoid antimicrobial resistance. Data suggest that strains of *L. acidophilus*, *L. rhamnosus* and *L. johnsonii* mitigate GV-vaginosis in mice (78). Kim et al. have demonstrated that administration of NK3 *(L. plantarum*), NK49 (*B. longum*), and their mixture alleviated GV-induced BV in mice by reducing TNF-α levels and myeloperoxidase activity and increasing IL-10 expression.

This treatment also contributed to COX-2, iNOS and NF-Kb suppression (77). In this study, the authors also investigated fecal microbiota composition in mice with GV-induced BV using qPCR. High levels of GV significantly increased Firmicutes and Proteobacteria, the main LPS-producers, and suppressed Bacteroidetes populations in the vagina. Moreover, GV infection was able to increase inflammatory molecules, such as TNF-α, NF-Kb, and myeloperoxidase, in the colon. However, treatment with NK3 and/or NK49 significantly decreased Proteobacteria and increased Bacteroidetes, contributing to the inhibition of gut microbiota LPS production (**Figure 2**). Crosstalk between the gut and vagina environment seems evident. Oral administration of probiotics, and then bacteria, might play a crucial role in the suppression of the pro-inflammatory cytokine expression in the vagina. This action influences the inflammatory status in the gut, acting on bacteria and immune cells, such as macrophages, dendritic cells and local lymphocytes. It could be interesting to hypothesize a similar action using FMT, which would allow a direct administration of bacteria and metabolites in the gut, bypassing stomach passage.

CONCLUDING REMARKS

Undoubtedly, the countless microbial communities present on the body's surface and in internal organs are directly involved in homeostasis (2). Knowledge into the interactions between the intestinal microbiota and health/disease balance, due to immune-related actions that influence extra-intestinal sites and diseases, is deepening. Concerning vaginal districts, evidence indicates that the immune system is involved in local disorders (59). Indeed, this relationship could be compellingly linked to gut microbes. On the other hand, vaginal microbiota represents a complex biological niche, contributing to the local health and coinciding with the anatomical intricacy of the female genital tract. Its proximity and connection to the intestine allows for strong interactions between gut and vaginal bacteria. These aspects must also consider the numerous chemical changes that the female reproductive tract undergoes with age and, periodically, with hormonal interactions (35). Then, a refined equilibrium between gut microbiota, immunity, vaginal microbiota, and hormones is essential in the physiological state of the female genital tract. Considering recent literature evidences, a direct action exerted by oral probiotics in the modulation of vaginal disorders such as BV appears clear (78). Another important finding was the association of specific patterns of intestinal bacteria with vaginal pathologies such as endometriosis and PCOS (67, 72). Alongside the synergistic action of intestinal and vaginal bacteria, the hormones, such as oestrogens, influence the cervicovaginal microenvironment (66). It is necessary to continue focusing

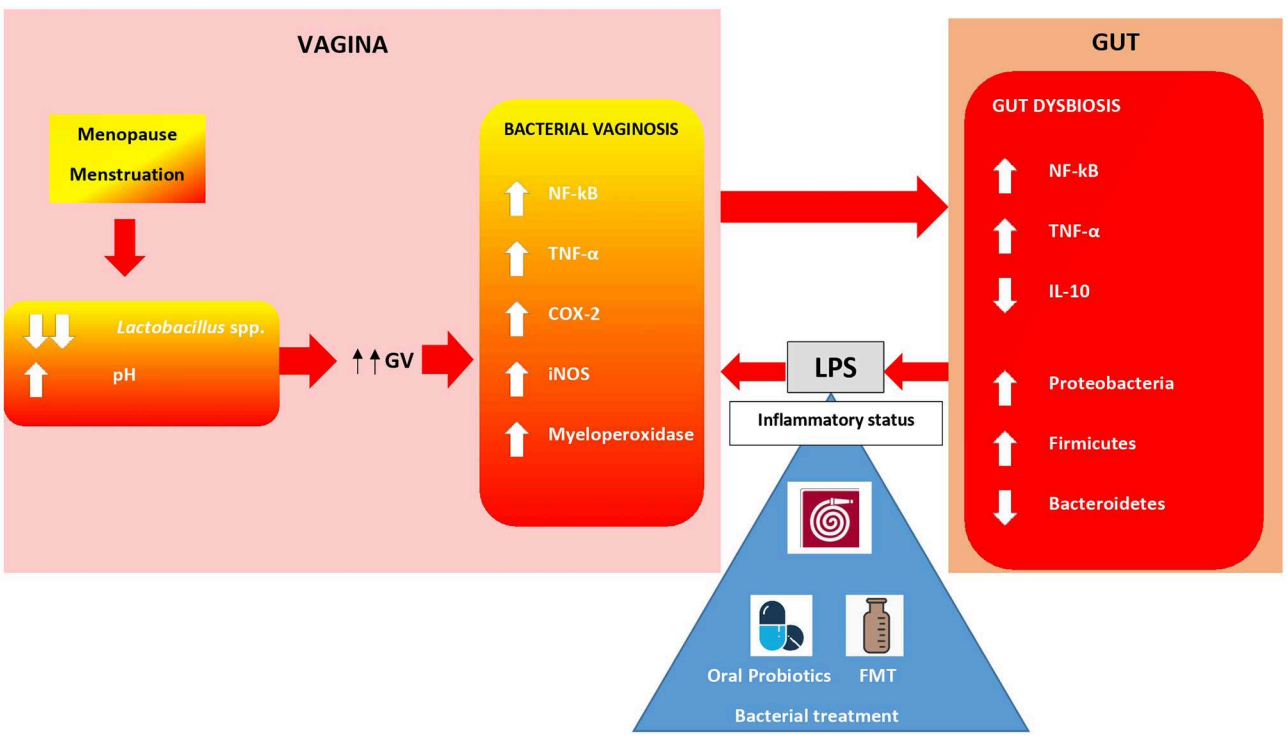

FIGURE 2 | Gut-Vagina cross-talking. Bacteria and immunity cells in vagina and gut are in close communication. Hormonal changes during menopause, menstruations lead to an overgrowth of anaerobic species such as *Gardnerella vaginalis* (GV). Bacterial vaginosis (BV), caused by GV, increases an immunity molecules release which exerts pro-inflammatory effects *in situ* and in the gut. Proteobacteria overgrowth leads to LPS iper-production establishing an inflammatory status. Oral probiotics alleviate BV in women. FMT might play a similar role in regulating both altered gut flora and vagina inflammation (74, 77).

on the dynamics of these interactions and on the effects on health status or diseases. The new tools developed in modern microbiology, such as metagenomic and culturomic analysis, must lead to a greater understanding of microorganism-host interactions and modify the simple concept of "commensal" or "pathogen" (8). The right way is to enhance the biological context in which the bacterial species live and the biological pathways they control. Gut bacteria are an "orchestra" exerting mechanisms of regulation on extra-intestinal bacteria, circulating hormone levels, and immunity. Indeed, manipulating the microbiome composition is a potential strategy to prevent or mitigate several diseases. The next step for the scientific community must be to clarify what is "normal" in the human microbiome, including cervicovaginal microbes, in order to define dysbiotic, disease-associated profiles, with the aim of providing therapies, or lifestyle interventions to return it to normal again.

A concrete future prospect could involve "driving" the bacterial populations that inhabit these organs through therapeutic procedures, such as fecal transplantation, as yet demonstrated in CDI resolution (7). FMT consists of a liquid feces suspension, infused into a recipient colon-site. In this way, bacteria and metabolites are able to modulate both local microbial community and systemic immunity, influencing other districts balances, as hopefully the female genital-tract one. An advanced, intriguing possibility could involve the infusion of a synthetic bacterial suspension consisting exclusively of selected and controlled bacterial populations in order to obtain an "*ad personam*" therapeutic approach.

AUTHOR CONTRIBUTIONS

GQ, MS, and LM drafted the manuscript and conducted the literature review.

REFERENCES

1. Johansson ME, Jakobsson HE, Holmen-Larsson J, Schutte A, Ermund A, Rodriguez-Pineiro AM, et al. Normalization of host intestinal mucus layers requires long-term microbial colonization. *Cell Host Microbe*. (2015) 18:582–92. doi: 10.1016/j.chom.2015.10.007
2. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. *Science*. (2005) 308:1635–8. doi: 10.1126/science.1110591
3. 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
4. Baker J, Chase D, Herbst-Kralovetz M. Uterine microbiota: residents, tourists, or invaders? *Front Immunol*. (2018) 9:208. doi: 10.3389/fimmu.2018.00208
5. Liu R, Zhang C, Shi Y, Zhang F, Li L, Wang X, et al. Dysbiosis of gut microbiota associated with clinical parameters in polycystic ovary syndrome. *Front Microbiol*. (2017) 28:324. doi: 10.3389/fmicb.2017.00324
6. Oren A, Rupnik M. Clostridium difficile and clostridioides difficile: two validly published and correct names. *Anaerobe*. (2018) 52:125–6. doi: 10.1016/j.anaerobe.2018.07.005
7. Cammarota G, Ianiro G, Tilg H, Rajilić-Stojanović M, Kump P, Satokari R, et al. European consensus conference on faecal microbiota transplantation in clinical practice. *Gut*. (2017) 66:569–80. doi: 10.1136/gutjnl-2016-313017
8. Lagier JC, Dubourg G, Million M, Cadoret F, Bilen M, Fenollar F, et al. Culturing the human microbiota and culturomics. *Nat Rev Microbiol*. (2018) 1:540–50. doi: 10.1038/s41579-018-0041-0
9. Scarpellini E, Ianiro G, Attili F, Bassanelli C, De Santis A, Gasbarrini A. The human gut microbiota and virome: potential therapeutic implications Dig. *Liver Dis*. (2015) 47:1007–12. doi: 10.1016/j.dld.2015.07.008
10. Yadav M, Verma M, Chauhan N. A review of metabolic potential of human gut microbiome in human nutrition. *Arch Microbiol*. (2018) 200:203–17. doi: 10.1007/s00203-017-1459-x
11. Kanwar SS, Walia S, Sharma S. Impact of probiotics and gut microbiota on host behavior. In: Garg N, Abdel-Aziz S, Aeron A, editors. *Microbes in Food and Health*. Cham: Springer (2016). p. 29–41. doi: 10.1007/978-3-319-25277-3_2
12. Andersson AF, Lindberg M, Jakobsson H, Bäckhed F, Nyrén P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE*. (2008) 3:e2836. doi: 10.1371/journal.pone.0002836
13. Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, et al. Molecular analysis of the bacterial microbiota in the human stomach. *Proc Natl Acad Sci USA*. (2006) 103:732–7. doi: 10.1073/pnas.0506655103
14. Paroni F, Palladini A, Masucci L, Cannistraci CV, Pastorino R, Ianiro G, et al. Effects of proton pump inhibitors on the gastric mucosa-associated microbiota in dyspeptic patients. *Appl Environ Microbiol*. 82:6633–44. doi: 10.1128/AEM.01437-16
15. Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benno Y. Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. *J Med Microbiol*. (2005) 54:1093–101. doi: 10.1099/jmm.0.45935-0
16. Kumar J, Kumar M, Gupta S, Bhambi M, Pandey R, et al. An improved methodology to overcome key issues in human fecal metagenomic DNA extraction. *Genom Proteom Bioinform*. (2016) 14:371–8. doi: 10.1016/j.gpb.2016.06.002
17. Cammarota G, Ianiro G, Cianci R, Bibbò S, Gasbarrini A, Currò D. The involvement of gut microbiota in inflammatory bowel disease pathogenesis: potential for therapy. *Pharmacol Ther*. (2015) 149:191–212. doi: 10.1016/j.pharmthera.2014.12.006
18. Jørgensen SMD, Hansen MM, Erikstrup C, Dahlerup JF, Hvas CL. Faecal microbiota transplantation: establishment of a clinical application framework. *Eur J Gastroenterol Hepatol*. (2017) 29:e36–45. doi: 10.1097/MEG.0000000000000958
19. Ianiro G, Masucci L, Quaranta G, Simonelli C, Lopetuso LR, Sanguinetti M, et al. Randomised clinical trial: faecal microbiota transplantation by colonoscopy plus vancomycin for the treatment of severe refractory *Clostridium difficile* infection-single versus multiple infusions. *Aliment Pharmacol Ther*. (2018) 48:152–9. doi: 10.1111/apt.14816
20. Filip M, Tzaneva V, Dumitrascu DL. Fecal transplantation: digestive and extradigestive clinical applications. *Clujul Med*. (2018) 91:259–65. doi: 10.15386/cjmed-946
21. Ramai D, Zakhia K, Ofosu A, Ofori E, Reddy M. Fecal microbiota transplantation: donor relation, fresh or frozen, delivery methods, cost-effectiveness. *Ann Gastroenterol*. (2019) 32:30–8. doi: 10.20524/aog.2018.0328
22. Takahashi M, Ishikawa D, Sasaki T, Lu YJ, Kuwahara-Arai K, Kamei M, et al. Faecal freezing preservation period influences colonization ability for faecal microbiota transplantation. *J Appl Microbiol*. (2019) 126:973–84. doi: 10.1111/jam.14167
23. Perez E, Lee CH, Petrof EO. A practical method for preparation of fecal microbiota transplantation. *Methods Mol Biol*. (2016) 1476:259–67. doi: 10.1007/978-1-4939-6361-4_19
24. Papanicolas LE, Choo JM, Wang Y, Leong LEX, Costello SP, Gordon DL, et al. Bacterial viability in faecal transplants: which bacteria survive? *EBioMedicine*. (2019) 41:509–16. doi: 10.1016/j.ebiom.2019.02.023
25. Kim P, Gadani A, Abdul-Baki H, Mitre R, Mitre M. Fecal microbiota transplantation in recurrent *Clostridium difficile* infection: a retrospective single-center chart review. *JGH Open*. (2018) 3:4–9. doi: 10.1002/jgh3.12093

26. Hyvärinen K, Mäntylä P, Buhlin K, Paju S, Nieminen MS, Sinisalo J, et al. A common peridontal pathogen has an adverse association with both acute and stable coronary artery disease. *Atherosclerosis*. (2012) 223:478–84. doi: 10.1016/j.atherosclerosis.2012.05.021
27. Ott SJ, El Mokhtari NE, Musfeldt M, Hellmig S, Freitag S, Rehman A. Detection of diverse bacterial signatures in atherosclerosis lesions of patients with coronary heart disease. *Circulation*. (2006) 113:929–37. doi: 10.1161/CIRCULATIONAHA.105.579979
28. Keshavarzian A, Green SJ, Engen PA, Voigt RM, Naqib A, Forsyth CB. Colonic bacterial composition in Parkinson's disease. *Mov Disord*. (2015) 30:1351–60. doi: 10.1002/mds.26307
29. Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, et al. Linking the human gut microbiome to inflammatory cytokine production capacity. *Cell*. (2016) 167:1125–36.e8. doi: 10.1016/j.cell.2016.10.020
30. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Soldan MM, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep*. (2016) 6:28484. doi: 10.1038/srep28484
31. Rubinstein MR, Wang X, Liu W. *Fusobacterium nucleatum* promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. *Cell Host Microbe*. (2013) 14:195–206. doi: 10.1016/j.chom.2013.07.012
32. Boleij A, Hechenbleikner EM, Goodwin AC. The *Bacteroides fragilis* toxin gene is prevalent in the colon mucosa of colorectal cancer patients. *Clin Infect Dis*. (2015) 60:208–15. doi: 10.1093/cid/ciu787
33. Scher JU, Sczesnak A, Longman R. Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. *Elife*. (2013) 5:e01202. doi: 10.7554/eLife.01202
34. Petrova MI, Lievens E, Malik S, Imholz N, Lebeer S. *Lactobacillus* species as biomarkers and agents that can promote various aspects of vaginal health. *Front Physiol*. (2015) 6:81. doi: 10.3389/fphys.2015.00081
35. Younes JA, Lievens E, Hummelen R, van der Westen R, Reid G, Petrova MI. Women and their microbes: the unexpected friendship. *Trends Microbiol*. (2018) 26:16–32. doi: 10.1016/j.tim.2017.07.008
36. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA*. (2011) 108 (Suppl. 1):4680–7. doi: 10.1073/pnas.1002611107
37. Paavonen J. Physiology and ecology of the vagina. *Scand J Infect Dis Suppl*. (1983) 40:31–5.
38. Randelovic G, Mladenović V, Ristić L, Otašević S, Branković S, Mladenović-Antić S, et al. Microbiological aspects of vulvovaginitis in prepubertal girls. *Eur J Pediatr*. (2012) 171:1203–8. doi: 10.1007/s00431-012-1705-9
39. Gupta S, Kumar N, Singhal N, Kaur R, Manektala U. Vaginal microflora in postmenopausal women on hormone replacement therapy. *Indian J Pathol Microbiol*. (2006) 49:457–61.
40. Delaney ML, Onderdonk AB. Nugent score related to vaginal culture in pregnant women. *Obstet Gynecol*. (2001) 98:79–84. doi: 10.1097/00006250-200107000-00015
41. Gilbert SF, Sapp J, Tauber AI. A symbiotic view of life: we have never been individuals. *Q Rev Biol*. (2012) 87:325–41. doi: 10.1086/668166
42. Reid G. Cervicovaginal microbiome threats and possibilities. *Trends Endocrinol Metab*. (2016) 27:446–54. doi: 10.1016/j.tem.2016.04.004
43. 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
44. Verstraelen H, Verhelst R, Claeys G, De Backer E, Temmerman M, Vaneechoutte M. Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L.* crispatus promotes the stability of the normal vaginal microflora and that L gasseri and/or L iners are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol*. (2009) 9:116. doi: 10.1186/1471-2180-9-116
45. Callahan BJ, Di Giulio DB, Goltsman DSA, Sun CL, Costello EK, Jeganathan P, et al. Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc Natl Acad Sci USA*. (2017) 114:9966–71. doi: 10.1073/pnas.1705899114
46. Anahtar MN, Gootenberg DB, Mitchell CM, Kwon DS. Cervicovaginal microbiota and reproductive health: the virtue of simplicity. *Cell Host Microbe*. (2018) 23:159–68. doi: 10.1016/j.chom.2018.01.013
47. Fang RL, Chen LX, Shu WS, Yao SZ, Wang SW, Chen YQ. Barcoded sequencing reveals diverse intrauterine microbiomes in patients suffering with endometrial polyps. *Am J Transl Res*. (2016) 8:1581–92.
48. Franasiak JM, Werner MD, Juneau CR, Tao X, Landis J, Zhan Y, et al. Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. *J Assist Reprod Genet*. (2016) 33:129–36. doi: 10.1007/s10815-015-0614-z
49. Haahr T, Jensen JS, Thomsen L, Duus L, Rygaard K, Humaidan P. Abnormal vaginal microbiota may be associated with poor reproductive outcomes: a prospective study in IVF patients. *Hum Reprod*. (2016) 31:795–803. doi: 10.1093/humrep/dew026
50. Hyman RW, Herndon CN, Jiang H, Palm C, Fukushima M, Bernstein D, et al. The dynamics of the vaginal microbiome during infertility therapy with in vitro fertilization-embryo transfer. *J Assist Reprod Genet*. (2012) 105–15. doi: 10.1007/s10815-011-9694-6
51. Chen C, Song XL, Wei WX, Zhong HZ, Dai JJ, Lan Z, et al. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat Commun*. (2017) 8:875. doi: 10.1038/s41467-017-00901-0
52. Cicinelli E, Matteo M, Tinelli R, Pinto V, Marinaccio M, Indraccolo U, et al. Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage as confirmed by improved pregnancy outcome after antibiotic treatment. *Reprod Sci*. (2014) 21:640–7. doi: 10.1177/1933719113508817
53. Moreno I, Codoñer FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazán J, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am J Obstet Gynecol*. (2016) 215:684–703. doi: 10.1016/j.ajog.2016.09.075
54. Antonio MA, Rabe LK, Hillier SL. Colonization of the rectum by *Lactobacillus* species and decreased risk of bacterial vaginosis. *J Infect Dis*. (2005) 192:394–8. doi: 10.1086/430926
55. van de Wijgert JH, Verwijs MC, Turner AN, Morrison CS. Hormonal contraception decreases bacterial vaginosis but oral contraception may increase candidiasis: implications for HIV transmission. *AIDS*. (2013) 27:2141–53. doi: 10.1097/QAD.0b013e32836290b6
56. Shen J, Song N, Williams CJ, Brown CJ, Yan Z, Xu C, et al. Effects of low dose estrogen therapy on the vaginal microbiomes of women with atrophic vaginitis. *Sci Rep*. (2016) 6:24380. doi: 10.1038/srep24380
57. Ferrer-Miralles N, Domingo-Espín J, Corchero JL, Vázquez E, Villaverde A. Microbial factories for recombinant pharmaceuticals. *Microb Cell Fact*. (2009) 24:17. doi: 10.1186/1475-2859-8-17
58. Cano-Garrido O, Seras-Franzoso J, Garcia-Fruitós E. Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. *Microb Cell Fact*. (2015) 16:137. doi: 10.1186/s12934-015-0313-6
59. Medaglini D, Rush CM, Sestini P, Pozzi G. Commensal bacteria as vectors for mucosal vaccines against sexually transmitted diseases: vaginal colonization with recombinant streptococci induces local and systemic antibodies in mice. *Vaccine*. (1997) 15:1330–7. doi: 10.1016/S0264-410X(97)00026-1
60. Kutteh WH, Kantele A, Moldoveanu Z, Crowley-Nowick PA, Mestecky J. Induction of specific immune responses in the genital tract of women after oral or rectal immunization and rectal boosting with Salmonella typhi Ty 21a vaccine. *J Reprod Immunol*. (2001) 52:61–75. doi: 10.1016/S0165-0378(01)00109-7
61. Kantele A, Häkkinen M, Moldoveanu Z, Lu A, Savilahti E, Alvarez RD, et al. Differences in immune responses induced by oral and rectal immunizations with Salmonella typhi Ty21a: evidence for compartmentalization within the common mucosal immune system in humans. *Infect Immun*. (1998) 66:5630–5.
62. Kishimoto TK, Jutila MA, Butcher EC. Identification of a human peripheral lymph node homing receptor: a rapidly down-regulated adhesion molecule. *Proc Natl Acad Sci USA*. (1990) 87:2244–8. doi: 10.1073/pnas.87.6.2244
63. Norman RJ, Dewailly D, Legro RS, Hickey TE. Polycystic ovary syndrome. *Lancet*. (2007) 370:685–97. doi: 10.1016/S0140-6736(07)61345-2

64. Balen A. The pathophysiology of polycystic ovary syndrome: trying to understand PCOS and its endocrinology. *Best Pract Res Clin Obstet Gynaecol*. (2004) 18:685–706. doi: 10.1016/j.bpobgyn.2004.05.004
65. Lindheim L, Bashir M, Münzker J, Trummer C, Zachhuber V, Leber B, et al. Alterations in gut microbiome composition and barrier function are associated with reproductive and metabolic defects in women with polycystic ovary syndrome (PCOS): a pilot study. *PLoS ONE*. (2017) 12:e0168390. doi: 10.1371/journal.pone.0168390
66. Flak MB, Neves JF, Blumberg RS. Immunology. Welcome to the microgenderome. *Science*. (2013) 339:1044–5. doi: 10.1126/science.1236226
67. Guo Y, Qi Y, Yang X, Zhao L, Wen S, Liu Y. Association between polycystic ovary syndrome and gut microbiota. *PLoS ONE*. (2016) 11:e0153196. doi: 10.1371/journal.pone.0153196
68. Tremellen K, Pearce K. Dysbiosis of Gut Microbiota (DOGMA)—a novel theory for the development ofPolycystic Ovarian Syndrome. *Med Hypotheses*. (2012) 79:104–12. doi: 10.1016/j.mehy.2012.04.016
69. Giudice LC. Clinical practice. Endometriosis. *N Engl J Med*. (2010) 362:2389–98. doi: 10.1056/NEJMcp1000274
70. Kobayashi H, Higashiura Y, Shigetomi H, Kajihara H. Pathogenesis of endometriosis: the role of initial infection and subsequent sterile inflammation. *Mol Med Rep*. (2014) 9:9–15. doi: 10.3892/mmr.2013.1755
71. Flores R, Shi J, Fuhrman B, Xu X, Veenstra TD, Gail MH, et al. Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: a cross-sectional study. *J Transl Med*. (2012) 10:253. doi: 10.1186/1479-5876-10-253
72. Ata B, Yildiz S, Turkgeldi E, Brocal VP, Dinleyici EC, Moya A, et al. The endobiota study: comparison of vaginal, cervical and gut microbiota between women with stage 3/4 endometriosis and healthy controls. *Sci Rep*. (2019) 18:2204. doi: 10.1038/s41598-019-39700-6
73. Marconi C, Cruciani F, Vitali B, Donders GG. Correlation of atopobium vaginae amount with bacterial vaginosis markers. *J Low Genit Tract Dis*. (2012) 16:127–32. doi: 10.1097/LGT.0b013e31823c79c4
74. Amabebe E, Anumba DOC. The vaginal microenvironment: the physiologic role of *Lactobacilli*. *Front Med*. (2018) 5:181. doi: 10.3389/fmed.2018.00181
75. Zariffard MR, Novak RM, Lurain N, Sha BE, Graham P, Spear GT. Induction of tumor necrosis factor-alpha secretion and toll-like receptor 2 and 4 mRNA expression by genital mucosal fluids from women with bacterial vaginosis. *J Infect Dis*. (2005) 191:1913–21. doi: 10.1086/429922
76. Trinh HT, Lee IA, Hyun YJ, Kim DH. Artemisia princeps Pamp. Essential oil and its constituents eucalyptol and α-terpineol ameliorate bacterial vaginosis and vulvovaginal candidiasis in mice by inhibiting bacterial growth and NF-κB activation. *Planta Med*. (2011) 77:1996–2002. doi: 10.1055/s-0031-1280094
77. Kim DE, Kim JK, Han SK, Jang SE, Han MJ, Kim DH. *Lactobacillus plantarum* NK3 and Bifidobacterium longum NK49 Alleviate Bacterial Vaginosis and Osteoporosis in Mice by Suppressing NF-κB-Linked TNF-α Expression. *J Med Food*. (2019) 22:1022–31. doi: 10.1089/jmf.2019.4419
78. Joo HM, Hyun YJ, Myoung KS, Ahn YT, Lee JH, Huh CS, et al. *Lactobacillus johnsonii* HY7042 ameliorates Gardnerella vaginalis-induced vaginosis by killing Gardnerella vaginalis and inhibiting NF-κB activation. *Int Immunopharmacol*. (2011) 11:1758–65. doi: 10.1016/j.intimp.2011.07.002

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REVIEW
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Gut and Lung Microbiota in Preterm Infants: Immunological Modulation and Implication in Neonatal Outcomes

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In recent years, an aberrant gastrointestinal colonization has been found to be associated with an higher risk for postnatal sepsis, necrotizing enterocolitis (NEC) and growth impairment in preterm infants. As a consequence, the reasons of intestinal dysbiosis in this population of newborns have increasingly become an object of interest. The presence of a link between the gut and lung microbiome's development (gut-lung axis) is emerging, and more data show as a gut-brain cross talking mediated by an inflammatory milieu, may affect the immunity system and influence neonatal outcomes. A revision of the studies which examined gut and lung microbiota in preterm infants and a qualitative analysis of data about characteristic patterns and related outcomes in terms of risk of growing impairment, Necrotizing Enterocolitis (NEC), Bronchopulmonary Dysplasia (BPD), and sepsis have been performed. Microbiota take part in the establishment of the gut barrier and many data suggest its immune-modulator role. Furthermore, the development of the gut and lung microbiome (gut-lung axis) appear to be connected and able to lead to abnormal inflammatory responses which have a key role in the pathogenesis of BPD. Dysbiosis and the gut predominance of facultative anaerobes appear to be crucial to the pathogenesis and subsequently to the prevention of such diseases.

Keywords: gut microbiota, lung microbiota, preterm infants' outcomes, gut-lung axis, late-onset sepsis, necrotizing enterocolitis, growth impairment, bronchopulmonary dysplasia

INTRODUCTION

All of the microorganisms that inhabit the human body constitutes the so-called human microbiota. The Human Microbiota Project was launched in 2008 to deepen our understanding of how the microbiome (the whole set of microorganisms, their genomes and the environmental conditions) influences human health and diseases. 16S ribosomal RNA (16S rRNA) sequencing allows

characterizing the complexity of the microbial population to study whether there is an "healthy microbiota" and potential implications of different patterns (1).

The importance of gut microbiome is due to the role it plays as a major interface to the external environment: it contemporarily protects against pathogens and toxins while housing beneficial commensal bacteria which are pivotal to maintain homeostasis, support digestion, protect from injury, regulate intestinal immune function (2).

Some studies (3, 4) show that at birth infants are nearly sterile but they subsequently acquire microbial colonists. This process progresses in the first 2–3 years of life, until reaching an "adult-like state." In at term newborns this evolution appears to be driven by nutritional, immunological, hormonal and prebiotic effect of maternal milk (5).

It is difficult to identify a "healthy" microbiota of a population such that of preterm infants. The preterm birth is a non-physiological condition, exposed to many early life clinical factors that alter the normal colonists acquisition process (6, 7).

The early life can be defined as a "critical window" during which the occurrence of dysbiosis can impact the health of preterm infants, especially influencing the developing immune systems (8–10).

Moreover, Olm et al. (11) underlined as our understanding of the habitat range and subpopulation complexity of founding strains is impaired by methodological limitations. These authors compared the *in situ* bacterial growth rates of multiple body sites by using metagenomics to reconstruct the genomes of strains that colonized the skin, mouth, and gut of two hospitalized premature infants. The results show an overlap of strains across body sites and imply that the premature infant microbiome is characterized by a low total microbial diversity of the early community when compared to full-term infants.

Anyway, in recent years, many studies regarding the features of gut and lung microbiota of preterm infants have followed.

Gut Microbiota in Preterm Infants

Altought newborns' gut was thought to be sterile and commensal microbes only acquired after birth, recently growing evidences seem to suggest that non-sterile intrauterine conditions could be the origin of this acquisition: Aagard et al. performed a whole-genome shotgun metagenomic study of placental specimens. Their results showed a unique placental microbial flora that comprises non-pathogenic commensal microbes belonging to the *Tenericutes*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Fusobacteria* phyla (12); Collado et al. reported that the amniotic fluid hosts a distinct microbial community characterized by a predominance of *Proteobacteria* (13).

Moreover, a recent microbial profiling study based on 16S rRNA sequencing shows that regardless of the delivery mode, the microbial population in the meconium is influenced by that in the correspondent maternal placenta (14).

Vaginally delivered newborns directly come into contact with vaginal microbial population and their fecal microbiota is dominated by *Prevotella* and *Lactobacillus* (15, 16) while newborns delivered by cesarean section are more likely to have a microbiota dominated by microbes derived from maternal skin, hospital environment and even hospital staff such as *Corynebacterium*, *Staphilococcus* and *Propionibacterium* spp. (6, 16–18). It is also well-known that neonatal gut microbiota is strongly influenced by food intake: stools of breast-fed are richer of *Lactobacilli* and *Bifidobacteria* and poorer of potential pathogens than formula-fed newborns whose stools contain a more diverse microbial flora with a prevalence of *Bacteroides*, *Staphilococci*, *Clostridia*, *Enterococci*, *Enterobacteria* (19–22).

Gestational age is another pivotal influencing factor, for different orders of reasons: preterm infants have immature gastrointestinal and immune systems; they are precociously exposed to extensive use of antibiotics and often long term hospitalized; they need mechanical ventilation and usually receive parenteral nutrition. Each one of these conditions may produce irreversible change into the natural process of colonization and development of the gut microbiota (23).

Particularly, in these newborns anaerobic colonization is delayed and their stools host higher levels of *Enterobacteriaceae*, *Enterococcus*, and opportunistic pathogens if compared with term newborns (24–29).

Recently, Tauchi and colleagues described a delayed *Bifidobacteriaceae* colonization, underlining the role of *Bifidobacterium* as probiotic to induce "normal" infant microbiota. Their data also suggest a longer predominance of *Staphilococcacae* in preterm infants with an increased risk of potentially pathogenetic methicillin resistant *Staphylococcus aureus* colonization in NICU infants (30).

Korpela et al. (31) performed a study on fecal samples from 45 preterm infants, in order to identify a pattern of development of the intestinal microbiota. Four phases were identified with a pattern of progression to a *Bifidobacterium*-dominated composition typical of the full-term non-hospitalized newborns. This normal-like microbiota development correlated with post-menstrual age, was achieved also in cesarean-delivery newborns and was favorited by administration of breast milk. Moreover, these authors observed as among the extremely premature infants the overgrowth of *Enterococcus* spp. inhibit the normal succession, while antibiotics administration causes temporary changes in intestinal microbiota composition that subsequently recovers after few days.

Table 1 summarizes different categories of typical gut microbial pattern that can be found in healthy newborns. The principal variables that can affect the rising abundance of a population over another are reported and it is clearly showed as there's not a single typical healthy microbiota, but gut microbiota is rather a spectrum, result of all the combination of variables that coexist in an infant.

Lung Microbiota in Preterm Infants

Lungs have historically been considered sterile in healthy people so that they were initially omitted from the priority organ system studied by the Human Microbiome Project; subsequent culture independent studies demonstrated that lungs host diverse communities of bacteria and the interest in this new field rapidly grew since the linkage of different microbiomes with specific respiratory diseases was observed (32).

TABLE 1 | Summary of different categories of typical gut microbial pattern that can be found in healthy newborns.

	Gut characteristic bacterial populations in healthy newborns
Term neonates	*Lactobacillus, Bifidobacterium*
Preterm neonates	*Enterobacteriaceae, Veillonella, Enterococcus, Staphylococcus*
Breast feed	*Lactobacillus, Bifidobacterium*
Formula feed	*Bacteroides, Staphylococcus, Clostridia, Enterococcus, Enterobacteria*
Vaginally delivered	*Prevotella, Lactobacillus*
C-section	*Corynebacterium, Staphylococcus, Propionibacterium*

We report the principal variables that can affect the rising abundance of a population over another: the correspondence may concern either a genus (such as Staphylococcus), either a family (such as Enterobacteriaceae). It clearly shows that there's not a single typical healthy microbiota, but gut microbiota is rather a spectrum, result of all the combination of variables that coexist in an infant.

Sampling from lungs and distal airways represents a major challenge in neonatal population because of low biomass with bacterial loads close to the detection limit of the assays and for the risk of contamination from upper airways (33).

Timing of colonization is debated. Mourani and colleagues noted that only 2 of 10 tracheal aspirates of intubated preterm infants contained detectable bacterial DNA in the first 72 h of life, whereas all samples from the same newborns were positive at day 7 (34). In contrast, Lohmann et al. reported that bacterial DNA was detectable in all tracheal aspirates taken immediately after intubation at day 1 of life from 25 preterm newborns (35) so that it appears that colonization of the airways begins very early or even before the delivery.

The development of lung microbiota in the preterm infant can be affected by several factors: first of all, maternal chorioamnionitis (which is a pivotal cause of premature birth); then exposure to mechanical ventilation, antibiotics, NICU environmental microbial population, feeding, gut microbiota composition.

An aberrant gastrointestinal colonization has been found to be associated with a higher risk for postnatal sepsis, necrotizing enterocolitis (NEC) and growth impairment in preterm infants. As a consequence, the reasons of intestinal dysbiosis in this population of newborns have increasingly become an object of interest.

Moreover, recently, there's a rising attention to the so-called "gut-lung axis." The presence of a link between the gut and lung microbiome's development is emerging. Gut and lung microbiota are known to be pivotal in the education of host immune system, as reported by Dang et al. (36). Both microbes and their products participate to this complex interaction. The intricate relationship between gut and lung microbiota is testified by the bidirectional association of gut dysbiosis with lung disease: Kalliomaki et al. (37) already observed an increased abundance of Clostridia and reduced Bifidobacteria in the gut of infants who developed early life asthma; the review by Dang (36) also shows evidences of gastrointestinal perturbations in patients with chronic lung disease. The "mucosal response theory "reported by Gallacher and Kotecha (33) implies that dendritic cells (DCs) in the intestine come into contact with the resident microbial population; it generates signals that result in phenotypic changes in the DCs which migrate to the mesenteric lymph nodes where they stimulate the production of regulatory cytokines such as IL-10, TGF-beta, INF-gamma, and IL-6. In the mucosa-associated lymphoid tissue, DCs present the bacterial derived antigens to T-cells, leading to their activation. T cells then acquire homing molecules like chemokine receptor 4 (CCR-4) and chemokine receptor 6 (CCR-6). Moreover, the activated T cells can reach the respiratory mucosa where they promote protective and anti-inflammatory responses. Other mechanisms of interaction have been highlighted: Short Chain Fatty Acids (SCFA), gut microbioma derived metabolites, have a kay role; they reach the blood stream and come to the bone marrow were they promote hematopoiesis stimulating the differentiation of the Hematopoietic Stem Cells (HSCs) toward an anti-inflammatory milieu which migrates in the airways (36).

More data show that an impairment of the lung microbiota could be implicated in the pathogenesis of BPD.

These new evidences are increasingly interesting, since the manipulation of maternal microbiota (during or even before pregnancy) might influence the pregnancy outcome and the fetal/infantile health.

OBJECTIVES

We evaluated the studies that examined gut and lung microbiota in preterm infants and summarized emerging evidences. Our objective was to perform a qualitative analysis of data about characteristic patterns leading to dysbiosis in different body sites and to evidence the actual knowledge of the role of dysbiosis on preterm infants' outcomes such as growing impairment, Necrotizing Enterocolitis (NEC), Bronchopulmonary Dysplasia (BPD) and sepsis.

MATERIALS AND METHODS

In order to identify papers considered in this qualitative review, we performed a literature search on PubMED. The research has been restricted to papers in English language. We limited the search by applying the filter of age "infants" and used the following search terms and logic: "preterm infants microbiota," "gastrointestinal microbiome *AND* Necrotizing Enterocolitis *OR* NEC," "breastfeeding *AND* enteral nutrition *AND* Necrotizing Enterocolitis *OR* NEC," "microbiota *AND* growth retardation," "intestinal microbiota *AND* weight gain," "intestinal microbiota *AND* growth," "gut microbiota *AND* extrauterine growth restriction," "preterm infants microbiota *AND* late onset sepsis *OR* LOS," "microbiota *OR* microbiome *OR* bacteria *OR* antibiotics *OR* gut *AND* lung *OR* airway *OR* BPD *OR* Bronchopulmonary Dysplasia," and "gut-lung axis." No limit about year of publication has been set, and the final search is updated to July 2019.

To identify any articles that may have been missed during the literature search, also reference lists of candidate articles have been carefully checked.

DISCUSSION

The Preterm Gut Microbiota and Growing Impairment

Postnatal growth failure is a frequent adverse outcome in preterm infants that occurs during a critical developmental period. The study of the extrauterine growth rates of extremely preterm (EPT) infants (i.e., birth gestational age ≤27 weeks) show that approximately half of those infants remain below the 10th percentile in weight of the reference *in utero* growth rates, at the time of neonatal intensive care unit (NICU) discharge (38, 39).

Gut microbiota, gastrointestinal tract, and immune system maturation appear to be affected by prematurity and nutrition. Particularly, the gut microbiota, with its distinct metabolic capacities, plays a role in the metabolism of dietary components, which appear to be indispensable for the host. Many factors are involved in the variation of gut microbiota in preterm infants, such as the hospital environment of the NICU and its associated common clinical practices and feeding regimens, with subsequent direct and indirect interference with energy harvest and storage, and thereby with weight gain (40–42). Indeed, the gut microbiota, with its complementary metabolic capacity to human gastrointestinal enzymes, is able to provide the host with nutrients and energy otherwise unavailable (43). It also interferes with the host body weight management (40, 44–46) by producing metabolites and by affecting the harvest, storage, and expenditure of energy from food components (43, 47).

The association between the gut microbiota, growth, and development in early life has been investigated by some studies in preterm infants (41). Of particular interest is the study of Grier et al. (42). These authors identified in preterm infants three phases (P1, P2, and P3) that correspond to the three states of the microbiota with distinct metabolic functions. Significant associations were found between nutrition, microbiota phase, and preterm infant growth (42). The microbiota analysis showed that P1 is associated to a low level of initial diversity with a predominance of facultative anaerobes. The transition out of P1, which could be identified with transition from meconium to normal postnatal stool, was characterized by increasing diversity and abundance of obligate anaerobes with a shift to fermentation-based metabolism in P3. A role of Paneth cells (PCs) AMPs in the modulation of the shift toward a community dominated by obligate anaerobes was suggested by the increase in the number of PC around a post-menstrual age (PMA) of 29 weeks, corresponding to the transition from P2 to P3. According to others studies (48, 49), *Bifidobacterium*, an Actinobacterium involved in the development and maintenance of the healthy infant gut microbiota, was significantly associated with lipid and protein intake in P3 as testified by its increased abundance with increased lipid in the diet and decreased abundance with greater amounts of protein. Moreover, an increased abundance of *Bifidobacterium* was also significantly associated with the use of corticosteroids and H2 receptor antagonists in P3. In addition, Proteobacteria resulted significantly associated with lipid intake, Firmicutes with protein and Actinobacteria, Proteobacteria, and Firmicutes with carbohydrates (42).

In contrast to these findings, in the study of Blankstad et al. (50), where the relationship between a fortified diet (with more energy, protein, fat, vitamin A, arachidonic acid and docosahexaenoic acid) and the intestinal microbiota development has been evaluated, *Bifidobacterium* was more represented among infants with standard nutrient supply and it was associated with an improved weight gain and, consequently, with overall better growth. Anyway, the authors postulated that the lower abundance of this bacterial genus in infants receiving the "fortified" nutrient supply could be explained as a consequence of a concomitant greater abundance of other microbes, and not necessarily less *Bifidobacterium* as a direct effect. Moreover, in this study (50), while the initial richness after birth did not appear to be influenced by nutrition, the maintenance of richness was observed only in the preterm infants who were fed with the fortified diet if compared with the standard nutrient supply. After the postnatal peak, all infants showed a decreased of microbial diversity, regardless of diet fortification.

Specific bacterial families and genera resulted to be associated with weight gain at 1 month of age also in the study of Arboleya et al. (41), with a correlation of growth rates and the levels of Enterobacteriaceae and Streptococcus at 2 days of age and of Bacteroides-group at 10 days of age. Moreover, some bacterial genera such as Staphylococcus and Enterococcus were negatively associated with weight gain, while Weissella was positively associated with weight gain in preterm infants (41). Concerning Weissella, there are interesting evidences that this genus could influence food digestion and energy harvest in infants (40, 43, 45). This aspect should be further explored, considering that lactobacilli, to which order Weissella belongs, are used as probiotics.

The results of a recent study (51), show that infants fed with the mother's own milk (MOM) had a greater abundance of *Bifidobacterium* and Bacteroides, each of that seems to be protecting against morbidities like NEC. Moreover, feeding primarily MOM was related to an increased gut microbiota diversity that had previously been related to healthier outcomes in VLBW infants and that resulted associated to a superior growth in comparison with infants fed with donor human milk.

Li et al. (52) demonstrated that preterm infants with growth failure have a distinct intestinal microbiome's profile when compared to infants with normal growth at postnatal days 1 and 28. At both time points, the sole highly abundant taxa in the EUGR group was the genus Parabacteroides, which is a gram-negative, anaerobic, non-spore forming genus. Some species of that genus turn out high quantities of acetic acid and propionic acid (53). Intestinal epithelial cells (IECs) can absorb these short fatty acids that can also enter the blood circulation, influencing the storage of sugar in muscle, liver, and fat. Also, the brain appears to be reached by acetic acid, with subsequent loss of appetite, and leading to reduced food intake (54). Thus, weight gain might relate to an excessive colonization of genus Parabacteroides in the intestinal tract of VLBW infants.

The study results of Yee et al. (55), complement previous data, showing an abundance of Proteobacteria in the preterm infants' microbiome (56, 57). These authors described a negative

correlation between infant weight gain during hospitalization and the relative abundances of Klebsiella and Staphylococcus. Both of these taxa are associated with known pathogens so that their enrichment could be a sign of dysbiosis, finally associated with reduced infant weight and length gain.

A deficit in anabolic metabolism of glucose and other non-lipid energy source, resulting in a greater reliance on fatty acids to satisfy metabolic demands, has been proposed in preterm infants with growth failure from Young et al. (58) From their data emerged an alteration of the microbiota maturation characterized by low diversity, persistent dominance of Enterobacteriaceae, and a deficiency of strictly anaerobic taxa such as Veillonella in preterm infants with postnatal growth failure. These infants also demonstrated a "metabolic signature," showing an increased lipolysis and fatty acid oxidation, with gain in multiple fatty acids, acylcarnitines, glycerol, and β-hydroxybutyric acid, that is distinctive of a fasted state.

Considering all the evidences reported (briefly summarized in **Table 2**), the knowledge of the mechanisms leading the infant microbiota to alter nutritional efficacy appears of great importance in the aim of predicting, preventing and treating growth failure in preterm infants.

The Preterm Gut Microbiota and NEC

Necrotising EnteroColitis (NEC) is a major cause of mortality and morbidity in premature newborns; it affects 7% of newborns with birth weight <1500 g, of whom 20–30% dies (2). It is characterized by submucosal hemorrhage and oedema, neutrophilic infiltration of the intestinal wall, disruption of intestinal villus architecture and finally full thickness necrosis or intestinal wall perforation (59). Typically, the involved newborn presents with abdominal distension, bloody stools and feeding intolerance, while pneumatosis intestinalis and portal venous gas are characteristic abdominal radiological findings (60).

The pathogenesis of the disease is unclear but probably multifactorial: enteral feeding, intestinal ischemia and aberrant microbial colonization are the principal involved factors.

Initial exposition to microbes and their metabolic products is normal part of development with a largely unexplored effect on immune system. Breastfeeding is known to protect against NEC (61, 62): the reason is that breastfeeding facilitates colonization by a balanced commensal flora which contrasts bacterial overgrowth; instead formula feeding is associated with harmful gut bacterial proliferation (63). Preterm infants early come into contact with different environmental microbic species and are also precociously exposed to antibiotic therapies which have shown to reduce the diversity of infant microbiota; moreover, perturbations in the composition of infant microbiota may let pathogenic microbes prevail over commensal species (64).

A broad spectrum of microbes has been involved in the pathogenesis of NEC across multiple studies. According to recent evidences certain microbial population such as *Bifidobacteria* and *Lactobacilli* are protective, while *Clostridia*, *Enterobacteriaceae,* and *Staphylococcus* have been associated to the development of the disease (65).

Emerging data suggest that a predominance of *Proteobacteria* is strictly associated with NEC. Wang et al. (66) showed that infants in the NEC group have more than 50% of gut bacterial colonization represented by the *Gammaproteobacteria* genera such as *Cronobacter sakazakii*, *Klebsiella sp*., and *Escherichia coli*. This result was not found in the healthy newborns. The same pattern of gut microbiota has been reported by other authors (57, 67).

But in the stool of infants with NEC are often found also *coagulase-negative staphylococci* (*CoNS*) from the phylum *Firmicutes* (68) and *Clostridium* spp. with the associated toxin (69). In the past, this last microbe, that is part of the

TABLE 2 | Summary of the changes in microbial resident population observed in the different pathologic conditions and the underlying pathogenetic mechanisms.

	Change in microbial populations	Pathogenetic mechanisms involved
BPD	Decreased abundance of *Lactobacillus*, reduction in the bacterial community turnover with increasing time from birth, decreased colonization by *Staphylococcus* in the first days after birth, increased colonization by *Ureaplasma*	A decreased abundance of protective species allows a relative overgrowth of potentially pathogen microbial populations; moreover, Ureaplasma-colonized newborns present peripheral blood leukocytosis and less severe RDS but early radiographic and histologic changes typical of BPD
EUGR	Low diversity, persistent dominance of *Enterobacteriaceae*, and a paucity of strictly anaerobic taxa including *Veillonella* compared to infants with appropriate growth. Relative abundance of *Klebsiella* and *Staphylococcus*. Increased rate of *Parabacteroides*.	Nutritional efficacy is dramatically affected by infant dysbiosis. In particular, *Parabacteroides* produce acetic and propionic acid that reach the blood circulation, affecting the storage of sugar in muscle, liver, and fat; acetic acid is also involved in reduced food intake due to loss of appetite.
LOS	Decreased bacterial diversity with a predominance of *Proteobacteria* and *Firmicutes*, whereas *Bifidobacteria* are usually found in healthy controls. A strong relation with *Fusobacteria* and *Tenericutes* has also been described.	Gut microbiota can translocate across a dysfunctional or immature intestinal barrier, overwhelm neonatal immune system and cause sepsis; moreover, intestinal dysbiosis is supposed to alter local and systemic immune function. Natural prebiotics, such as raffinose, inhibit the growth of potentially pathogenic bacteria and promote proliferation of *Bifidobacterium*.
NEC	Decreased bacterial diversity with increased rate of *Clostridia*, *Enterobacteriaceae,* and *Staphylococcus* are frequently observed; a predominance of *Proteobacteria* (such as *Escherichia coli* and *Klebsiella*) is considered strictly associated with the pathogenesis of the disease.	Antibiotics pressure and enteral feeding generate changes in gut microbiota which may lead to an overgrowth of potential pathogens; moreover, LPS of Gram Negative activates TLR-4 and premature gut reacts with an exaggerated cytokine mediated inflammatory response, which may be due to a deficient expression of inhibitors of the NFkB pathway.

BPD, Bronchopulmonary Dysplasia; EUGR, Extra-Uterine Growth Restriction; LOS, Late-Onset sepsis; NEC, Necrotising Enterocolitis.

commensal microbiome of preterm infants, was associated with the progression of NEC (70, 71).

Concerning this aspect, it is known how bacterial products are recognized via Microbial Associated Molecular Patterns (MAMPs) by Pattern Recognition Receptors (PRRs) and in particular by the more studied Toll Like Receptor (TLRs) expressed on the intestinal mucosa: this interaction results in the activation of Nuclear Factor- kappa B (NFkB) and its inflammatory pathway and caspases. These propagate the apoptosis and induce the production of cytokines (INF-1, TNF-alfa, IL-1, IL-6, IL-8) via activation of transcription genes (72). TLRs are pivotal to maintain the equilibrium between adequate inflammatory response and homeostasis, performing crucial functions like regulation of cell proliferation and growth, antimicrobial agents secretion, and control of the barrier function (2).

After birth MAMPs start to interact with TLRs; moreover Lipopolysaccharide (LPS) of Gram Negative activates TLR-4 (73).

Premature gut reacts to the TLR-4 activation with a more accentuated cytokine mediated inflammatory response if compared to a full-term gut; IL-8 is especially involved in neutrophil chemotaxis increase and inflammation, leading in most severe cases to tissue injury and NEC (74, 75). This exaggerated inflammatory response may be due to a deficient expression of inhibitors of the NFkB pathway (76, 77). Commensal bacteria seem to have a beneficial role in the repression of the continuative stimulation occurring in the infant gut by blocking the degradation of NFkB inhibitors; they also contribute to the neonatal gut homeostasis generating a low-level stimulation of TLR-4 (78, 79) (**Table 2**).

Probably, a single pathogen entirely responsible for NEC will never be isolated, since there is not a single microorganism predictive of the risk of NEC. Reasonably, it is the limited diversity of the whole microbiota and the rising abundance of pathogenetic bacteria to be together responsible for the premature susceptibility to NEC (66).

Moreover, a better comprehension in this field is reached considering the influence of gut microbiota on the host immune system.

The Preterm Lung Microbiota and BPD

A so called "gut-lung axis," that is a connection between the development of the gut and lung microbiome, has been described (80).

Commensal bacteria in the gut and lungs are necessary for the normal development of the immune homeostasis (80). Therefore, microbial dysbiosis may lead to abnormal inflammatory responses, which are referred to the pathogenesis of BPD.

Airways of the newborn develop during pregnancy influenced by amniotic fluid, placenta, and vagina with its own microbiome (12, 81).

The described lung microbiome at birth evolves over the first weeks and months of postnatal life (82).

Several factors are involved in the development of the lung microbiome. Among them, chorioamnionitis, transplacental infection, or abnormal colonization appear to be able to create an inflammatory process, first step in the pathogenesis of BPD (83). Exposure to prenatal and postnatal antibiotics, respiratory support devices, sepsis, feeding and nutrition, concurrent development of the intestinal microbiome, and the surrounding environmental microbiome, can also be also implicated (82, 84, 85).

In order to support the important role of intrauterine infection as determinant of preterm labor and neonatal diseases, already in 2005 the presence of 16S rRNA was higher in samples of placenta, fetal membranes and cord blood serum from mothers presenting with preterm prelabor rupture of membranes (pPROM) or in spontaneous idiopathic preterm labor, and in the BALF samples or gastric aspirates of their newborns, collected within 24 h of life. BALF samples from these newborns showed a reduction of the prevalence of *Lactobacillus* and a decrease in α-diversity, suggestive for a state of precoscious disbiosys (86).

Most studies described the presence of an airway microbiome early at birth dominated by *Staphylococcus* and *Ureaplasma* (87, 88) and a longitudinal change in the first weeks of life with increasing bacterial loads (34, 35, 89, 90). Lal et al. described the composition of the airway microbiota by analyzing the tracheal aspirates of newborns in the first day of life: a predominance of *Firmicutes* and *Proteobacteria* and the presence of *Actinobacteria*, *Bacteroidetes*, *Tenericutes*, *Fusobacterium*, *Cyanobacteria*, and *Verrucomicrobia* was observed, without differences between preterm and full term infants (89). Anyway, other authors didn't find adequate bacterial DNA for successful sequence analysis in the tracheal aspirates of 8/10 preterm infants, to sustain the sterility of the airways of intubated preterm infants at birth, with the evidence of a subsequent bacterial colonization after the first 3 days of life (34).

It seems difficult to compare studies about airway microbiome for clinical and methodological heterogeneity. However, the airway colonization pattern appears to be also influenced by lung diseases, particularly by evolution on BPD. In infants who developed BPD compared with those who did not, differences in abundance and a decreased bacterial diversity have been reported (90) (**Table 2**).

In a study on 94 preterm infants, Wagner et al. found that those who developed severe BPD acquired less initial *Staphylococcus* and high *Ureaplasma* in the first days after birth (90). A possible explanation is that *Ureaplasma* intrauterine exposure downregulates the host response to acute LPS exposure in the preterm sheep model (91). Moreover, host bactericidal activity is related to functional complement and seems to be directly correlated to gestational age (92) (**Table 2**).

Lohmann and Lal described changes in the relative abundance of *Proteobacteria* and *Firmicutes* but with conflicting data on the preponderance of one or the other (35, 89).

Lohmann et al. (35), at time of intubation, showed a lower bacterial diversity (in terms of lower species count and Shannon diversity index) in newborns who subsequently developed BPD than in those who did not. Concerning the evolution of the lung microbiota, these authors showed a trend to an increase in *Firmicutes* and a decrease in *Proteobacteria* in infants who developed BPD in contrast to the relatively diverse and stable community in the non-BPD group. At the genera level, in both

groups *Acinetobacter* was the predominant genus, but its relative abundance decreased longitudinally in the BPD group, while an increasing amount of *Staphylococcus* and *Klebsiella* was observed. Anyway, the changes in bacterial composition do not correlate with the levels of inflammatory cytokines, leaving unanswered the question of the clinical relevance of such findings.

The study of Lal et al. (89) present methodological differences and the airway microbiome of infants after diagnosis of BPD was compared to that of full term newborns matching for post-menstrual age. In contrast to the findings of Lohmann et al., the analysis shows an increase in the phylum *Proteobacteria* and a decrease in the phyla *Firmicutes* and *Fusobacteria* in association with BPD diagnosis. When compared with ELBW newborns and full-term infants, *Gamma Proteobacteria* resulted more abundant whereas *Alpha Proteobacteria* were in lower abundance in BPD infants. At the genus level, the most abundant *Proteobacteria* in BPD patients were *Enterobacteriaceae*.

An interesting finding was the decrease in *Firmicutes* such as *Lactobacillus* in airway microbiome of infants with chorioamnionitis and in preterm infants who went on to develop lung disease (90). This could be an important finding for the association of BPD and chorioamnionitis. Previously, a beneficial role of *Lactobacillus* in other airway diseases has also been reported (93–95).

There are lots of potential determinants of the airway microbiome, but it is difficult to determine whether these changes are causal or established because of a change in treatment or in other factors that lead to BPD.

Even if an association of "antibiotics induced dysbiosis" with BPD has been reported (90), apparently, there are no significant differences in the initial specimen between infants who received empiric antibiotic treatment and those who did not (89). The comparison of the airway microbiome of infants whose mothers received prenatal antibiotics and the infants of mothers who didn't show differences either (90).

The variability in treatments among preterm infants, especially related to antibiotic administration appears to be able to influence these findings (90).

Even if studies which demonstrate a link between dysbiosis and BPD exist, there is a lack in the description of a causal relationship between airway injury during development and respiratory colonization with microorganisms.

Further metabolic analysis of the lung microbiota, including metagenomic, and metabolomic assessments, are necessary to define their role as the cause of lung injury sequence and inflammation.

The Preterm Gut Microbiota and the Risk of Late Onset Sepsis

It is known that many of the organisms that are responsible for late onset sepsis (LOS) in extremely preterm infants, including *staphylococci*, arise from the intestinal tract (96). Indeed, several studies carried out in extremely preterm infants, have demonstrated the presence of organisms in the feces prior to or concurrent with the onset of LOS which resulted to be caused by the same organism (82, 97, 98).

Puri et al. evaluated infants exposed to intra-amniotic infection and suggested a causative role of specific alterations in the early neonatal microbiome in the development of later sepsis or death (99).

These authors associated an aberrant gastrointestinal colonization with chorioamnionitis or funisitis. The analysis of meconium samples from extremely preterm infants showed, according to the results reported by Moles et al. (100), a definite flora in the first week of life dependent on the exposition to chorioamnionitis or funisitis. A restricted set of taxa was studied in reference to LOS, with a strongest correlation to later sepsis with *Genus Sneathia* (*Phylum Fusobacteria*) and bacteria family (*Phylum Tenericutes*) (99).

Data reported from other authors suggest that in preterm infants is a dysbiosis instead of a predominance of potential pathogens to be associated with sepsis (97) (**Table 2**). In their case control study, Mai et al. (101) found a predominance of *Firmicutes* in stool samples of newborns with LOS, while Madan et al. (102), by analyzing the early fecal samples of preterm infants with a gestational age between 24 and 27 weeks, highlighted a decreased bacterial diversity with a prevalence of *Proteobacteria* and *Firmicutes* (*Staphylococcus*).

Korpela et al. (31) correlated the risk of sepsis with a disrupted intestinal microbial development in preterm infants, characterized by a predominance of aerobic cocci and a reduction of *bifidobacterial*. In their cohort of preterm newborns, *Enterobacter* was abundant in some sepsis cases, but it was detected also in many cases not developing sepsis.

These data support two possible hypothesis: the translocation across the intestine to blood could be the cause of sepsis or, alternatively, since in some cases the organism responsible of sepsis is not detected in the intestine before the onset of sepsis, it is possible that an intestinal dysbiosis could be responsible for an alteration of the local and systemic immune function (101, 102).

An aberrant immune function could be involved also in the connection between chorioamnionitis, intestinal dysbiosis, and increased susceptibility to LOS. Indeed, a decreased function of the anti-inflammatory T-regulatory cell subset has been found in preterm infants of mother with a diagnosis of chorioamnionitis and/or funisitis (103) (**Table 2**).

Stewart et al. performed a study showing the association of the gut microbiome and metabolome with the pathogenesis of LOS (104). The gut microbiome of infants with a diagnosis of LOS appeared to be specific for each patient and highly dynamic trough time. Moreover, the identification of one of the most abundant operational taxonomic units in the gut microbiota at diagnosis of LOS with the pathogen in the blood, suggested the translocation trough the gut epithelium as a first element in the pathogenesis of LOS. On the other hand, control infants showed a predominance of *Bifidobacteria*, a taxa that is correlated with some metabolites including raffinose, sucrose, and acetic acid. Among them, a significant role as prebiotic, able to inhibit the growth of potential pathogenic bacteria and to enhance the presence of *Bifidobacterium* spp. (105–107), is conducted by raffinose. It is a α-galactosyl (α-GAL) oligosaccharide that is fermented in the gut by bacteria containing the α-GAL enzyme. While it is reduced in LOS infants before the diagnosis and

increased after treatment, in control infants its concentration is constantly high (104).

Neonatal microbiota has been studied also in relation to *GBS* sepsis, which represents a global problem with an estimate overall incidence of about 0.5/1,000 live births. To date, intrapartum antibiotic prophylaxis (IAP) represents a good strategy which lead to a decrement in the incidence of early-onset sepsis (EOS) while LOS rates remained unchanged (108–110).

The route of infection in EOS is well-known, implicating the vertical transmission of *GBS* from the colonized maternal vaginal tract to the infant at birth. In many cases, bacteria entry the respiratory tract trough the aspiration of contaminated fluids, resulting in sepsis or pneumonia during the first days of life (111). The route of infection in LOS is less well-understood. Particularly, it is unknown how the colonization remains stable from the first contact with *GBS* until the disease onset. Also in this case, there are data that show as an aberrant co-development of microbiota and host immunity represent a risk for *GBS* LOS, rather than genetic variations in immune genes alone (112). Affected infants show a decreased presence of anaerobic *Bacteroides* and *Bifidobacterium* spp., while aerobic *Enterobacteria* appear to be increased, when compared to non-septic twin controls (113).

Those observations, together with the evidence of a reduction of intestinal *Bifidobacterium* density in infants whose mothers received IAP, stress how clinicians should be careful in the usage of antibiotics in the early period of life (114). Consistent with this observation, in preterm infants an empirical antibiotic treatment results to be associated to a 3-fold higher risk for LOS caused by various pathogens including *GBS* in preterm infants (115).

Both the depletion of competitive microbes and a delayed immune cell maturation and dysbiosis with the subsequent emergence of pathogenic bacteria, represent a possible way by which antibiotics can influence the microbiome composition.

Recently, concerning the immunological implications, Josefsdottir et al. (116) observed as in antibiotic-treated mice the microbiota is the cause of neutropenia and general depletion of hematopoietic stem cells across multiple lineages. Newborns represent a sensitive population also for its smaller granulocyte pool that may further propagate the negative effects of antibiotics (117), and for the immaturity of phagocytes and adaptive immune cells that reduced the strength to fight against pathogens. Hence, while the adult immune system can respond to a pathogen invasion through the muco-cutaneous barrier, the neonatal immunity may be overwhelmed, resulting in bacterial spread and sepsis.

CONCLUSIONS

The analysis of the knowledge about the preterm infant's microbiota and its relationship with clinical outcomes, show that facultative anaerobes dominated the preterm infant gut, including Enterobacteriaceae, Enterococcus, and Staphylococcus. These are communities that count commonly antibiotic-resistant organisms.

Microbiota plays a key role in the establishment of the gut barrier and many data suggest its immune-modulator role. For all these reasons, the understanding and the prevention of dysbiosis is crucial for the prevention of diseases such as sepsis, NEC and BPD, but may also impact growth rates, immune function, and the risk for various chronic diseases and conditions.

Differences in patient population or care/feeding practices are to take into account in the analysis of the studies conducted in this field. Future studies should be addressed in order to explore differences in the gut/lung microbiota in sub-selected populations, based on specific treatments and probiotic administration. Moreover, concerning the actual acknowledgment about the relation between gut microbiota and the onset of LOS, a deepen description of this link could lead to realized screening approaches able to drive early intervention for the prevention of LOS in the high-risk preterm infants population.

AUTHOR CONTRIBUTIONS

CTi, GV, and LP conceptualized and designed the review. CTi and LP analyzed the references material and wrote the manuscript. AP, MT, CA, AL, SD'I, and CTe contributed to the writing of the manuscript. BP, MS, ND, and GV supervised the work and contributed to the writing of the manuscript. All authors reviewed and approved the final version of the manuscript.

REFERENCES

1. Integrative HMP (iHMP) Research Network Consortium. The integrative human microbiome project. *Nature*. (2019) 569:641–8. doi: 10.1038/s41586-019-1238-8
2. Patel RM, Denning PW. Intestinal microbiota and its relationship with necrotizing enterocolitis. *Pediatr Res*. (2015) 78:232–8. doi: 10.1038/pr.2015.97
3. Cilieborg MS, Boye M, Sangild PT. Bacterial colonization and gut development in preterm neonates. *Early Hum Dev*. (2012) 88:S41–9. doi: 10.1016/j.earlhumdev.2011.12.027
4. Faith JJ, Colombel JF, Gordon JI. Identifying strains that contribute to complex diseases through the study of microbial inheritance. *Proc Natl Acad Sci USA*. (2015) 112:633–40. doi: 10.1073/pnas.1418781112
5. Pacheco AR, Barile D, Underwood MA, Mills DA. The impact of the milk glycobiome on the neonate gut microbiota. *Annu Rev Anim Biosci*. (2015) 3:419–45. doi: 10.1146/annurev-animal-022114-111112
6. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe*. (2015) 17:690–703. doi: 10.1016/j.chom.2015.04.004
7. Mueller NT, Bakacs E, Combellick J, Grigoryan Z, Dominguez-Bello MG. The infant microbiome development: mom matters. *Trends Mol Med*. (2015) 21:109–17. doi: 10.1016/j.molmed.2014.12.002
8. Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM, Relman DA. The application of ecological theory toward an understanding of the human microbiome. *Science*. (2012) 336:1255–62. doi: 10.1126/science.1224203

9. Cahenzli J, Köller Y, Wyss M, Geuking MB, McCoy KD. Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host Microbe.* (2013) 14:559–70. doi: 10.1016/j.chom.2013.10.004
10. Sim K, Powell E, Shaw AG, McClure Z, Bangham M, Kroll JS. The neonatal gastrointestinal microbiota: the foundation of future health? *Arch Dis Child Fetal Neonatal Ed.* (2013) 98:F362–4. doi: 10.1136/archdischild-2012-302872
11. Olm MR, Brown CT, Brooks B, Firek B, Baker R, Burstein D, et al. Identical bacterial populations colonize premature infant gut, skin, and oral microbiomes and exhibit different *in situ* growth rates. *Genome Res.* (2017) 27:601–12. doi: 10.1101/gr.213256.116
12. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Sci Transl Med.* (2014) 6:237ra65. doi: 10.1126/scitranslmed.3008599
13. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci Rep.* (2016) 6:23129. doi: 10.1038/srep23129
14. Dong XD, Li XR, Luan JJ, Liu XF, Peng J, Luo YY, et al. Bacterial communities in neonatal feces are similar to mothers' placentae. *Can J Infect Dis Med Microbiol.* (2015) 26:90–4. doi: 10.1155/2015/737294
15. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci USA.* (2010) 107:11971–5. doi: 10.1073/pnas.1002601107
16. Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C. Mode of delivery affects the bacterial community in the newborn gut. *Early Hum Dev.* (2010) 86:13–5. doi: 10.1016/j.earlhumdev.2010.01.004
17. Bokulich NA, Chung J, Battaglia T, Henderson N, Jay M, Li H, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Sci Transl Med.* (2016) 8:343ra82. doi: 10.1126/scitranslmed.aad7121
18. Fouhy F, Ross RP, Fitzgerald GF, Stanton C, Cotter PD. Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps. *Gut Microbes.* (2012) 3:203–20. doi: 10.4161/gmic.20169
19. Martin R, Makino H, Cetinyurek YA, Ben-Amor K, Roelofs M, Ishikawa E, et al. Early-life events, including mode of delivery and type of feeding, siblings and gender, shape the developing gut microbiota. *PLoS ONE.* (2016) 11:e0158498. doi: 10.1371/journal.pone.0158498
20. Guaraldi F, Salvatori G. Effect of breast and formula feeding on gut microbiota shaping in newborns. *Front Cell Infect Microbiol.* (2012) 2:94. doi: 10.3389/fcimb.2012.00094
21. Gritz EC, Bhandari V. Corrigendum: the human neonatal gut microbiome: a brief review. *Front Pediatr.* (2015) 3:60. doi: 10.3389/fped.2015.00060
22. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr.* (2000) 30:61–7. doi: 10.1097/00005176-200001000-00019
23. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol Mol Biol Rev.* (2017) 81:e00036–17. doi: 10.1128/MMBR.00036-17
24. Hill CJ, Lynch DB, Murphy K, Ulaszewska M, Jeffery IB, O'Shea CA, et al. Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET cohort. *Microbiome.* (2017) 5:4. doi: 10.1186/s40168-016-0213-y
25. Rouge C, Goldenberg O, Ferraris L, Berger B, Rochat F, Legrand A, et al. Investigation of the intestinal microbiota in preterm infants using different methods. *Anaerobe.* (2010) 16:362–70. doi: 10.1016/j.anaerobe.2010.06.002
26. Jacquot A, Neveu D, Aujoulat F, Mercier G, Marchandin H, Jumas-Bilak E, et al. Dynamics and clinical evolution of bacterial gut microflora in extremely premature patients. *J Pediatr.* (2011)158:390–6. doi: 10.1016/j.jpeds.2010.09.007
27. Arboleya S, Binetti A, Salazar N, Fernandez N, Solis G, Hernandez-Barranco A, et al. Establishment and development of intestinal microbiota in preterm neonates. *FEMS Microbiol Ecol.* (2012) 79:763–72. doi: 10.1111/j.1574-6941.2011.01261.x
28. Arboleya S, Sanchez B, Milani C, Duranti S, Solis G, Fernandez N, et al. Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. *J Pediatr.* (2015) 166:538–44. doi: 10.1016/j.jpeds.2014.09.041
29. Cong X, Xu W, Janton S, Henderson WA, Matson A, McGrath JM, et al. Gut microbiome developmental patterns in early life of preterm infants: impacts of feeding and gender. *PLoS ONE.* (2016) 11:e0152751. doi: 10.1371/journal.pone.0152751
30. Tauchi H, Yahagi K, Yamauchi T, Hara T, Yamaoka R, Tsukuda N, et al. Gut microbiota development of preterm infants hospitalised in intensive care units. *Benef Microbes.* (2019) 10:641–51. doi: 10.3920/BM2019.0003
31. Korpela K, Blakstad EW, Moltu SJ, Strømmen K, Nakstad B, Rønnestad AE et al. Intestinal microbiota development and gestational age in preterm neonates. *Sci Rep.* (2018) 8:2453. doi: 10.1038/s41598-018-20827-x
32. Dickson RP, Erb-Downward JR, Huffnagle GB. The role of the bacterial microbiome in lung disease. *Expert Rev Respir Med.* (2013) 7:245–57. doi: 10.1586/ers.13.24
33. Gallacher DJ, Kotecha S. Respiratory microbiome of new-born infants. *Front Pediatr.* (2016) 4:10. doi: 10.3389/fped.2016.00010
34. Mourani PM, Harris JK, Sontag MK, Robertson CE, Abman SH. Molecular identification of bacteria in tracheal aspirate fluid from mechanically ventilated preterm infants. *PLoS ONE.* (2011) 6:e25959. doi: 10.1371/journal.pone.0025959
35. Lohmann P, Luna RA, Hollister EB, Devaraj S, Mistretta TA, Welty SE, et al. The airway microbiome of intubated premature infants: characteristics and changes that predict the development of bronchopulmonary dysplasia. *Pediatr Res.* (2014) 76:294–301. doi: 10.1038/pr.2014.85
36. Dang AT, Marsland BJ. Microbes, metabolites, and the gut-lung axis. *Mucosal Immunol.* (2019) 12:843–50. doi: 10.1038/s41385-019-0160-6
37. Kalliomäki M, Kirjavainen P, Eerola E, Kero P, Salminen S, Isolauri E. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol.* (2001) 107:129–34. doi: 10.1067/mai.2001.111237
38. Ehrenkranz RA, Younes N, Lemons JA, Fanaroff AA, Donovan EF, Wright LL, et al. Longitudinal growth of hospitalized very low birth weight infants. *Pediatrics.* (1999) 104:280–9. doi: 10.1542/peds.104.2.280
39. Horbar JD, Ehrenkranz RA, Badger GJ, Edwards EM, Morrow KA, Soll RF, et al. Weight growth velocity and postnatal growth failure in infants 501 to 1500 grams: 2000–2013. *Pediatrics.* (2015) 136:e84–92. doi: 10.1542/peds.2015-0129
40. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature.* (2006) 444:1027–131. doi: 10.1038/nature05414
41. Arboleya S, Martinez-Camblor P, Solís G, Suárez M, Fernández N, de Los Reyes-Gavilán CG, et al. Intestinal microbiota and weight gain in preterm neonates. *Front Microbiol.* (2017) 8:183. doi: 10.3389/fmicb.2017.00183
42. Grier A, Qiu X, Bandyopadhyay S, Holden-Wiltse J, Kessler HA, Gill AL, et al. Impact of prematurity and nutrition on the developing gut microbiome and preterm infant growth. *Microbiome.* (2017) 5:158. doi: 10.1186/s40168-017-0377-0
43. Krajmalnik-Brown R, Ilhan ZE, Kang DW, DiBaise JK. Effects of gut microbes on nutrient absorption and energy regulation. *Nutr Clin Pract.* (2012) 27:201–14. doi: 10.1177/0884533611436116
44. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA.* (2005) 102:11070–5. doi: 10.1073/pnas.0504978102
45. Jumpertz R, Le DS, Turnbaugh PJ, Trinidad C, Bogardus C, Gordon JI, et al. Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. *Am J Clin Nutr.* (2011) 94:58–65. doi: 10.3945/ajcn.110.010132
46. Blanton LV, Charbonneau MR, Salih T, Barratt MJ, Venkatesh S, Ilkaveya O, et al. Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. *Science.* (2016) 351:aad3311. doi: 10.1126/science.aad3311
47. Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu.Rev Nutr.* (2002) 22:283–307. doi: 10.1146/annurev.nutr.22.011602.092259

48. Butel MJ, Suau A, Campeotto F, Magne F, Aires J, Ferraris L, et al. Conditions of bifidobacterial colonization in preterm infants: a prospective analysis. *J Pediatr Gastroenterol Nutr*. (2007) 44:577–82. doi: 10.1097/MPG.0b013e3180406b20
49. O'Callaghan A, vanSinderen D. Bifidobacteria and their role as members of the human gut microbiota. *Front Microbiol*. (2016) 7:925. doi: 10.3389/fmicb.2016.00925
50. Blakstad EW, Korpela K, Lee S, Nakstad B, Moltu SJ, Strømmen K, et al. Enhanced nutrient supply and intestinal microbiota development in very low birth weight infants. *Pediatr Res*. (2019) 86:323–32. doi: 10.1038/s41390-019-0412-x
51. Ford SL, Lohmann P, Preidis GA, Gordon PS, O'Donnell A, Hagan J, et al. Improved feeding tolerance and growth are linked to increased gut microbial community diversity in very-low-birth-weight infants fed mother's own milk compared with donor breast milk. *Am J Clin Nutr*. (2019) 109:1088–97. doi: 10.1093/ajcn/nqz006
52. Li H, He Z, Gao D, Lv Y, Zhou Q, Xiao B, et al. Characteristics of the intestinal microbiota in very low birth weight infants with extrauterine growth restriction. *Front Pediatr*. (2019) 7:99. doi: 10.3389/fped.2019.00099
53. Grotoh A, Nara M, Sugiyama Y, Sakanaka M, Yachi H, Kitakata A, et al. Use of Gifu anaerobic medium for culturing 32 dominant species of human gut microbes and its evaluation based on short-chain fatty acids fermentation profiles. *Biosci Biotechnol Biochem*. (2017) 81:2009–17. doi: 10.1080/09168451.2017.1359486
54. Kim CH. Microbiota or short-chain fatty acids: which regulated diabetes? *Cell Mol Immunol*. (2017) 15:88–91. doi: 10.1038/cmi.2017.57
55. Yee AL, Miller E, Dishaw LJ, Gordon JM, Ji M, Dutra S, et al. Longitudinal microbiome composition and stability correlate with increased weight and length of very-low-birth-weight infants. *mSystems*. (2019) 4:e00229–18. doi: 10.1128/mSystems.00229-18
56. Dahl C, Stigum H, Valeur J, Iszatt N, Lenters V, Peddada S, et al. Preterm infants have distinct microbiomes not explained by mode of delivery, breastfeeding duration or antibiotic exposure. *Int J Epidemiol.* (2018) 47:1658–69. doi: 10.1093/ije/dyy064
57. Pammi M, Cope J, Tarr PI, Warner BB, Morrow AL, Mai V, et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome.* (2017) 5:31. doi: 10.1186/s40168-017-0248-8
58. Younge NE, Newgard CB, Cotten CM, Goldberg RN, Muehlbauer MJ, Bain JR, et al. Disrupted maturation of the microbiota and metabolome among extremely preterm infants with postnatal growth failure. *Sci Rep*. (2019) 9:8167. doi: 10.1038/s41598-019-44547-y
59. Grishin A, Papillon S, Bell B, Wang J, Ford HR. The role of the intestinal microbiota in the pathogenesis of necrotizing enterocolitis. *Semin Pediatr Surg*. (2013) 22:69–75. doi: 10.1053/j.sempedsurg.2013.01.002
60. Neu J, Walker WA. Necrotizing enterocolitis. *N Engl J Med*. (2011) 364:255–64. doi: 10.1056/NEJMra1005408
61. Schanler RJ. The use of human milk for premature infants. *Pediatr Clin North Am*. (2001) 48:207–19. doi: 10.1016/S0031-3955(05)70295-9
62. Lucas A, Cole T. Breast milk and neonatal necrotising enterocolitis. *Lancet*. (1990) 336:1519–23. doi: 10.1016/0140-6736(90)93304-8
63. Gregory KE1, Deforge CE, Natale KM, Phillips M, Van Marter LJ. Necrotizing enterocolitis in the premature infant: neonatal nursing assessment, disease pathogenesis, and clinical presentation. *Adv Neonatal Care*. (2011) 11:155–66. doi: 10.1097/ANC.0b013e31821baaf4
64. Wandro S, Osborne S, Enriquez C, Bixby C, Arrieta A, Whiteson K. The microbiome and metabolome of preterm infant stool are personalized and not driven by health outcomes, includ-ing necrotizing enterocolitis and late-onset sepsis. *mSphere.* (2018) 3:e00104–18. doi: 10.1128/mSphere.00104-18
65. Hunter CJ, Upperman JS, Ford HR, Camerini V. Understanding the susceptibility of the premature infant to necrotizing enterocolitis (NEC). *Ped Res*. (2008) 63:117–23. doi: 10.1203/PDR.0b013e31815ed64c
66. Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *ISME J*. (2009) 3:944–54. doi: 10.1038/ismej.2009.37
67. Coggins SA, Wynn JL, Weitkamp JH. Infectious causes of necrotizing enterocolitis. *Clin Perinatol.* (2015) 42:133–54, ix. doi: 10.1016/j.clp.2014.10.012
68. Stewart CJ, Marrs EC, Magorrian S, Nelson A, Lanyon C, Perry JD, et al. The preterm gut microbiota: changes associated with necrotizing enterocolitis and infection. *Acta Paediatr*. (2012) 101:1121–7. doi: 10.1111/j.1651-2227.2012.02801.x
69. Ferraris L, Butel MJ, Campeotto F, Vodovar M, Rozé JC, Aires J. Clostridia in premature neonates' gut: incidence, antibiotic susceptibility, and perinatal determinants influencing colonization. *PLoS ONE.* (2012) 7:e30594. doi: 10.1371/journal.pone.0030594
70. Kosloske AM, Ulrich JA. A bacteriologic basis for the clinical presentations of necrotizing enterocolitis. *J Pediatr Surg.* (1980) 15:558–64. doi: 10.1016/S0022-3468(80)80773-1
71. Kosloske AM, Ulrich JA, Hoffman H. Fulminant necrotising enterocolitis associated with Clostridia. *Lancet.* (1978) 2:1014–6. doi: 10.1016/S0140-6736(78)92337-1
72. Torrazza RM, Neu J. The altered gut microbiome and necrotizing enterocolitis. *Clin Perinatol*. (2013) 40:93–108. doi: 10.1016/j.clp.2012.12.009
73. Neal MD, Leaphart C, Levy R, Prince J, Billiar TR, Watkins S, et al. Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. *J Immunol*. (2006) 176:3070–9. doi: 10.4049/jimmunol.176.5.3070
74. Markel TA, Crisostomo PR, Wairiuko GM, Pitcher J, Tsai BM, Meldrum DR. Cytokines in necrotizing enterocolitis. *Shock*. (2006) 25:329–37. doi: 10.1097/01.shk.0000192126.33823.87
75. Sharma R, Tepas JJ, Hudak ML, Mollitt DL, Wludyka PS, Teng RJ, et al. Neonatal gut barrier and multiple organ failure: role of endotoxin and proinflammatory cytokines in sepsis and necrotizing enterocolitis. *J Pediatr Surg*. (2007) 42:454–61. doi: 10.1016/j.jpedsurg.2006.10.038
76. Claud EC, Lu L, Anton PM, Savidge T, Walker WA, Cherayil BJ. Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. *Proc Natl Acad Sci USA*. (2004) 101:7404–8. doi: 10.1073/pnas.0401710101
77. Spehlmann ME, Eckmann L. Nuclear factor-kappa B in intestinal protection and destruction. *Curr Opin Gastroenterol*. (2009) 25:92–9. doi: 10.1097/MOG.0b013e328324f857
78. Neish AS. Molecular aspects of intestinal epithelial cell-bacterial interactions that determine the development of intestinal inflammation. *Inflamm Bowel Dis*. (2004) 10:159–68. doi: 10.1097/00054725-200403000-00015
79. Neish AS, Gewirtz AT, Zeng H, Young AN, Hobert ME, Karmali V, et al. Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science.* (2000) 289:1560–3. doi: 10.1126/science.289.5484.1560
80. Marsland BJ, Trompette A, Gollwitzer ES. The gut-lung axis in respiratory disease. *Ann Am Thorac Soc.* (2015) 12(Suppl. 2):S150–6. doi: 10.1513/AnnalsATS.201503-133AW
81. DiGiulio DB. Diversity of microbes in amniotic fluid. *Semin Fetal Neonatal Med*. (2012) 17:2–11. doi: 10.1016/j.siny.2011.10.001
82. Taft DH, Ambalavanan N, Schibler KR, Yu Z, Newburg DS, Deshmukh H, et al. Center variation in intestinal microbiota prior to late-onset sepsis in preterm infants. *PLoS ONE.* (2015) 10:e0130604. doi: 10.1371/journal.pone.0130604
83. Gantert M, Been J, Gavilanes AW, Garnier Y, Zimmermann LJ, Kramer BW. Chorioamnionitis: a multiorgan disease of the fetus? *J Perinatol.* (2010) 30:S21–30. doi: 10.1038/jp.2010.96
84. Novitsky A, Tuttle D, Locke RG, Saiman L, Mackley A, Paul DA. Pro- longed early antibiotic use and bronchopulmonary dysplasia in very low birth weight infants. *Am J Perinatol.* (2015) 32:43–8. doi: 10.1055/s-0034-1373844
85. Cantey JB, Huffman LW, Subramanian A, Marshall AS, Ballard AR, Lefevre C, et al. Antibiotic exposure and risk for death or bronchopulmonary dysplasia in very low birth weight infants. *J Pediatr*. (2017) 181:289–93.e1. doi: 10.1016/j.jpeds.2016.11.002
86. Miralles R, Hodge R, McParland PC, Field DJ, Bell SC, Taylor DJ, et al. Relationship between antenatal inflammation and antenatal infection identified by detection of microbial genes by polymerase chain reaction. *Pediatr Res.* (2005) 57:570–7. doi: 10.1203/01.PDR.0000155944.48195.97

87. Stressmann FA, Connett GJ, Goss K, Kollamparambil TG, Patel N, Payne MS, et al. The use of culture-independent tools to characterize bacteria in endo-tracheal aspirates from pre-term infants at risk of bronchopul- monary dysplasia. *J Perinat Med.* (2010) 38:333–7. doi: 10.1515/jpm.2010.026
88. Payne MS, Goss KC, Connett GJ, Kollamparambil T, Legg JP, Thwaites R, et al. Molecular microbiological characterization of preterm neo- nates at risk of bronchopulmonary dysplasia. *Pediatr Res.* (2010) 67:412–8. doi: 10.1203/PDR.0b013e3181d026c3
89. Lal CV, Travers C, Aghai ZH, Eipers P, Jilling T, Halloran B, et al. The airway microbiome at birth. *Sci Rep.* (2016) 6:31023. doi: 10.1038/srep31023
90. Wagner BD, Sontag MK, Harris JK, Miller JI, Morrow L, Robertson CE, et al. Airway microbial community turnover differs by BPD severity in ventilated preterm infants. *PLoS ONE.* (2017) 12:e0170120. doi: 10.1371/journal.pone.0170120
91. Kallapur SG, Kramer BW, Knox CL, Berry CA, Collins JJ, Kemp MW, et al. Chronic fetal exposure to *Ureaplasma parvum* suppresses innate immune responses in sheep. *J Immunol.* (2011) 187:2688–95. doi: 10.4049/jimmunol.1100779
92. Kemp MW, Ahmed S, Beeton ML, Payne MS, Saito M, Miura Y, et al. Foetal *Ureaplasma parvumbacteraemia* as a function of gestation-dependent complement insufficiency: evidence from a sheep model of pregnancy. *Am J Reprod Immunol.* (2017) 77:e12599. doi: 10.1111/aji.12599
93. Cox MJ, Huang YJ, Fujimura KE, Liu JT, McKean M, Boushey HA, et al. *Lactobacillus* casei abundance is associated with profound shifts in the infant gut microbiome. *PLoS ONE.* (2010) 5:e8745. doi: 10.1371/journal.pone.0008745
94. Fujimura KE, Demoor T, Rauch M, Faruqi AA, Jang S, Johnson CC, et al. House dust exposure mediates gut microbiome *Lactobacillus* enrichment and airway immune defense against allergens and virus infection. *Proc Natl Acad Sci USA.* (2014) 111:805–10. doi: 10.1073/pnas.1310750111
95. Cope EK, Lynch SV. Novel microbiome-based therapeutics for chronic rhinosinusitis. *Curr Allergy Asthma Rep.* (2015) 15:504. doi: 10.1007/s11882-014-0504-y
96. Tarr PI, Warner BB. Gut bacteria and late-onset neonatal bloodstream infections in preterm infants. *Semin Fetal Neonatal Med.* (2016) 21:388–93. doi: 10.1016/j.siny.2016.06.002
97. Shaw AG, Sim K, Randell P, Cox MJ, McClure ZE, Li MS, et al. Late-Onset bloodstream infection and perturbed maturation of the gastrointestinal microbiota in premature infants. *PloS ONE.* (2015) 10:e0132923. doi: 10.1371/journal.pone.0132923
98. Carl MA, Ndao IM, Springman AC, Manning SD, Johnson JR, Johnston BD, et al. Sepsis from the gut: the enteric habitat of bacteria that cause late-onset neonatal bloodstream infections. *Clin Infect Dis.* (2014) 58:1211–8. doi: 10.1093/cid/ciu084
99. Puri K, Taft DH, Ambalavanan N, Schibler KR, Morrow AL, Kallapur SG. Association of chorioamnionitis with aberrant neonatal gut colonization. *PLoS ONE.* (2016) 11:e0162734. doi: 10.1371/journal.pone.0162734
100. Moles L, Gomez M, Heilig H, Bustos G, Fuentes S, de Vos W, et al. Bacterial diversity in meconium of pretermneonates and evolution of their fecal microbiota during the first month of life. *PLoS ONE.* (2013) 8:e66986. doi: 10.1371/journal.pone.0066986
101. Mai V, Torrazza RM, Ukhanova M,Wang X, Sun Y, Li N, et al. Distortions in development of intestinal microbiota associated with late onset sepsis in preterminfants. *PLoS ONE.* (2013) 8:e52876. doi: 10.1371/journal.pone.0052876
102. Madan JC, Salari RC, Saxena D, Davidson L, O'Toole GA, Moore JH, et al. Gut microbial colonisation in premature neonates predicts neonatal sepsis. *Arch Dis Child Fetal Neonatal Ed.* (2012) 97:F456–62. doi: 10.1136/fetalneonatal-2011-301373
103. Rueda CM,Wells CB, Gisslen T, Jobe AH, Kallapur SG, Chougnet CA. Effect of chorioamnionitis on regulatoryT cells in moderate/late pretermneonates. *Hum Immunol.* (2015) 76:65–73. doi: 10.1016/j.humimm.2014.10.016
104. Stewart CJ, Embleton ND, Marrs ECL, Smith DP, Fofanova T, Nelson A, et al. Longitudinal development of the gut microbiome and metabolome in preterm neonates with late onset sepsis and healthy control. *Microbiome.* (2017) 5:75. doi: 10.1186/s40168-017-0295-1
105. Patel S, Goyal A. The current trends and future perspectives of prebiotics research: a review. *3 Biotech.* (2012) 2:115–25. doi: 10.1007/s13205-012-0044-x
106. Vamanu E, Vamanu A. The influence of prebiotics on bacteriocin synthesis using the strain *Lactobacillus* paracasei CMGB16. *Afr J Microbiol Res.* (2010) 4:534–7.
107. Dinoto A, Marques TM, Sakamoto K, Fukiya S, Watanabe J, Ito S, et al. Population dynamics of *Bifidobacterium* species in human feces during raffinose administration monitored by fluorescence *in situ* hybridizationflow cytometry. *Appl Environ Microbiol.* (2006) 72:7739–47. doi: 10.1128/AEM.01777-06
108. Cohen-Wolkowiez M, Moran C, Benjamin DK, Cotten CM, Clark RH, Benjamin DK Jr, et al. Early and late onset sepsis in late preterm infants. *Pediatr Infect Dis J.* (2009) 28:1052–6. doi: 10.1097/INF.0b013e3181acf6bd
109. Bekker V, Bijlsma MW, Van De Beek D, Kuijpers TW, Van Der Ende A. Incidence of invasive group B streptococcal disease and pathogen genotype distribution in newborn babies in the Netherlands over 25 years: a nationwide surveillance study. *Lancet Infect Dis.* (2014) 14:1083–9. doi: 10.1016/S1473-3099(14)70919-3
110. Langley G, Schaffner W, Farley MM, Lynfield R, Bennett NM, Reingold A, et al. Twenty years of active bacterial core surveillance. *Emerg Infect Dis.* (2015) 21:1520–8. doi: 10.3201/eid2109.141333
111. Doran KS, Nizet V. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. *Mol Microbiol.* (2004) 54:23–31. doi: 10.1111/j.1365-2958.2004.04266.x
112. Kolter J, Henneke P. Codevelopment of Microbiota and innate immunity and the risk for group B streptococcal disease. *Front Immunol.* (2017) 8:1497. doi: 10.3389/fimmu.2017.01497
113. Cernada M, Bauerl C, Serna E, Collado MC, Martinez GP, Vento M. Sepsis in preterm infants causes alterations in mucosal gene expression and microbiota profiles compared to non-septic twins. *Sci Rep.* (2016) 6:25497. doi: 10.1038/srep25497
114. Aloisio I, Mazzola G, Corvaglia LT, Tonti G, Faldella G, Biavati B, et al. Influence of intrapartum antibiotic prophylaxis against group B *Streptococcus* on the early newborn gut composition and evaluation of the anti-Streptococcus activity of *Bifidobacterium* strains. *Appl Microbiol Biotechnol.* (2014) 98:6051–60. doi: 10.1007/s00253-014-5712-9
115. Kuppala VS, Meinzen-Derr J, Morrow AL, Schibler KR. Prolonged initial empirical antibiotic treatment is associated with adverse outcomes in premature infants. *J Pediatr.* (2011) 159:720–5. doi: 10.1016/j.jpeds.2011.05.033
116. Josefsdottir K, Baldridge M, Kadmon C, King K. Antibiotics impair murine hematopoiesis by depleting the intestinal microbiota. *Blood.* (2017) 129:729–39. doi: 10.1182/blood-2016-03-708594
117. Basha S, Surendran N, Pichichero M. Immune responses in neonates. *Expert Rev Clin Immunol.* (2014) 10:1171–84. doi: 10.1586/1744666X.2014.942288

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REVIEW
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Immunological Role of the Maternal Uterine Microbiome in Pregnancy: Pregnancies Pathologies and Alterated Microbiota

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Understanding what happens at the time of embryo implantation has been the subject of significant research. Investigators from many differing fields including maternal fetal medicine, microbiology, genetics, reproductive endocrinology and immunology have all been studying the moment the embryo interacts with the maternal endometrium. A perfect relationship between the uterus and the embryo, mediated by a tightly controlled interaction between the embryo and the endometrium, is required for successful implantation. Any factors affecting this communication, such as altered microbiome may lead to poor reproductive outcomes. Current theories suggest that altered microbiota may trigger an inflammatory response in the endometrium that affects the success of embryo implantation, as inflammatory mediators are tightly regulated during the adhesion of the blastocyst to the epithelial endometrial wall. In this review, we will highlight the various microbiome found during the periconceptual period, the microbiomes interaction with immunological responses surrounding the time of implantation, its effect on implantation, placentation and ultimately maternal and neonatal outcomes.

Keywords: uterine microbiome, pregnancy failure, pre-eclampsia, IUGR, early pregnancy immunology, endometrial micorbiome

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INTRODUCTION

The human body is colonized with over ten times more bacteria than the number of cells (1). Most prior medical research has been focused on disease causing bacteria and only recently, has there been a coordinated focus on studying the resident bacteria, viruses, and fungi collectively called the microbiome. In 2008, the NIH undertook a large human microbiome project in an effort to characterize all the microorganisms living in association with the human body in 300 healthy volunteers (2). The focus was on nasal passages, oral cavity, skin, GI tract, and the urogenital tract including the vagina. Few studies have focused on uterine microbiome as it was originally thought to be a sterile environment. Prior microbiome studies utilized either culture or 16S sequence-based technology in determining the bacterial environment. Early work describing the reproductive microbiome came from culture-based approaches (3). However, data from the next generation sequencing of the vaginal microbiome show that many organisms are not identified when culture only based approaches are utilized (4). Early studies suggested only about 1–2% of bacteria that is present can be picked up on culture (5), while recent studies using next generation sequencing suggest 20–60% depending on body site (1). More recent reproductive studies utilized

16S RNA hypervariable region gene sequencing, which can determine the genus and species level of bacteria, but not sub-speciation (6). There are both advantages and disadvantages to 16S sequencing. 16S is significantly cheaper and can be performed on smaller amount of DNA sample since an amplification process is required. However, the amplification process can also introduce an inherent amplification bias making it harder to accurately determine relative abundance of the bacterial species. Additionally, 16S technology utilizes a mapping database, meaning the species must have been characterized before by others. When compared with 16S-based sequencing, shotgun metagenomics can help with the identification of lower taxonomic resolution meaning detecting low abundance microbial communities and can better differentiate between closely related species (7). Essentially, prior work with culture and 16S was potentially missing key factors of the reproductive tract microbiome, however there are no studies to date published on shotgun sequencing of the endometrium at the time of an embryo transfer.

Recently there has been a new focus on determining uterine microbiome after a pilot study in 2016 showed that women with a non-lactobacillus dominant (NLD) uterine environment had an almost 40% drop off in pregnancy rates (8). Another study in 2015, utilized targeted sequencing and microarray data focused on the hypervariable regions of 16S rRNA and showed that uterine microbiome at the time of transfer can be characterized by sequencing the tip of the transfer catheter from the embryo transfer (9). Since then, many authors have been attempting to characterize the natural microbiome of the uterus to determine what affect the uterine microbiome has on pregnancy outcomes (10). Current theories suggest that NLD microbiota may trigger an inflammatory response in the endometrium that affects the success of embryo implantation, as inflammatory mediators are closely regulated during the adhesion of the blastocyst to the epithelial endometrial wall.

A perfect relationship between the uterus and the embryo, mediated by a tightly controlled interaction between the embryo and the endometrium, is required for successful implantation. With almost 4 million births annually it is important to continue researching and discovering key factors of healthy pregnancies. Any factors affecting this communication, such as altered microbiome may lead to poor reproductive outcomes.

NORMAL MICROBIOME

Uterine Microbiome

For many decades, the uterus was thought to be a sterile environment. Despite being adjacent to the bacterially colonized vagina, it was thought that the cervical mucous maintains uterine sterility. It wasn't until recently that this dogma was challenged. In 1996, Egbase et al. demonstrated that the reproductive tract microbiota can have an effect on IVF outcomes (11).

Is there a healthy "normal" uterine microbiome? This question has been hard to answer. Over the last 15 years about 10 studies have examined uterine microbiome, and over 60 have looked at the reproductive tract microbiome, however most studies to date involved women with pathology (12, 13). Prior to 2014 most studies had attempted to utilize culture techniques which has been shown to miss the majority of the pathogens present, as only bacteria whose metabolic needs are met will grow (14). Additionally, highly abundant and fast growing bacteria will dominate and suppress others (15). Thus, it was not until the expansion of next generation sequencing that a better picture of the reproductive microbiome was even possible. In 2015, Franasiak et al. was the first to measure uterine microbiome from catheter tips at the time of an embryo transfer (6). In the studies of healthy women the most consistent phyla have been Firmicutes, Bacteroidetes proteobacteria, and actinobacteria (6, 8–10, 16–20). The most common genera found in multiple studies have been *Lactobacillus* and *Streptococcus* both of which can be found in the vagina and cervix (12). Some studies have found that *Lactobacillus* is more prominent in women with endometrial polyps or chronic endometritis (21). Multiple studies have suggested that chronic endometritis is associated with recurrent pregnancy loss (22, 23). Chronic endometritis (CE) is typically defined as a chronic inflammation of the uterine lining and is associated with the presence of plasma cells on endometrial biopsy (24, 25). Numerous microbes have been found in patients with CE including *Gonorrhea*, *Chlamydia*, *Escherichia coli*, *Streptococcus*, *Staphylococcus*, *Enterococcus fecalis*, and non-microbial causes, such as retained tissue. Additionally, endometriosis has been hypothesized to alter the endometrium through increased inflammation and progesterone resistance which can affect implantation, increase risk of miscarriage, poor pregnancy outcomes including pregnancy induce hypertension and preterm birth (26). It would appear that increased inflammation at the endometrial level regardless of the cause may affect implantation and pregnancy outcomes.

A landmark study by Moreno et al. in 2016 suggested that *Lactobacillus* dominance (>90%) conferred a protective benefit resulting in increasing implantation rates (8). However, given that only 16S methodology was used it is unclear whether certain species or subspecies of *Lactobacillus* may be capable of conferring this benefit. A recent study on the endometrial microbiota and chronic endometritis reported that *Lactobacillus crispatus* was less abundant in patients with CE suggesting that there may be certain *Lactobacillus* spp that is protective (27). More comprehensive whole genome shotgun sequencing (WGS) may help answer this question.

Where does the uterine microbiome come from? There are currently a few theories. The primary theory is ascension from the vagina. While there is a known cervical plug that does protect the uterine environment, we know that, during intercourse, semen is able to ascend into the uterus through small channels in the cervical mucus. Studies have shown evidence of a uterine pump moving radio tagged isotopes from the vagina into the uterus within 15 min of intercourse (28). Other possible methods include hematogenous spread from the gut and transmembrane gut leakage into the peritoneal cavity with retrograde ascension via the fallopian tubes. Dendritic cells and leukocytes traffic bacteria found in the gut and can hematogenously spread bacteria to other locations, such as the uterus (29). One study showed that when genetically labeled *Enterococcus fecium* was placed in the oral cavity of a mouse it could be detected in

the placenta (30). Given these possible origins of the uterine microbiome, it is important to understand the microbiome of various anatomical locations.

Vaginal Microbiome

Since the most likely explanation is ascension, it is important to spend some time understanding the vaginal microbiome. Over the last decade studies involving both 16S and metagenomics have examined the microbiome of the human vagina. The human vagina has been shown to harbor predominantly *Lactobacillus* spp in concentrations as high as 10^7-10^9 per gram of vaginal fluid (10, 31, 32). The high levels of *Lactobacillus* are known to secrete lactic acid creating the characteristic low PH found in the vagina. This low PH has been shown to help protect against cervico-vaginal infections (33, 34). While the exact reason for *Lactobacillus* predominance is not known, there are some beneficial aspects of eubiosis. One benefit of the native microbiome is a concept called competitive exclusion. Competitive exclusion is where native microbiome can adapt to be the best nutrient scavenger in that environment, competing with potential invaders for nutrients and in turn starving other pathogens. In reproductive aged women there are five major types of vaginal microbiota or community state types (CST). CST I II, III, and V are all predominantly *Lactobacillus*. Whereas, CST IV is predominantly a mixture of strict and facultative anaerobes including *Gardnerella*, *Atopobium*, *Mobiluncus*, and *Prevotella* (35, 36). CST IV is commonly broken down into CST IV-A and CST IV-B which is associated with bacterial vaginitis (BV) (36). CST I appears to be more common in Caucasian women and protective against BV, while CST IV is more common in African American and Hispanic women (37, 38).

Studies have suggested that the vaginal microbiota is subject to frequent fluctuations (39). In some women, menses or sexual behaviors may trigger transitions between the CSTs at different points in time (36). During times of elevated estrogen, such as immediately prior to ovulation, *Lactobacillus* tends to stabilize, while during menstruation *Lactobacillus* tends to decrease (40). While menses appears to alter the composition of the vaginal microbiome, the change appears to depend on the initial CST and other factors, such as the use of pads or tampons (41). The above studies suggest the dynamic nature of the vaginal microbiome, questioning whether one can reliability predict microbiome between menstrual cycles.

There is debate in the literature regarding whether contraceptive options have an effect on the vaginal microbiome. One study suggested that hormonal contraceptives did not have an effect on vaginal microbiota while copper IUDs are associated with an increase in BV (42). Another study suggested that women who took combined oral contraceptives were more likely to have *Lactobacillus* dominance compared to women who used barrier methods (43). One study suggested that LNG-IUD may increase the risk of candida and decrease *Lactobacillus* dominance (44). Still another study suggested it was not contraceptive options which drove vaginal microbiome changes but rather the number of sexual partners and the woman's ethnicity (37). Additionally, elevated hormones at the time of implantation could affect the microbiome, for example during a fresh embryo transfer.

A number of other conditions have been associated with an altered vaginal microbiome. Some studies have suggested that *Lactobacillus* dominated microbiota can be protective against PID (32). CST I appears to decrease the risk of STIs, such has *Chlamydia*, while CST III appears to be associated with an increased risk of *Chlamydia* infection (45). CSTs II and V appear to compete with Gonorrhea thereby possibly decreasing the risk of infection (46).

Alterations of the vaginal microbiota may also be responsible for various pregnancy outcomes. During pregnancy menses cease and there is a consistently elevated level of estrogen with a concomitant increase in *Lactobacillus* dominance (47). Several studies have suggested that altered microbiota are associated with preterm birth (48–50). Preterm premature rupture of membranes (PPROM), chorioamnionitis and early or late miscarriages have also been associated with changes in the vaginal microbiome (51–56). One study of the vaginal microbiome on the day of embryo transfer suggested that a lower microbiota index is associated with better IVF outcomes. A recent study suggested that higher levels of vaginal *L. crispatus* is associated with higher chance of pregnancy when utilizing ICSI (57). Neither of these studies included sampling of the upper reproductive tract; therefore, it is hard to draw conclusive results (58). The literature appears to support the concept that alterations in the vaginal microbiome are associated with various poor outcomes and that examining the vaginal along with the upper reproductive tract microbiome at the time of an IVF transfer may shed additional information into the causes of these poor outcomes.

Placental Microbiome?

Numerous studies have suggested that bacterial infections are the cause of PPROM and preterm labor (59–61). However, until 2014, it was thought that the placenta did not contain its own microbiome. In chorioamnionitis, an inflammatory infectious process of the fetal side of placenta, the most commonly isolated pathogens are *Bacteroides* species, *E. coli*, *Gardnerella vaginalis*, *Mycoplasma hominis*, *Peptostreptococci*, *Streptococci*, and *Ureaplasma urealyticum* (62). This would suggest that pathologic bacteria can invade the amnion, and chorion from the vagina. Additionally, there are theories of oral placental transmission in patients with poor periodontal disease associated with poor pregnancy outcomes (63). However, the idea that a placenta contains its own healthy microbiome only came about recently after the seminal publication by Aagaard et al. in 2014 (64). This study found multiple phyla in the placental microbiome namely *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria.* The initial results were called into question as the techniques did not account for live vs. dead bacteria and did not provide a maternal blood sample to determine if the microbiome was from the maternal villi or from the fetal side (65). Additional studies utilizing high throughput sequencing technology confirmed the presence of a placental microbiome (66, 67). Given that the prevailing opinion for many years was that the uterus was sterile, there were theories proposed that the findings were contaminations in either technique or processing. Later studies suggested that DNA reagent kits have their own distinct microbiome called a "kitome"

(68). Researchers suggested that, in locations with a high biomass, such as the gut, low level contamination of the "kitome" would not be detected, which would explain why many of the gut studies did not have this issue. However, when looking at ultra-low biomass locations, such as the placenta or uterus, the results may represent the kit without proper controls (69). A recent seminal study in which the researchers carefully controlled for possible contaminants by utilizing multiple detection methods including culture, qPCR, 16S rRNA gene sequencing and shotgun metagenomics, demonstrated that no resident microbiota could be identified in the placenta. While this study was done with the best technology currently available, if a uterine microbiome exists, as many studies have suggested, it is highly unlikely that the placental and uterine microbiome are not related. It is possible that the placental microbiome is currently undetectable due to ultra-low biomass or the limitations of current technology, however dismissing it as non-existent may be premature.

NORMAL AND ABNORMAL IMMUNOLOGICAL RESPONSES TO PREGNANCY

The embryo is initially fertilized in the fallopian tube and must evade the immune system, grow and move into the uterus before interacting with the endometrium for implantation. The process of implantation is divided into three key steps: apposition, attachment (adhesion), and penetration (invasion). The immune system plays a role in each step of the embryo's development through the delivery of a live healthy fetus.

An egg is released as a maternal cell containing maternal antigens and would be recognized by the immune system as "self." Sperm then enters the egg typically in the ampullary region of the fallopian tube, triggering multiple changes including production of its own antigens comprised of both maternal and paternal components. Until the embryo starts presenting its own foreign antigens it is viewed as a maternal cell. Once it starts presenting its own antigens it is surrounded by the zona pellucida, a hard shell protecting the embryo from maternal immune cells. Additionally, there are maternal cumulus oophorus cells that provide some protection for the first few days. After the first few days, the embryo must interact with the maternal system. Given that donor embryos are able to implant in surrogates, there must be communication from the embryo that ultimately prevents the maternal immune system from attacking it. It would appear that this communication does not occur immediately given that it takes 4–5 days post-embryo transfer for implantation to occur. During that time a series of events occur, including endometrial priming, immune tolerance and ultimately implantation.

The immune system is usually thought of as a mechanism for the body to defend itself against invaders. Although the egg comes from the woman and would be considered "self," the sperm displays paternal antigens. Shortly after conception the fetus begins displaying major histocompatibility complex (MHC) genes that are a combination of both mother (self) and father (foreign) (70). How then are embryos not attacked and destroyed by the immune system in every pregnancy? The immune system plays an important role in pregnancy success. A combination of multiple strategies are employed to selectively circumvent or engage different aspects of the immune response.

The endometrium has an innate immune system called pattern recognition receptors (PRR) that can detect certain pathogenic receptor patterns locally and mount an inflammatory response. Examples include toll like receptors 1–10, Nod like receptors, and others (71). Some have suggested that local bacteria can also use the PRR as a way to communicate with the host and induce a safe environment (72). A prime example is the way that T-cells are differentiated.

T-Cells

There are many different types of T cells that are part of the adaptive immune response. T-helper cells (Th) start out from the same precursor as a Th0 cell. Dendritic cells, a type of antigen producing cell (APC), presents antigens to the naïve Th0 cells and depending on the environmental milieu of cytokines, chemokines, and bacteria the Th0 will differentiate into one of 4 main cell types: Th1, Th2, Th17, and T-regulatory (Treg) (73). Th1 cells are stimulated due to the presence of bacterial DNA and produce pro-inflammatory cytokines, such as TNF and IL12. Th2 cells are stimulated by IL4, IL6, IL10, and IL11 and produce anti-inflammatory cytokines, such as IL4 (74). Th17 is induced based on TGFβ, IL6, and IL21 and produces pro inflammatory cytokines (74). Treg cells are induced by TCR and TGFβ, express Foxp3, CD25 and are key players in inducing tolerance and negative regulation of immune-mediated inflammation (75). Local microbiome can affect whether the TGFβ increased Th17 or Treg cell population (76). Treg cell dysfunction is associated with autoimmune, auto-inflammatory disorders, allergies, as well as acute and chronic infections (77).

The embryo must convince the body that it should be tolerated. Treg cells take "non-self" antigens that are presented to it and promote active tolerance to those antigens by down-regulating the Th1 and Th17 responses (70, 78, 79). Treg cells inhibit additional T-cell proliferation and proinflammatory cytokine productions by producing TGFβ and IL10 while suppressing B cell proliferation, antibody production, NK cell cytotoxicity, as well as dendritic and macrophage maturation and activation (80–82).

Treg cells help down-regulate immune response against the embryo to allow for intimate interaction with the endometrial lining (70). Treg cells are recruited to the uterus shortly after conception which is an important time for the fledgling embryo's existence. Apposition, implantation and early placental morphogenesis is a key time in which the embryo begins to interact directly with the maternal immune system. Poor immune tolerance at this point could result in shallow implantation which has been implicated as a possible cause of IUGR and miscarriage (83, 84). Additionally, problems in early placental development have been linked with pre-eclampsia, an increasingly common morbidity of late pregnancy (85). There is evidence that the local microbiome can induce a shift to Th1 and away from Treg cell dominance. For example, *Bacteroides* is thought to increase the presence of Th1 cells (86). An upregulation of Th1 will cause an

increase in local inflammation and an augmented local immune response. As mentioned prior, it would appear that increased inflammation at the endometrial level regardless of the cause may affect implantation and pregnancy outcomes. However, beneficial microbiome can increase uterine Treg cells locally thereby downregulating the immune response and inducing tolerance to a specific species or a pregnancy (13).

Blast Development

In vivo, in the first 48 h post-fertilization the embryo is encased in a hard-outer shell called the zona pellucida. Within 2 days, the embryo moves from the fallopian tubes into the uterus where it floats while undergoing cleavage and differentiation into a blastocyst. About 5–6 days post-fertilization, the embryo hatches from the zona pellucida exposing its outer trophoblasts to the endometrial lining (87). Embryos display cytokine receptors from conception until implantation. Cytokines play a key role in embryo development, gene expression, cell number, embryo competence and viability (88, 89). In healthy women, embryotrophic factors, such as GM-CSF, CSF1, LIF, HB-EGF, IGF1, and IGF2 all act to promote blastocyst development, increase blast cell numbers and gene expression. Decreased cell number and poor trophoblast development can cause changes in placental structure and nutrient transport functions leading to pregnancy loss or developmental damage (90, 91). GM-CSF is produced by the uterine epithelial cells, exerting a pro survival and anti-stress effect required for fetal viability and offspring health (92). Embryotoxic cytokines, such as TNF, IFNG, and TRAIL are stimulated during inflammation or local infections via toll like receptors and induce apoptosis and inhibit embryo development (93). Other environmental factors, such as hyperglycemia and obesity can also induce embryotoxic chemokines which may explain why these conditions are associated with poor pregnancy outcomes. This complex balancing act between embryotrophic vs. embryotoxic cytokines acts a physiologic control enabling pregnancy when the uterus is under optimal conditions and inhibiting pregnancy when the conditions are sub-optimal. Obviously, these alone are not the only determinants, as obese diabetic women do get pregnant, however, the delicate cytokine balance, controlled by many factors including the local microbiome, can have a large impact on reproductive outcomes.

Endometrial Receptivity

Sex steroids also play an important role in modifying the immune response and preparing the uterine lining for implantation. In the proliferative phase, estrogen stimulates proliferation and differentiation of endometrial cells as well as pro inflammatory cytokines, such as CSF1, GM-CSF, INFG, and TNF. During the luteal phase, progesterone stimulates the mesenchymal cells to prepare for implantation and suppresses the pro inflammatory cytokines by inhibiting epithelial cell productions of GM-CSF 1 and IL1. Progesterone also enhances the expression of IL8 and attracts uterine NK cells (70). Estrogen increases Treg attracting chemokines, CCL3, 4, and 5, while progesterone sustains the Treg population and increases their suppressive function (94). Additional endometrial chemokines, such as LIF and IL11 are essential for decidual and vascular changes required to allow proper trophoblastic invasion (95). It has been suggested that in patients with endometriosis the endometrial tissue may have progesterone resistance which could explain the increased inflammation and poor pregnancy outcomes (96). Placental trophoblasts secrete TGFβ which helps suppress immunity, and assists in inducing Treg cells at the placental maternal interface (70, 97). Excess inflammatory cytokines, such as TNF, IFNG, and IL2, during the implantation period can act to skew the adaptive immune response away from Treg cells and toward cytotoxicity (98). Excess inflammatory factors are found during local infection and times of nutritional or metabolic stress, thereby helping the body suppress pregnancy during unfavorable conditions; leading to failed implantation or pregnancy loss. Excess inflammation may also be found when the host microbiome is altered thereby conferring negative effects on implantation and outcomes.

Other cells also play a role in controlling immune response at the uterine level. Uterine macrophages produce Treg stimulatory cytokines TGFβ, PGE2, and IL10 (99). Decidual macrophages also secrete matrix proteinases which can help with trophoblast invasion (100). Dendritic cells present antigens to naive T cells to stimulate Treg cell activation before implantation (101). Uterine NK cells are involved in the endometrial remodeling needed for implantation (102). Seminal fluid appears to play a role in driving leukocytes to the uterus. Seminal fluid can stimulate the expansion of the Treg population via TGFβ and PGE2 along with paternal antigens and help inhibit the activated Treg cells from leaving the uterus, inducing tolerance to the embryo.

The embryo also plays a role in its own fate by secreting factors. PreImplantation Factor (PIF) is secreted only by viable embryos and can be measured in embryo culture media. PIF can be seen in maternal circulation as soon as 4 days post-embryo transfer indicating that it does come from the embryo. When PIF is seen in maternal plasma, the chance of normal pregnancy is significantly higher (103). PIF appears to have anti inflammatory effects. It provokes global immune regulation by binding ligands to CD14 monocytes/neutrophils and to T and B cells promoting the required Th2/Th1 cytokine ratio. It also appears to affect genes involved in oxidative stress, protein misfolding, and platelet activation.

Human chorionic gonadotropin (hCG) has many different roles during early pregnancy including immunosuppression. Its' most well-known and crucial role is to promote corpus luteal progesterone production for the first 3–4 weeks after implantation (104). In addition, hCG is thought to help promote placentation and angiogenesis by recruiting and promoting VegF and Treg function necessary for decreasing local inflammation (105).

The immune environment during the peri-conceptual period is controlled by many factors, including cytokines, chemokines and leukocyte lineages. These factors are intertwined with ovarian steroid hormones, seminal fluid, diet, nutrition, metabolism, obesity, infections, and the microbiome to influence the balance between embryotrophic and embryotoxic milieus and ultimately a successful or unsuccessful pregnancy.

UTERINE EMBRYO SIGNALING AND IMPLANTATION

As mentioned previously there are three stages to implantation: apposition, adhesion, and invasion. We have discussed how the microbiome cytokine influence can affect the competency of the embryo. We will now briefly discuss each stage of implantation to understand how the microbiome could help or hinder the successful progression of pregnancy. Researchers still do not fully understand how implantation works. Decidualization is the transformation of the endometrium into a receptive state prior to the presence of the embryo. Between 6 and 10 days after ovulation the point in which the endometrium is receptive occurs, called the window of implantation. Once the embryo reaches the primed endometrium there is a process of selection, apposition, attachment, implantation, and ultimately placentation that requires intricate embryo uterine interactions to occur in a synchronized fashion for pregnancy to be sustained. While the main drivers of these processes are hormonal control, additional local cytokine factors may play an important role. Alterations from the natural microbiome may affect the local cytokine/chemokine profile thereby affecting implantation.

Apposition and Adhesion

Approximately 6–7 days after fertilization the blastocyst makes contact with the uterine wall in a transient dynamic process called apposition. Microvilli extending from the syncytiotrophoblast interact with small microprotrusions from the uterine epithelial cells called pinopodes. This step is unstable and must occur first before the embryo can firmly attach to the wall. The interaction between the uterine cell wall and the embryo activates cytokine signaling and remodeling of the cytoskeleton of the epithelial layer. Decidual macrophages secrete matrix proteinases which can help with trophoblast invasion (100). Matrix metalloproteinases are important players in trophoblast invasion and are regulated by cytokines and tissue inhibitors (TIMPs) (106). TIMPs are upregulated by TGFβ inhibiting proper matrix degradation which can affect implantation (107). Increased TGFβ can be caused by colonizing microbes (76). Tight junctions are disrupted mediated by local cellular communication (108). Uterine NK cells are also involved in the endometrial remodeling needed for implantation (109).

Invasion

The final stage is invasion during which the syncytiotrophoblast invade the uterine epithelium. By approximately day 10 after fertilization, the blastocyst is surrounded by uterine tissue and the epithelium regrows over the implantation site. The cytotrophoblasts continue to expand until they reach the myometrium and the uterine spiral arteries leading to the establishment of uteroplacental circulation (110). The resulting uterine arterioles are composed of both maternal and fetal cells, underscoring the importance of immune tolerance in proper placentation. Implantation is the result of an intricate bi-directional dialogue between the embryo and endometrium mediated by a host of factors regulating the invading cells from cytokines and growth factors to steroid hormones and proteinases (111). Dysfunctional placentation can have clinical implications due to either excessive or inadequate invasion. Excessive invasion of the cytotrophoblasts can lead to abnormally strong attachment or a morbidly adherent placenta, such as an accrete, increta, or percreta depending on depth of invasion. Inadequate invasion has been implicated in pre-eclampsia, and IUGR (112, 113).

CURRENT UNDERSTANDING OF PRE-ECLAMPSIA

After years of study, the mechanisms by which pregnancy incites or aggravates hypertension remain unknown and hypertensive disorders continue to play an important role in maternal morbidity and mortality worldwide. Preeclampsia is more likely to occur in women who are exposed to chorionic villi for the first time (nulliparous women); are genetically predisposed to hypertensive disorders of pregnancy; have preexisting conditions associated with endothelial cell activation or inflammation including diabetes, cardiovascular or renal disease, or immunologic disorders; and in women who are exposed to a superabundance of chorionic villi (as in the cases of twins or molar pregnancies). Additionally, while a fetus is not required for the development of preeclampsia (as in molar pregnancies), the presence of chorionic villi is. A case series published in 2008, reported that PEC may develop even when the chorionic villi are extra-uterine as in the case of an abdominal pregnancy (114). Regardless of the underlying cause, the events leading to PEC all result in systemic vascular endothelial damage leading to transudation of plasma, vasospasm, and thrombotic sequelae.

Currently, the four most likely explanations for the development of PEC include: immunological maladaptive tolerance between maternal, paternal, and fetal tissues; placental implantation with abnormal trophoblastic invasion; oxidative stressors resulting in endothelial cell dysfunction; or genetic factors including predisposing genes and epigenetic influences.

Loss of maternal immune tolerance to paternally derived antigens is a possible etiology of preeclampsia (115). This hypothesis is supported by the fact that preeclampsia is more likely to occur when the formation of blocking antibodies to paternal antigens might be impaired. For instance, there is an increased risk in first pregnancies or pregnancies with a new partner and molar pregnancies which have an increased paternal antigenic load. Additionally, pregnancies with a fetus with trisomy 13 who have elevated antiangiogenic factors arising from the presence of an extra copy of *soluble fms-like tyrosine kinase 1* which is located on chromosome 13 have increased risk of preeclampsia (116, 117). A recently published study indicated that the angiogenic factors placental growth factor, *soluble fms-like tyrosine kinase 1,* and soluble endoglin are biomarkers with predictive potential for preeclampsia. The soluble *fms-like tyrosine kinase 1*/placental growth factor ratio is able to accurately predict the short term absence of preeclampsia and suggest the likelihood of adverse events within 4 weeks (118). Further, another study from April 2019 demonstrated that $CD3^+$,

$CD8^-$, $FoxP3^-$ T cells were associated with uteroplacental acute atherosis which is a common lesion of the maternal spiral arteries in the decidua basalis in preeclampsia (119). The decidua basalis layer forms the maternal-fetal immunologic interface where fetal extra-villous trophoblasts interact with maternal immune cells. Immune maladaptation may also play a role in the pathophysiology of preeclampsia. Extra-villous trophoblasts express HLA-C which is a ligand for killer immunoglobulin-like receptors (KIR) on NK- and T-cells. The combination of maternal KIR-B haplotype and fetal HLA-C2 has been shown to be significantly associated with acute atherosis. Thus, it seems that interactions between fetal HLA and activating KIRs on maternal decidual NK-or T-cells may promote local decidual vascular inflammation and trigger the formation of acute atherosis (120). This supports the theory that inadequate maternal tolerance of invasive trophoblast, which can be due to a shift in the immune system against tolerance, i.e., the Th1/Th2 ratio, can trigger poor trophoblast invasion and the occurrence of preeclampsia (121).

As previously discussed, normal implantation requires invasion of the syncytiotrophoblast into the uterine epithelium, ultimately resulting in remodeling of the uterine spiral arteries to create a dilated low resistance vessel. In pregnancies complicated by preeclampsia, there is thought to be incomplete invasion of the spiral arteriolar wall leading to small caliber, high resistance vessels with correlation shown between the degree of syncytiotrophoblast dysfunction and the severity of the resulting preeclampsia (122, 123). These high resistance vessels impair placental blood flow resulting in diminished perfusion and a hypoxic environment. There is subsequently a release of placenta microparticles which incites a systemic inflammatory response (124, 125).

Decreased placental perfusion from dysfunctional placenta implantation results in repeated ischemia/reperfusion episodes which creates a favorable environment for developing oxidative stress and stimulates the production and secretion of pro-inflammatory cytokines as well as vasoactive compounds. Cytokines, such as tumor necrosis factor alpha and interleukins contribute to systemic oxidative stress which is characterized by reactive oxygen species and free radicals that lead to the formation of lipid peroxides (126). These lipid peroxides then generate highly toxic radicals that injure vascular endothelial cells, modify nitric oxide production by the cells, and interfere with prostaglandin balance. The above cascade results in systemic endothelial dysfunction characterized by vascular inflammation and constriction. Other consequences of oxidative stress include production of lipid-laden macrophage foam cells that are seen in acute atherosis, activation of microvascular coagulation, and increased capillary permeability. The important role of oxidative stress in the pathophysiology of preeclampsia is further supported by a study in which concentrations of maternal oxygen free radical were measured in 52 women with and without preeclampsia. Maternal serum concentrations of oxygen free radicals were significantly increased in the preeclampsia group relative to the normal group (127). Some researchers conclude that oxidative stress appears to be the central component of both placental and endothelial dysfunction, the causative etiology of preeclampsia (128).

Preeclampsia is a multifactorial condition with a strong genetic component. Immune maladaptation, endothelial function, and oxidative stress all encompass genetic factors that could be responsible for the pathogenic changes that take place in preeclampsia. Additionally, there is evidence that paternal genes significantly increase the risk of preeclampsia (129). In a study of ∼1.2 million births in Sweden, a genetic association for preeclampsia was noted (130). The hereditary risk of preeclampsia most often cited is 20–40% for daughters of pre-eclamptic mothers and 11–37% for sisters of pre-eclamptic women (131). The hereditary predisposition for preeclampsia most likely results from the interactions of hundreds of genes and it is doubtful that any one gene will be found responsible. Epigenetic alterations have also been noted in preeclampsia including alterations of methylation in the placenta of pre-eclamptic patients. Additionally, it is hypothesized that antiangiogenic and cytotoxic factors released by the placenta in preeclampsia have the potential to induce epigenetic modifications in maternal tissues (132).

CURRENT UNDERSTANDING IN CAUSES OF IUGR

Fetal growth restriction, also known as intrauterine growth restriction (IUGR), is a common pregnancy complication that has been linked with a variety of adverse perinatal outcomes. The precise mechanism by which normal growth occurs is unknown, but IUGR is usually the end result of maternal, fetal and placental causes or a combination thereof. Although the primary pathophysiologic mechanisms underlying these conditions are different, they often result in the same final common pathway of suboptimal uterine–placental perfusion and fetal nutrition.

Multiple placental, cord, and uterine anomalies are associated with poor fetal growth. Placental insufficiency may be due to abnormal placental development or placental damage. Several placental abnormalities including chronic abruption, infarction, circumvallate shape, chorioangioma, velamentous cord insertion, and umbilical artery thrombosis have been shown to lead to uteroplacental insufficiency and IUGR (133). Inflammation may contribute to placental damage and abnormal development as inflammatory mediators promote thrombosis (133). Hypoperfusion of the placental site may also arise secondary to implantation site disorders. Brosens et al. postulated that there is a partial progesterone resistance in the fetal uterus at the time of birth that may persist into the adolescent years resulting in compromised physiological transformation of the spiral arteries. This theory is supported by the fact that major obstetric syndromes due to impaired placental bed spiral artery remodeling, including preeclampsia, growth restriction, and preterm labor, are all more prevalent in teenage pregnancies (134).

Maternal medical comorbidities, especially those with vascular disease or thrombosis, are also associated with IUGR via poor placental perfusion. Chronic vascular disease,

including maternal ischemic heart disease, is associated with higher rates of preeclampsia and IUGR (135). Chronic renal insufficiency is frequently accompanied by underlying hypertension and vascular disease and thus, often also results in IUGR. Additionally, pre-gestational diabetes, especially when complicated by vascular or renal disease, and disease states resulting in chronic uteroplacental hypoxia like pre-eclampsia, chronic hypertension and asthma can lead to significantly reduced birthweight. Maternal conditions, like antiphospholipid syndrome, increase the risk of ischemic placental dysfunction resulting in a similar outcome of poor perfusion and decreased growth (136). Other maternal factors may contribute to IUGR without directly impacting the placenta. These include poor maternal nutrition and eating disorders; constitutionally small mothers, particularly when combined with poor gestational weight gain, and social issues (137–139).

Exposure to drugs and teratogens during pregnancy are associated with IUGR. Cigarette, opiate, alcohol, and cocaine use cause fetal growth restriction directly and by decreasing maternal food intake (140, 141). Even prescription medications like anticonvulsants, antineoplastic agents, and antithrombotic drugs are teratogens and can result in fetal growth restriction (142, 143). Multifetal gestation and fetal malformations are also seen in association with intrauterine growth restriction. In one study of pregnancies complicated by gastroschisis, one-third of neonates had birthweights less than the 10th percentile (143).

Still other etiologies of fetal growth restriction interact with the placental, maternal, and fetal compartments. The most important of these include infections and genetic anomalies. In their text book Maternal Fetal Medicine: Principles and Practice, Creasy et al. postulated that fetal infections account for 5–10% of IUGR with malaria accounting for most cases of infection-related fetal growth restriction worldwide (144, 145). Additionally, rubella, CMV, toxoplasmosis, varicella, and syphilis have all been shown to have a causal relationship with fetal growth restriction (146–149). Under normal conditions, maternal genes have the main influence on birthweight (150). Fetal aneuploidy is responsible for up to 5% of IUGR diagnosed at any point of pregnancy and up to 20% of IUGR diagnosed in the first half of pregnancy (151). Trisomy 13 and 18 are usually associated with more severe IUGR while in Trisomy 21 the growth restriction is typically mild (152). Even confined placental mosaicism is associated with low birth weight and adverse pregnancy outcomes (153). In summary there are many possible etiologies including inflammation and abnormal initial placentation all causing the same final pathway of IUGR.

HOW MICROBIOME AFFECTS IMMUNITY IN OTHER AREAS OF THE BODY AND IMPLICATIONS FOR THE UTERUS

Studies looking at germ free mice revealed that without bacteria there are profound effects on mouse lymphoid tissue, suggesting that bacteria is important for immune development (154). The GI tract is the most studied area of human microbiome to date as the majority of immune system interactions occur within the gut (76). It contains the most abundant population of microorganisms with over 5,000 bacteria taxa (155). The gut microbiome is colonized at birth with initial differences noted depending on mode of delivery (156). Unlike the uterine microbiome that undergoes changes with menses, once a child is about 2.5 years old their microbiome remains relatively stable until age 65 (156). Although it is stable, it can be quickly altered by host factors, such as antibiotics, travel or high fat diets (157). Dysbiosis in the gut has been linked to many diseases, ranging from liver disease and GI cancer to metabolic disease, respiratory disease, mental health disorders and autoimmune disorders (158). The suggested reasoning is that the changes in the microbiome can increase immune system sensitivity to pathogens thereby causing increased inflammation (159). The most potent causes of dysbiosis are pathogens in the gut. There is evidence that altered oral flora, such as during periodontal disease can cause many systemic disorders, such as atherosclerosis and poor pregnancy outcomes (160, 161). Chronic low-level periodontal disease may induce low level chronic systemic inflammation (162). There are theories as to why dysbiosis causes these changes. Alterations in gut microbiota has profound effects on the T-cell mediated autoimmune and inflammatory responses. The gut microbiota is known to provide signaling for proper development, differentiation and epigenetic influences of immune cells (154). Dysbiosis can transition the chemokine profile to cause a Th17 pro inflammatory dominance, which can trigger not only local, but also systemic inflammatory responses and has been linked to Alzheimer's disease (159, 163).

In 2012, Hooper and Macpherson described immunological benefits of host intestinal microbial homeostasis: (1) Host microbiome restricts direct contact between epithelia and pathogenic microbes, (2) Host microbiome anatomically limits the exposure of pathogenic bacteria to the systemic immune system, and (3) Host microbiome can aid in the rapid detection and killing of bacteria upon barrier breach (154).

Some of the suggested theories is that is that host bacteria (microbiome) have the chance to adapt to their environment. The adaptation process allows them to specialize in nutrient utilization thereby depleting nutrients that would otherwise be available for invading pathogens. This process, called colonization resistance, could support the potential importance of a healthy uterine microbiome (164). Additionally, symbiotic bacteria may also compete for the cell's receptors. For example, one study utilizing an *in vitro* model found that the presence of lactobacillus in the reproductive tract prevents gonorrhea from attaching to endometrial cells (46).

The host microbiome may also help support a healthy and intact epithelial barrier thereby preventing pathogen access to the cell. In the gut, studies have suggested that the microbiome can affect epithelial cell differentiation, maintenance and adaptation and modulate epithelial cell permeability (165, 166). Given that the uterus frequently undergoes shedding and regrowth, microbial support could play an important role in

maintenance of the epithelial tissue. Conversely, dysbiosis could be an underlying cause of poor endometrial thickening and abnormal placentation.

HYPOTHESIS FOR HOW ALTERED MICROBIOME AFFECTS PEC AND IUGR

As discussed above, studies have shown that changes in the microbiome can prompt inflammation. For example, specific oral pathogenic bacteria including *Fusobacterium nucleatum, Porphyromonas gingivalis, Filifactor alocis, Campylobacter rectus* are associated with both periodontitis and the development of pregnancy disease (167). The involvement of systemic inflammatory responses in pregnancies complicated by PEC and IUGR has led to the theory that maternal infections may be important factors in the pathogenesis of pregnancy complications. At the base of all possible etiologies of preeclampsia, there exists the same common result of systemic vascular endothelial damage. The remodeling of the spiral arteries in the decidua basalis is a critical step in the establishment of a healthy pregnancy. The decidua basalis is the maternal fetal immunologic interface and it has been shown that local inflammation in area can lead to acute atherosis and poor trophoblast invasion. Additionally, studies have shown that women with asymptomatic bacteriuria, urinary tract infection, and chronic pyelonephritis are at increased risk for preeclampsia (168, 169). Another study by den Hollander et al. found that *Helicobacter pylori,* as a cause of chronic inflammatory conditions, is associated with an increased risk of PEC (170). Further, Li Juan et al. demonstrated that preeclampsia is associated with a disrupted gut microbiota composition compared with that of women who had uncomplicated pregnancies (171). In a recent review of the current knowledge about the possible association between the microbiome and the development of preeclampsia, Dunn et al. did a comprehensive literature search and reported that overall, five groups of investigators studied the microbiome of PEC (172). In two of the studies, the placenta site was analyzed; in the remaining three, the mouth, gut, or an intra-amniotic site was examined. Some findings supported the association between pathogenic bacteria and PEC, but specific pathogenic organisms were not identified and further research is warranted. In a 2015 study, placental tissue samples from women with and without preeclampsia were collected and screened for the presence of bacteria by PCR for 16s rRNA and next generation sequencing. 12.7% of the tissue from women with PEC was PCR positive, while all of the placentas of the control group were negative (173).

While the etiology of growth restriction has also not been elucidated precisely, it seems to result from poor uterine-placental perfusion. It is known that inflammation may contribute to placental damage and that any maternal disease state that can lead to utero-placental hypoxia, like preeclampsia, can result in the development of intrauterine growth restriction. In this way, it is possible that the maternal microbiome could modulate the development of growth restriction by influencing the inflammatory state of the placenta and uterus. One example of systemic inflammation contributing to the development of pregnancy complications can be seen in the study of Den Hollander et al. They reported that *H. pylori* seropositivity with CagA-positive strains, which are associated with higher levels of systemic inflammation than CagA-negative strains, is associated with IUGR (170). Additionally, a study analyzing the characteristics of gut microbiota in IUGR and normal birth weight piglets in the first 12 h of life found an imbalanced inflammatory and plasma metabolome profile in the IUGR piglets (174). The gut microbiome is believed to be colonized at birth with differences seen based on mode of delivery and thus, presumably, exposure to the maternal microbiome. To our knowledge no studies have been done, to date, evaluating the uterine microbiome at the time of implantation and its effect on the development of obstetrical complications of pregnancy including preeclampsia and growth restriction.

SUMMARY OF EFFECTS OF MICROBIOME ON UTERINE IMMUNE SYSTEM

Based on both *in vitro* mouse and gut studies there are a likely four possible ways in which local microbiome can affect the clinical sequalae of pregnancy. It is possible that the microbiome cause alterations in regional signaling pathways. For example, as mentioned before, alterated microbiome can cause an abnormal inflammatory response through the uterine toll like receptors. When these receptors are activated they can alter the cytokine milieu swaying the local response to a pro inflammatory and anti-tolerance immune cell response. A second method as previously mentioned can be through the alteration of the endometrial epithelial barrier integrity. Certain pathogens cause a localized decrease in matrix degradation proteins which may affect placentation. A third possible method is that the local microbiome has a competitive advantage, as it has adapted to be the best nutrient scavenger in that area and can usually starve out possible invading species in a process called competitive exclusion. Lastly, microbes can secrete metabolites, such as short chain fatty acids that suppress growth of certain species. Taken together, the natural microbiome can have an impressive effect on the local interactions between the embryo and endometrium with implications on implantation, placentation and embryonic growth, ultimately affecting pregnancy and pregnancy outcomes.

CONCLUSION–FUTURE AREAS OF RESEARCH

Current data has yet to support what the characterization of a normal uterine microbiome. However, it is hard to believe that a mucosa that is in close proximity to the vagina with a well-characterized microbiome with regular sperm penetration that microbes do not get through the cervix. The question remains as to whether those microbes are transient and whether a host microbiome controls that transience. Prior studies have been limited so far by 16S only sequencing with an amplification bias and potentially missing any species not currently in

the bioinformatics database. It is highly likely that a normal endometrial microbiome does exist. It is possible that it is of very low level and therefore easily subject to sampling contamination which could explain why we have not yet discovered the "normal microbiome," however it is likely present and given what we know about gut microbiome, it likely plays a role in immunoregulation, endometrial remodeling, pregnancy implantation and placentation. Future research should include sampling of multiple possible contamination sites as well as utilizing shotgun metagenomics for a better understanding of all the pathogens at play.

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REFERENCES

1. NIH Hmp Working Group, Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, et al. The NIH human microbiome project. *Genome Res.* (2009) 19:2317–23. doi: 10.1101/gr.096651.109
2. *About The Human Microbiome. NIH Human Microbiome Project–About the Human Microbiome*. Available online at: https://hmpdacc.org/hmp/overview/ (accessed November 8, 2018).
3. Selman H, Mariani M, Barnocchi N, Mencacci A, Bistoni F, Arena S, et al. Examination of bacterial contamination at the time of embryo transfer, and its impact on the IVF/pregnancy outcome. *J Assist Reprod Genet.* (2007) 24:395–9. doi: 10.1007/s10815-007-9146-5
4. Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Delanghe J, Van Simaey L, et al. Cloning of 16s rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between atopobium vaginae, *Gardnerella vaginalis* and bacterial vaginosis. *BMC Microbiol.* (2004) 4:16. doi: 10.1186/1471-2180-4-16
5. Wade W. Unculturable bacteria–the uncharacterized organisms that cause oral infections. *J R Soc Med.* (2002) 95:81–3. doi: 10.1258/jrsm.95.2.81
6. Franasiak JM, Werner MD, Juneau CR, Tao X, Landis J, Zhan Y, et al. Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16s ribosomal subunit. *J Assist Reprod Genet.* (2016) 33:129–36. doi: 10.1007/s10815-015-0614-z
7. Li F, Chen C, Wei W, Wang Z, Dai J, Hao L, et al. The metagenome of the female upper reproductive tract. *Gigascience.* (2018) 7:Giy107. doi: 10.1093/Gigascience/Giy107
8. Moreno I, Codoñer FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazán J, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am J Obstet Gynecol.* (2016) 215:684–701. doi: 10.1016/j.ajog.2016.09.075
9. Tao X, Franasiak JM, Zhan Y, Scott RT III, Rajchel J, Bedard J, et al. Characterizing the endometrial microbiome by analyzing the ultra-low bacteria from embryo transfer catheter tips in IVF cycles: next generation sequencing (NGS) analysis of the 16s ribosomal gene. *Hum Microb J.* (2017) 3:15–21. doi: 10.1016/j.humic.2017.01.004
10. Chen C, Song X, Wei W, Zhong H, Dai J, Lan Z, et al. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat Commun.* (2017) 8:875. doi: 10.1038/s41467-017-00901-0
11. Egbase PE, al-Sharhan M, al-Othman S, al-Mutawa M, Udo EE, Grudzinskas JG. Incidence of microbial growth from the tip of the embaryo transfer catheter after embryo transfer in relation to clinical pregnancy rate following *in-vitro* fertilization. *Hum Reprod.* (1996) 11:1687–9.
12. Koedooder R, Mackens S, Budding A, Fares D, Blockeel C, Laven J, et al. Identification and evaluation of the microbiome in the female and male reproductive tracts. *Hum Reprod Update.* (2019) 25:298–325. doi: 10.1093/humupd/dmy048
13. Baker JM, Chase DM, Herbst-Kralovetz MM. Uterine microbiota: residents, tourists or invaders? *Front Immunol.* (2018) 9:208. doi: 10.3389/fimmu.2018.00208
14. Nadkarni MA, Martin FE, Hunter N, Jacques NA. Methods for optimizing DNA extraction before quantifying oral bacterial numbers by real-time PCR. *FEMS Microbiol Lett.* (2009) 296:45–51. doi: 10.1111/j.1574-6968.2009.01629.x
15. Hiergeist A, Gläsner J, Reischl U, Gessner A. Analyses of intestinal microbiota: culture versus sequencing. *ILAR J.* (2015) 56:228–40. doi: 10.1093/ilar/ilv017
16. Fang RL, Chen LX, Shu WS, Yao SZ, Wang SW, Chen YQ. Barcoded sequencing reveals diverse intrauterine microbiomes in patients suffering with endometrial polyps. *Am J Transl Res.* (2016) 8:1581–92.
17. Verstraelen H, Vilchez-Vargas R, Desimpel F, Jauregui R, Vankeirsbilck N, Weyers S, et al. Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16s rRNA gene. *PeerJ.* (2016) 4:E1602. doi: 10.7717/peerj.1602
18. Walther-António MR, Chen J, Multinu F, Hokenstad A, Distad TJ, Cheek EH, et al. Potential contribution of the uterine microbiome in the development of endometrial cancer. *Genome Med.* (2016) 8:122. doi: 10.1186/s13073-016-0368-y
19. Miles SM, Hardy BL, Merrell DS. Investigation of the microbiota of the reproductive tract in women undergoing a total hysterectomy and bilateral salpingo-oopherectomy. *Fertil Steril.* (2017) 107:813–20.e1. doi: 10.1016/j.fertnstert.2016.11.028
20. Khan KN, Fujishita A, Masumoto H, Muto H, Kitajima M, Masuzaki H, et al. Molecular detection of intrauterine microbial colonization in women with endometriosis. *Eur J Obstet Gynecol Reprod Biol.* (2016) 199:69–75. doi: 10.1016/j.ejogrb.2016.01.040
21. Fang RL, Chen LX, Shu WS, Yao SZ, Wang SW, Chen YQ. Barcoded sequencing reveals diverse intrauterine microbiomes in patients suffering with endometrial polyps. *Am J Transl Res.* (2016) 8:1581–92.
22. D'Ippolito S, Di Nicuolo F, Pontecorvi A, Gratta M, Scambia G, Di Simone N. Endometrial microbes and microbiome: recent insights on the inflammatory and immune players of the human endometrium. *Am J Reprod Immunol.* (2018) 80:e13065. doi: 10.1111/aji.13065
23. McQueen DB, Perfetto CO, Hazard FK, Lathi RB. Pregnancy outcomes in women with chronic endometritis and recurrent pregnancy loss. *Fertil Steril.* (2015) 104:927–31. doi: 10.1016/j.fertnstert.2015.06.044
24. McQueen DB, Bernardi LA, Stephenson MD. Chronic endometritis in women with recurrent early pregnancy loss and/or fetal demise. *Fertil Steril.* (2014) 101:1026–30. doi: 10.1016/j.fertnstert.2013.12.031
25. Maria M, Cicinelli E, Greco P, Massenzio F, Baldini D, Falagario T, et al. Abnormal pattern of lymphocyte subpopulations in the endometrium of infertile women with chronic endometritis. *Am J Reprod Immunol.* (2009) 61:322–9. doi: 10.1111/j.1600-0897.2009.00698.x
26. Farland LV, Prescott J, Sasamoto N, Tobias DK, Gaskins AJ, Stuart JJ, et al. Endometriosis and risk of adverse pregnancy outcomes. *Obstet Gynecol.* (2019) 134:527–36. doi: 10.1097/AOG.0000000000003410
27. Liu Y, Ko EY, Wong KK, Chen X, Cheung WC, Law TS, et al. Endometrial microbiota in infertile women with and without chronic endometritis as diagnosed using a quantitative and reference range-based method. *Fertil Steril.* (2019) 112:707–17.e1. doi: 10.1016/j.fertnstert.2019.05.015
28. Kunz G, Beil D, Deiniger H, Einspanier A, Mall G, Leyendecker G. The uterine peristaltic pump – normal and impeded sperm transport within the female genital tract. *Adv Exp Med Biol.* (1997) 424:267–77. doi: 10.1007/978-1-4615-5913-9_49

29. Donnet-Hughes A, Perez PF, Dore J, Leclerc M, Levenez F, Benyacoub J, et al. Potential role of the intestinal microbiota of the mother in neonatal immune education. *Proc Nutr Soc*. (2010) 69:407–15. doi: 10.1017/S0029665110001898
30. Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, et al. Is meconium from healthy newborns actually sterile? *Res Microbiol*. (2008) 159:187–9. doi: 10.1016/j.resmic.2007.12.007
31. Srinivasan S, Liu C, Mitchell CM, Fiedler TL, Thomas KK, Agnew KJ, et al. Temporal variability of human vaginal bacteria and relationship with bacterial. *PLoS ONE*. (2010) 5:e10197. doi: 10.1371/journal.pone.0010197
32. Kroon SJ, Ravel J, Huston WM. Cervicovaginal microbiota, womens health and reproductive outcomes. *Fertil Steril*. (2018) 110:327–36. doi: 10.1016/j.fertnstert.2018.06.036
33. Ronnqvist PD, Forsgren-Brusk UB, Grahn-Hakansson EE. Lactobacilli in the female genital tract in relation to other genital microbes and vaginal pH. *Acta Obstet Gynecol Scand*. (2006) 85:726–35. doi: 10.1080/00016340600578357
34. Aldunate M, Srbinovski D, Hearps AC, Latham CF, Ramsland PA, Gugasyan R, et al. Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. *Front Physiol*. (2015) 6:164. doi: 10.3389/fphys.2015.00164
35. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, Mcculle SL, et al. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA*. (2011) 108:4680–7. doi: 10.1073/pnas.1002611107
36. Gajer P, Brotman RM, Bai G, Sakamoto J, Schutte UM, Zhong X, et al. Temporal dynamics of the human vaginal microbiota. *Sci Transl Med*. (2012) 4:132ra52. doi: 10.1126/scitranslmed.3003605
37. Borgdorff H, Verwijs MC, Wit FW, Tsivtsivadze E, Ndayisaba GF, Verhelst R, et al. The impact of hormonal contraception and pregnancy on sexually transmitted infections and on cervicovaginal microbiota in African sex workers. *Sex Transm Dis*. (2015) 42:143–52. doi: 10.1097/OLQ.0000000000000245
38. Marrazzo JM, Thomas KK, Fiedler TL, Ringwood K, Fredricks DN. Risks for acquisition of bacterial vaginosis among women who report sex with women: a cohort study. *PLoS ONE*. (2010) 5:e11139. doi: 10.1371/journal.pone.0011139
39. Schwebke JR, Richey CM, Weiss HL. Correlation of behaviors with microbiological changes in vaginal flora. *J Infect Dis*. (1999) 180:1632–6.
40. Dos Santos Santiago GL, Tency I, Verstraelen H, Verhelst R, Trog M, Temmerman M, et al. Longitudinal qPCR study of the dynamics of *L. crispatus*, *L. iners*, *A. vaginae* (sialidase positive) *G. vaginalis*, and *P. bivia* in the vagina. *PLoS ONE*. (2012) 7:e45281. doi: 10.1371/journal.pone.0045281
41. Hickey RJ, Abdo Z, Zhou X, Nemeth K, Hansmann M, Osborn TW III, et al. Effects of tampons and menses on the composition and diversity of vaginal microbial communities over time. *BJOG*. (2013) 120:695–706. doi: 10.1111/1471-0528.12151
42. 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.e1–10. doi: 10.1016/j.ajog.2018.02.017
43. 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. doi: 10.1016/j.contraception.2016.11.006
44. Donders GGG, Bellen G, Ruban K, Van Bulck B. Short- and long-term influence of the levonorgestrel-releasing intrauterine system (Mirena®) on vaginal microbiota and candida. *J Med Microbiol*. (2018) 67:308–13. doi: 10.1099/jmm.0.000657
45. Van Houdt R, Ma B, Bruisten SM, Speksnijder A, 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. doi: 10.1136/sextrans-2017-053133
46. Spurbeck RR, Arvidson CG. Inhibition of *Neisseria gonorrhoeae* epithelial cell interactions by vaginal *Lactobacillus* species. *Infect Immun*. (2008) 76:3124–30. doi: 10.1128/IAI.00101-08
47. Digiulio DB, Callahan BJ, Mcmurdie PJ, Costello EK, Lyell DJ, Robaczewska A, et al. Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci USA*. (2015) 112:11060–5. doi: 10.1073/pnas.1502875112
48. Elovitz MA, Gajer P, Riis V, Brown AG, Humphrys MS, Holm JB, et al. Cervicovaginal microbiota and local immune response modulate the risk of spontaneous preterm delivery. *Nat Commun*. (2019) 10:1305. doi: 10.1038/s41467-019-09285-9
49. Hyman RW, Fukushima M, Jiang H, Fung E, Rand L, Johnson B, et al. Diversity of the vaginal microbiome correlates with preterm birth. *Reprod Sci*. (2014) 21:32–40. doi: 10.1177/1933719113488838
50. Stout MJ, Zhou Y, Wylie KM, Tarr PI, Macones GA, Tuuli MG. Early pregnancy vaginal microbiome trends and preterm birth. *Am J Obstet Gynecol*. (2017) 217:356.e1–18. doi: 10.1016/j.ajog.2017.05.030
51. Donders GG, Van Bulck B, Caudron J, Londers L, Vereecken A, Spitz B. Relationship of bacterial vaginosis and mycoplasmas to the risk of spontaneous abortion. *Am J Obstet Gynecol*. (2000) 183:4317. doi: 10.1067/mob.2000.105738
52. Hay PE, Lamont RF, Taylor-Robinson D, Morgan DJ, Ison C, Pearson J. Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage. *BMJ*. (1994) 308:295–8.
53. Llahi-Camp JM, Rai R, Ison C, Regan L, Taylor-Robinson D. Association of bacterial vaginosis with a history of second trimester miscarriage. *Hum Reprod*. (1996) 11:1575–8.
54. Gibbs RS. Chorioamnionitis and bacterial vaginosis. *Am J Obstet Gynecol*. (1993) 169:460–2. doi: 10.1016/0002-9378(93)90341-F
55. Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, et al. Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. The Vaginal Infections and Prematurity Study Group. *N Engl J Med*. (1995) 333:1737–42.
56. Paramel Jayaprakash T, Wagner EC, Van Schalkwyk J, Albert AY, Hill JE, Money DM, et al. High diversity and variability in the vaginal microbiome in women following preterm premature rupture of membranes (PPROM): a prospective cohort study. *PLoS ONE*. (2016) 11:e0166794. doi: 10.1371/journal.pone.0166794
57. Koedooder R, Singer M, Schoenmakers S, Savelkoul PHM, Morré SA, de Jonge JD, et al. The vaginal microbiome as a predictor for outcome of *in vitro* fertilization with or without intracytoplasmic sperm injection: a prospective study. *Hum Reprod*. (2019) 34:1042–54. doi: 10.1093/humrep/dez065
58. Hyman RW, Herndon CN, Jiang H, Palm C, Fukushima M, Bernstein D, et al. The dynamics of the vaginal microbiome during infertility therapy with invitro fertilization embryo transfer. *J Assist Reprod Genet*. (2012) 29:105–15. doi: 10.1007/s10815-011-9694-6
59. Garmi G, Okopnik M, Keness Y, Zafran N, Berkowitz E, Salim R. Correlation between clinical, placental histology and microbiological findings in spontaneous preterm births. *Fetal Diagn Ther*. (2016) 40:141–9. doi: 10.1159/000441518
60. Sweeney EL, Kallapur SG, Gisslen T, Lambers DS, Chougnet CA, Stephenson SA, et al. Placental infection with ureaplasma species is associated with histologic chorioamnionitis and adverse outcomes in moderately preterm and late-preterm infants. *J Infect Dis*. (2016) 213:1340–7. doi: 10.1093/infdis/jiv587
61. Quinn PA, Butany J, Taylor J, Hannah W. Chorioamnionitis: its association with pregnancy outcome and microbial infection. *Am J Obstet Gynecol*. (1987) 156:379–87.
62. Moreno I, Simon C. Deciphering the effect of reproductive tract microbiota on human reproduction. *Rprod Med Biol*. (2019) 18:40–50. doi: 10.1002/rmb2.12249
63. Han YW. Oral health and adverse pregnancy outcomes–what's next? *J Dent Res*. (2011) 90:289–93. doi: 10.1177/0022034510381905
64. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Sci Transl Med*. (2014) 6: 237ra65. doi: 10.1126/scitranslmed.3008599
65. Kliman HJ. Comment on the placenta harbors a unique microbiome. *Sci Transl Med*. (2014) 6:254le4. doi: 10.1126/scitranslmed.3009864
66. Doyle RM, Alber DG, Jones HE, Harris K, Fitzgerald F, Peebles D, et al. Term and preterm labour are associated with distinct microbial community structures in placental membranes which are independent of mode of delivery. *Placenta*. (2014) 35:1099–101. doi: 10.1016/j.placenta.2014.10.007

67. Parnell LA, Briggs CM, Cao B, Delannoy-Bruno O, Schrieffer AE, Mysorekar IU. Microbial communities in placentas from term normal pregnancy exhibit spatially variable profiles. *Sci Rep*. (2017) 7:11200. doi: 10.1038/s41598-017-11514-4
68. De Goffau MC, Lager S, Salter SJ, Wagner J, Kronbichler A, Charnock-Jones DS, et al. Recognizing the reagent microbiome. *Nat Microbiol.* (2018) 3:851–3. doi: 10.1038/s41564-018-0202-y
69. Kim D, Hofstaedter CE, Zhao C, Mattei L, Tanes C, Clarke E, et al. Optimizing methods and dodging pitfalls in microbiome research. *Microbiome*. (2017) 5:52. doi: 10.1186/s40168-017-0267-5
70. Robertson SA, Moldenhauer LM. Immunological determinants of implantation success. *Int J Dev Biol*. (2014) 58:205–17. doi: 10.1387/ijdb.140096sr
71. Benner M, Ferwerda G, Joosten I, van der Molen RG. How uterine microbiota might be responsible for a receptive, fertile endometrium. *Hum Reprod Update.* (2018) 24:393–415. doi: 10.1093/humupd/dmy012
72. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science*. (2011) 332:974–7. doi: 10.1126/science.1206095
73. Zhou L, Chong MM, Littman DR. Plasticity of $Cd4^+$ T cell lineage differentiation. *Immunity.* (2009) 30:646–55. doi: 10.1016/j.immuni.2009.05.001
74. Kaiko GE, Horvat JC, Beagley KW, Hansbro PM. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology.* (2008) 123:326–38. doi: 10.1111/j.1365-2567.2007.02719.x
75. Vaeth M, Wang YH, Eckstein M, Yang J, Silverman GJ, Lacruz RS, et al. Tissue resident and follicular Treg cell differentiation is regulated by CRAC channels. *Nat Commun.* (2019) 10:1183. doi: 10.1038/s41467-019-08959-8
76. Brown EM, Kenny DJ, Xavier RJ. Gut microbiota regulation of T cells during inflammation and autoimmunity. *Annu Rev Immunol.* (2019) 37:599–624. doi: 10.1146/annurev-immunol-042718-041841
77. Plitas G, Rudensky AY. Regulatory T cells: differentiation and function. *Cancer Immunol Res.* (2016) 4:721–5. doi: 10.1158/2326-6066.CIR-16-0193
78. Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev.* (2011) 241:260–8. doi: 10.1111/j.1600-065X.2011.01018.x
79. Shevach EM. $Cd4^+$ $Cd25^+$ suppressor T cells: more questions than answers. *Nat Rev Immunol.* (2002) 2:389–400. doi: 10.1038/nri821
80. Ghiringhelli F, Menard C, Terme M, Flament C, Taieb J, Chaput N, et al. $Cd4^+Cd25^+$ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J Exp Med.* (2005) 202:1075–85. doi: 10.1084/jem.20051511
81. Taams LS, Van Amelsfort JMR, Tiemessen MM, Jacobs KMG, De Jong EC, Akbar AN, et al. Modulation of monocyte/macrophage function by human $Cd4^+Cd25^+$ regulatory T cells. *Hum Immunol.* (2005) 66:222–30. doi: 10.1016/j.humimm.2004.12.006
82. Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting edge: direct suppression of B cells by $Cd4^+$ $Cd25^+$ regulatory T cells. *J Immunol.* (2005) 175:4180–3. doi: 10.4049/jimmunol.175.7.4180
83. Maltepe E, Bakardjiev AI, Fisher SJ. The placenta: transcriptional, epigenetic, and physiological integration during development. *J Clin Invest.* (2010) 120:1016–25. doi: 10.1172/JCI41211
84. Fowden AL, Forhead AJ, Coan PM, Burton GJ. The placenta and intrauterine programming. *J Neuroendocrinol.* (2008) 20:439–50. doi: 10.1111/j.1365-2826.2008.01663.x
85. Padidas K, Norwitz E. Implantation. Chapter 4. In: Farquharson RG, Stephenson MD, editors. *Early Pregnancy*. Cambridge, UK: Cambridge University Press (2017). p. 27–42.
86. Troy E, Kasper D. Beneficial effects of *Bacteroides fragilis* polysaccharides on the immune system. *Front Biosci.* (2010) 15:25–34. doi: 10.2741/3603
87. Padidas K, Norwitz E. Implantation. Chapter 4. In: Farquharson RG, Stephenson MD, editors. *Early Pregnancy*. Cambridge University Press (2017). p. 27–42.
88. Sharkey AM, Dellow K, Blayney M, Macnamee M, Charnock Jones S, Smith SK. Stage-specific expression of cytokine and receptor messenger ribonucleic acids in human preimplantation embryos. *Biol Reprod.* (1995) 53:974–81.
89. Guzeloglu-Kayisli O, Kayisli UA, Taylor HS. The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. *Semin Reprod Med.* (2009) 27:62–79. doi: 10.1055/s-0028-1108011
90. Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development.* (2000) 127:4195–202.
91. Sjoblom C, Roberts CT, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology.* (2005) 146:2142–53. doi: 10.1210/en.2004-1260
92. Robertson SA. GM-CSF regulation of embryo development and pregnancy. *Cytokine Growth Factor Rev.* (2007) 18:287–98. doi: 10.1016/j.cytogfr.2007.04.008
93. Chin PY, Thompson JG, Robertson SA. *A Modest Inflammatory Insult in the Pre-implantation Period Alters Oviduct Cytokine Expression and Programs Fetal Development*. Sydney, NSW: Society For Reproductive Biology (2010). p. Abstract 172.
94. Mao G, Wang J, Kang Y, Tai P, Wen J, Zou Q, et al. Progesterone increases systemic and local uterine proportions of $Cd4^+Cd25^+$ Treg cells during midterm pregnancy in mice. *Endocrinology.* (2010) 151:5477–88. doi: 10.1210/en.2010-0426
95. Dimitriadis E, White CA, Jones RL, Salamonsen LA. Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum Reprod Update.* (2005) 11:613–30. doi: 10.1093/humupd/dmi023
96. Vannuccini S, Clifton VL, Fraser IS, Taylor HS, Critchley H, Giudice LC, et al. Infertility and reproductive disorders: impact of hormonal and inflammatory mechanisms on pregnancy outcome. *Hum Reprod Update*. (2016) 22:104–15. doi: 10.1093/humupd/dmv044
97. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell.* (2000) 101:455–8. doi: 10.1016/S0092-8674(00)80856-9
98. Moldenhauer LM, Hayball JD, Robertson SA. Immune-deviating cytokines determine the maternal T cell response and conceptus tolerance or rejection. *Biol Reprod.* (2009) 2:86. doi: 10.1093/biolreprod/81.s1.86
99. Mcintire RH, Hunt JS. Antigen presenting cells and Hla-G–a review. *Placenta.* (2005) 26:S104–9. doi: 10.1016/j.placenta.2005.01.006
100. Renaud SJ, Graham CH. The role of macrophages in utero-placental interactions during normal and pathological pregnancy. *Immunol Invest.* (2008) 37:535–64. doi: 10.1080/08820130802191375
101. Rowe JH, Ertelt JM, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. *Nature.* (2012) 490:102–6. doi: 10.1038/nature11462
102. Searle RF, Jones RK, Bulmer JN. Phenotypic analysis and proliferative responses of human endometrial granulated lymphocytes during the menstrual cycle. *Biol Reprod.* (1999) 60:871–8.
103. Barnea E. Signaling between embryo and mother in early pregnancy: basis for development of tolerance. In: Carp HJA, editor *Recurrent Pregnancy Loss–Causes, Controversies, and Treatment, 2nd edn.* Boca Raton, FL: CRC Press (2014). p. 17–28.
104. Cole LA. Biological functions of HCG and HCG-related molecules. *Reprod Biol Endocrinol.* (2010) 8:102. doi: 10.1186/1477-7827-8-102
105. Berndt S, Blacher S, D'hauterive PS, Thiry M, Tsampalas M, Cruz A, et al. Chorionic gonadotropin stimulation of angiogenesis and pericyte recruitment. *J Clin Endocrinol Metab.* (2009) 94:4567–74. doi: 10.1210/jc.2009-0443
106. Raga F, Casañ EM, Wen Y, Huang H-Y, Bonilla-Musoles F, Plan ML. Independent regulation of matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-3 in human endometrial stromal cells by gonadotropin-releasing hormone: implications in early human implantation. *J Clin Endocrinol Metab*. (1999) 84:636. doi: 10.1210/jc.84.2.636
107. Taylor HS, Fritz MA, Pal L, Seli E. Sperm and egg transport, fertilization and implantation. Chapter 6. In: Taylor H, Pal L, Seli E, editors. *Speroffs Clinical Gynecologic Endocrinology and Infertility*. 9th ed. Philadelphia, PA: Wolters Kluwer (2019). p. 175–95.
108. Pagidas K, Norwitz E. Implantation. Chapter 4. In: Farquharson RG, Stephenson MD, editors. *Early Pregnancy*. Cambridge University Press (2017). p. 27–42.

109. Searle RF, Jones RK, Bulmer JN. Phenotypic analysis and proliferative responses of human endometrial granulated lymphocytes during the menstrual cycle. *Biol Reprod.* (1999) 60:871–8.
110. Pagidas K, Norwitz E. Implantation. Chapter 4. In: Farquharson RG, Stephenson MD, editors. *Early Pregnancy*. Cambridge University Press (2017). p. 27–42.
111. Pagidas K, Norwitz E. Implantation. Chapter 4. In: Farquharson RG, Stephenson MD, editors. *Early Pregnancy*. Cambridge University Press (2017). p. 27–42.
112. Norwitz ER. Defective implantation of placentation laying the blueprint for pregnancy complications. *Reprod Biomed Online*. (2006) 13:591–9. doi: 10.1016/S1472-6483(10)60649-9
113. Krebs C, Macara LM, Leiser R, Bowman AW, Greer IA, Kingdom JC. Intrauterine growth restriction with absent end diastolic flow velocity in the umbilical artery is associated with maldevelopment of the placental terminal villous tree. *Am J Obstet Gynecol.* (1996) 175:1534–42.
114. Worley LC, Hnat MD, Cunningham FG. Advanced extrauterine pregnancy: diagnostic and therapeutic challenges. *Am J Obstet Gynecol.* (2008) 198:297.e1. doi: 10.1016/j.ajog.2007.09.044
115. Erlebacher A. Immunology of the maternal-fetal interface. *Annu Rev Immunol*. (2013) 31:387. doi: 10.1146/annurev-immunol-032712-100003
116. Bdolah Y, Palomaki GE, Yaron Y, Bdolah-Abram T, Goldman M, Levine RJ, et al. Circulating angiogenic proteins in trisomy 13. *Am J Obstet Gynecol.* (2006) 194:239. doi: 10.1016/j.ajog.2005.06.031
117. Mostello D, Catlin TK, Roman L, Holcomb WL Jr, Leet T. Preeclampsia in the parous woman: who is at risk? *Am J Obstet Gynecol.* (2002) 187:425. doi: 10.1067/mob.2002.123608
118. Flint EJ, Cerdeira AS, Redman CW, Vatish M. The role of angiogenic factors in the management of preeclampsia. *Acta Obstet Gynecol Scand.* (2019) 98:700–7. doi: 10.1111/aogs.13540
119. Johnsen GM, Storvold GL, Drabbels JJM, Haasnoot GW, Eikmans M, Spruyt-Gerritse MJ, et al. The combination of maternal KIR-B and fetal HLA-C2 is associated with decidua basalis acute atherosis in pregnancies with preeclampsia. *J Reprod Immunol.* (2018) 129:23–9. doi: 10.1016/j.jri.2018.07.005
120. Johnsen GM, Storvold GL, Alnaes-Katjavivi PH, Roald B, Golic M, Dechend R, et al. Lymphocyte characterization of decidua basalis spiral arteries with acute atherosis in preeclamptic and normotensive pregnancies. *J Reprod Immunol*. (2019) 132:42–8. doi: 10.1016/j.jri.2019.03.003
121. Tersigni C, Redman CW, Dragovic R, Tannetta D, Scambia G, Di Simone N, et al. HLA-DR is aberrantly expressed at feto-maternal interface in preeclampsia. *J Reprod Immunol.* (2018) 129:48–52. doi: 10.1016/j.jri.2018.06.024
122. Madazli R, Budak E, Calay Z, Aksu MF. Correlation between placental bed biopsy findings, vascular cell adhesion molecule and fibronectin levels in preeclampsia. *BJOG.* (2000) 107:514. doi: 10.1111/j.1471-0528.2000.tb13271.x
123. Fisher S, Roberts JM. The placenta in normal pregnancy and preeclampsia. In: Taylor RN, Roberts JM, Cunningham FG, editors. *Chesley's Hypertensive Disorders in Pregnancy*, 4th ed. Amsterdam: Academic Press (2015). p. 81–112.
124. Redman CW, Tannetta DS, Dragovic RA, Gardiner C, Southcombe JH, Collett GP, et al. Review: does size matter? Placental debris and the pathophysiology of pre-eclampsia. *Placenta*. (2012) 33:S48. doi: 10.1016/j.placenta.2011.12.006
125. Lee SM, Romero R, Lee YJ, Park IS, Park CW, Yoon BH. Systemic inflammatory stimulation by microparticles derived from hypoxic trophoblast as a model for inflammatory response in preeclampsia. *Am J Obstet Gynecol*. (2012) 207:337.e1. doi: 10.1016/j.ajog.2012.06.047
126. Manten GT, van der Hoek YY, Marko Sikkema J, Voorbij HA, Hameeteman TM, Visser GH, et al. The role of lipoprotein (a) in pregnancies complicated by pre-eclampsia. *Med Hypotheses*. (2005) 64:162. doi: 10.1016/j.mehy.2004.04.026
127. Owaki Y, Watanabe K, Iwasaki A, Saitou T, Matsushita H, Wakatsuki A. Placental hypoplasia and maternal organic vascular disorder in pregnant women with gestational hypertension and preeclampsia. *J Matern Fetal Neonatal Med*. (2019) 2:1–7. doi: 10.1080/14767058.2019.1608175
128. Aouache R, Biquard L, Vaiman D, Miralles F. Oxidative stress in preeclampsia and placental diseases. *Int J Mol Sci*. (2018) 19:e1496. doi: 10.3390/ijms19051496
129. Aquis A, Sultana R, Camenzuli C, Calleja-Agius J, Balzan R. An update of the genetics of preeclampsia. *Minerva Ginecol.* (2018) 70:465–79. doi: 10.23736/S0026-4784.17.04150-8
130. Nilsson E, Ros HS, Cnattingius S, Lichtenstein P. The importance of genetic and environmental effects for pre-eclampsia and gestational hypertension: a family study. *BJOG*. (2004) 111:200–6. doi: 10.1111/j.1471-0528.2004.00042x.x
131. Ward K, Taylor RN. Genetic factors in the etiology of preeclampsia. In: Taylor RN, Roberts JM, Cunningham FG, editors. *Chesley's Hypertensive Disorders in Pregnancy*, 4th ed. Amsterdam: Academic Press (2015). p. 57–80.
132. Apicella C, Ruano CSM, Mehats C, Miralles F, Vaiman D. The role of epigenetics in placental development and the etiology of preeclampsia. *Int J Mol Sci*. (2019) 20:e2837. doi: 10.3390/ijms20112837
133. Silver RM. Examining the link between placental pathology, growth restriction, and stillbirth. *Best Pract Res Clin Obstet Gynaecol.* (2018) 49:89–102. doi: 10.1016/j.bpobgyn.2018.03.004
134. Brosens I, Benagiano G, Brosens JJ. The potential origin of placentation disorders in the young primi gravida. *Am J Obstet Gynecol.* (2015) 212:580. doi: 10.1016/j.ajog.2015.01.013
135. Leary PJ, Leary SE, Stout KK, Schwartz SM, Easterling TR. Maternal, perinatal, and post-neonatal outcomes n women with chronic heart disease in Washington State. *Obstet Gynecol*. (2012) 120:1283–90. doi: 10.1097/AOG.0b013e3182733d56
136. Schreiber K, Hunt BJ. Pregnancy and antiphospholipid syndrome. *Semin Thromb Hemost.* (2016) 42:780–8. doi: 10.1055/s-0036-1592336
137. Durie DE, Thronburg LL, Glantz JC. Effect of second-trimester and third-trimester rate of gestational weight gain on maternal and neonatal outcomes. *Obstet Gynecol.* (2011) 118:569–75. doi: 10.1097/AOG.0b013e3182289f42
138. Micali N, Stemann Larsen P, Strandberg-Larsen K, Nybo Andersen AM. Size at birth and preterm birth in women with lifetime eating disorders: a prospective population-based study. *BJOG*. (2016) 123:1301–10. doi: 10.1111/1471-0528.13825
139. Coker AL, Garcia LS, Williams CM, Crawford TN, Clear ER, McFarlane J, et al. Universal psychosocial screening and adverse pregnancy outcomes in an academic obstetric clinic. *Obstet Gynecol*. (2012) 119:1180–9. doi: 10.1097/AOG.0b013e318253d76c
140. Holbrook BD, Rayburn WF. Teratogenic risks from exposure to illicit drugs. *Obstet Gynecol Clin North Am.* (2014) 41:229–39. doi: 10.1016/j.ogc.2014.02.008
141. Sebastiani G, Borras-Novell C, Casanova MA, Tutusaus MP, Martínez SF, Gómez Roig MD, et al. The effects of alcohol and drugs of abuse on maternal nutritional profile during pregnancy. *Nutrients.* (2018) 10:1008. doi: 10.3390/nu10081008
142. Nie Q, Su B, Wei J. Neurological teratogenic effects of antiepileptic drugs during pregnancy. *Exp Ther Med.* (2016) 12:2400–4. doi: 10.3892/etm.2016.3628
143. Cyganek A, Pietrzak B, Kociszewska-Najman B. Intrauterine growth restriction in pregnant renal and liver transplant recipients: risk factors assessment. *Transplant Proc.* (2014) 46:2794–7. doi: 10.1016/j.transproceed.2014.09.001
144. Creasy RK, Resnik R, Iams JD, Lockwood C, Moore T, Greene M, et al. *Creasy and Resnik's Maternal–Fetal Medicine: Principles and Practice*. 8th ed. Philadelphia, PA: Saunders Elsevier (2019).
145. Desai M, ter Kuile FO, Nosten F, McGready R, Asamoa K, Brabin B, et al. Epidemiology and burden of malaria in pregnancy. *Lancet Infect Dis*. (2007) 7:93–104. doi: 10.1016/S1473-3099(07)70021-X
146. Toda K, Reef S, Tsuruoka M, Iijima M, Dang TH, Duong TH, et al. Congenital rubella syndrome (CRS) in Vietnam 2011–2012–CRS epidemic after rubella epidemic in 2010–2011. *Vaccine*. (2015) 33:3673–7. doi: 10.1016/j.vaccine.2015.06.035
147. Picone O, Teissier N, Cordier AG, Vauloup-Fellous C, Adle-Biassette H, Martinovic J, et al. Detailed *in utero* ultrasound description of 30 cases of congenital cytomegalovirus infection. *Prenat Diagn*. (2014) 34:518. doi: 10.1002/pd.4340

148. Sheffield JS, Sánchez PJ, Morris G, Maberry M, Zeray F, McIntire DD, et al. Congenital syphilis after maternal treatment for syphilis during pregnancy. *Am J Obstet Gynecol*. (2002) 186:569–73. doi: 10.1067/mob.2002.121541
149. Paquet C, Yudin MH. Toxoplasmosis in pregnancy: prevention, screening, and treatment. *J Obstet Gynaecol Can*. (2013) 35:78–81. doi: 10.1016/S1701-2163(15)31053-7
150. Selling KE, Carstensen J, Finnstrom O, Sydsjö G. Intergenerational effects of preterm birth and reduced intrauterine growth: a population based-study of Swedish mother-offspring pairs. *BJOG*. (2006) 113:430–40. doi: 10.1111/j.1471-0528.2006.00872.x
151. Snijders RJ, Sherrod C, Gosden CM, Nicolaides KH. Fetal growth retardation: associated malformations and chromosomal abnormalities. *Am J Obstet Gynecol*. (1993) 168:547–55.
152. Schemmer G, Wapner RJ, Johnson A, Schemmer M, Norton HJ, Anderson WE. First-trimester growth patterns of aneuploid fetuses. *Prenat Diagn*. (1997) 17:155–9.
153. Toutain J, Labeau-Gaüzere C, Barnetche T, Horovitz J, Saura R. Confined placental mosaicism and pregnancy outcome: a distinction needs to be made between types 2 and 3. *Prenat Diagn*. (2010) 30:1155–64. doi: 10.1002/pd.2631
154. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science*. (2012) 336:1268–73. doi: 10.1126/science.1223490
155. Thursby E, Juge N. Introduction to the human gut microbiota. *Biochem J*. (2017) 474:1823–36. doi: 10.1042/BCJ20160510
156. Grice EA, Segre JA. The human microbiome: our second genome. *Annu Rev Genomics Hum Genet*. (2012) 13:151–70. doi: 10.1146/annurev-genom-090711-163814
157. David LA, Materna AC, Friedman J, Campos-Baptista MI, Blackburn MC, Perrotta A, et al. Host lifestyle affects human microbiota on daily timescales. *Genome Biol*. (2014) 15:R89. doi: 10.1186/gb-2014-15-7-r89
158. Wang B, Yao M, Lv L, Ling Z, Li L. The human microbiota in health and disease. *Engineering*. (2017) 3:71–82. doi: 10.1016/J.ENG.2017.01.008
159. Sochocka M, Donskow-Łysoniewska K, Diniz BS, Kurpas D, Brzozowska E, Leszek J. The gut microbiome alterations and inflammation-driven pathogenesis of Alzheimer's disease—a critical review. *Mol Neurobiol*. (2019) 56:1841–51. doi: 10.1007/s12035-018-1188-4
160. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol*. (2015) 15:30–44. doi: 10.1038/nri3785
161. Slocum C, Kramer C, Genco CA. Immune dysregulation mediated by the oral microbiome: potential link to chronic inflammation and atherosclerosis. *J Intern Med*. (2016) 280:114–28. doi: 10.1111/joim.12476
162. Arimatsu K, Yamada H, Miyazawa H, Minagawa T, Nakajima M, Ryder MI, et al. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. *Sci Rep*. (2014) 4:4828. doi: 10.1038/srep04828
163. Sato K, Takahashi N, Kato T, Matsuda Y, Yokoji M, Yamada M, et al. Aggravation of collagen-induced arthritis by orally administered *Porphyromonas gingivalis* through modulation of the gut microbiota and gut immune system. *Sci Rep*. (2017) 7:6955. doi: 10.1038/s41598-017-07196-7
164. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol*. (2013) 14:685–90. doi: 10.1038/ni.2608
165. Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. *Gastroenterology*. (2007) 132:1359–74. doi: 10.1053/j.gastro.2007.02.056
166. Yu Y, Lu L, Sun J, Petrof EO, Claud EC. Preterm infant gut microbiota affects intestinal epithelial development in a humanized microbiome gnotobiotic mouse model. *Am J Physiol Gastrointest Liver Physiol*. (2016) 311:G521–32. doi: 10.1152/ajpgi.00022.2016
167. Cobb CM, Kelly PJ, Williams KB, Babbar S, Angolkar M, Derman RJ. The oral microbiome and adverse pregnancy outcomes. *Int J Womens Health*. (2017) 9:551–9. doi: 10.2147/IJWH.S142730
168. Minassian C, Thomas SL, Williams DJ, Campbell O, Smeeth L. Acute maternal infection and risk of preeclampsia: a population based case control study. (2013) 8:e73047. doi: 10.1371/journal.pone.0073047
169. Kalinderi K, Delkos D, Kalinderis M, Athanasiadis A, Kalogiannidis I. Urinary tract infection during pregnancy: current concepts on a common multifaceted problem. (2018) 38:448–53. doi: 10.1080/01443615.2017.1370579
170. Den Hollander W, Schalekamp-Timmermans S, Holster IL, Jaddoe VW, Hofman A, Moll HA, et al. *Helicobacter pylori* colonization and pregnancies complicated by preeclampsia, spontaneous prematurity, and small for gestational age birth. *Helicobacter*. (2017) 22:e12364. doi: 10.1111/hel.12364
171. Lv LJ, Li SH, Li SC, Zhong ZC, Duan HL, Tian C, et al. Early onset preeclampsia is associated with gut microbial alterations in antepartum and postpartum women. *Front Cell Infect Microbiol*. (2019) 9:224. doi: 10.3389/fcimb.2019.00224
172. Dunn AB, Hanson L, VandeVusse L, Leslie S. Through the microbial looking glass: premature labor, preclampsia, and gestational diabetes: a scoping review. *J Perinat Neonatal Nurs*. (2019) 33:35–51. doi: 10.1097/JPN.0000000000000375
173. Amarasekara R, Jayasekara RW, Senanayake H, Dissanayake VH. Microbiome of the placenta in pre-eclampsia supports the role of bacteria in the multifactorial cause of pre-eclampsia. *J Obstet Gynaecol Res*. (2015) 41:662–9. doi: 10.1111/jog.12619
174. Huang S, Li N, Liu C, Li T, Wang W, Jiang L, et al. Characteristics of the gut microbiota colonization, inflammatory profile, and plasma metabolome in intrauterine growth restricted piglets during the first 12 hours after birth. *J Microbiol*. (2019) 57:748–58. doi: 10.1007/s12275-019-8690-x

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Microbiota Induced Changes in the Immune Response in Pregnant Mice

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Pregnancy is associated with adaptations of the immune response and with changes in the gutmicrobiota. We hypothesized the gut microbiota are involved in inducing (part of) the immunological adaptations during pregnancy. To test this hypothesis, we collected feces from pregnant conventional mice before and during pregnancy (days 7, 14, and 18) and microbiota were measured using 16S RNA sequencing. At day 18, mice were sacrificed and splenic (various Th cell populations) and blood immune cells (monocyte subsets) were measured by flow cytometry. The data were compared with splenic and blood immune cell populations from pregnant (day 18) germfree mice and non-pregnant conventional and germfree mice. Finally, the abundances of the individual gut bacteria in the microbiota of each conventional pregnant mouse were correlated to the parameters of the immune response of the same mouse. The microbiota of conventional mice were significantly different at the end of pregnancy (day 18) as compared with pre-pregnancy (Permanova, $p < 0.05$). The Shannon index was decreased and the Firmicutes/Bacteroidetes ratio was increased (Friedman followed by Dunn's test, $p < 0.05$), while abundances of various species (such as *Allobaculum stercoricanis*, *Barnesiella intestihominis,* and *Roseburia faecis)* were significantly different at day 18 compared with pre-pregnancy. In pregnant conventional mice, the percentage of Th1 cells was decreased, while the percentages of Treg cells and Th2 cells were or tended to be increased vs. non-pregnant mice. In germfree mice, only the percentage of Th1 cells was decreased in pregnant vs. non-pregnant mice, with no effect of pregnancy on Treg and Th2 cells. The percentages of monocyte subsets were affected by pregnancy similarly in conventional and germfree mice. However, the activation status of monocytes (expression of CD80 and MHCII) was affected by pregnancy mainly in conventional mice, and not in germfree mice. Correlation (Spearman's coefficient) of pregnancy affected microbiota with pregnancy affected immune cells, i.e., immune cells that were only affected differently in conventional mice and germfree mice, showed 4 clusters of bacteria and 4 clusters of immune cells, some of these clusters were correlated with each other. For instance, the microbiota in cluster 1 and 2 (in which there were various short chain fatty acid producing microbiota) are positively correlated with immune cells in cluster B, containing Treg cells and Th2 cells. Microbiota and immune cells are affected by

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pregnancy in mice. The different immunological adaptations to pregnancy between conventional and germfree mice, such as the increase in Treg and tendency to an increase in Th2 cells in conventional pregnant mice only, may suggest that the microbiota may play a role in adapting the maternal immune response to pregnancy.

Keywords: pregnancy, gut microbiota, immune response, monocytes, lymphocytes

INTRODUCTION

Pregnancy is characterized by many changes in the immune response in order to tolerate the semi-allogeneic fetus (1). Changes are observed in the peripheral immune response as well as locally at the maternal-fetal interface. At the maternal fetal interface, there is an increased number of uterine NK cells and macrophages (2), which are important for placenta development. Peripherally, the pregnant immune response is amongst others associated with a shift away from a Th1 immune response (3, 4) and increased numbers of regulatory T cells (Treg cells) as well as a decreased number of Th17 cells (5–7). In the periphery, also changes in the innate immune response can be found, such as an increased number monocytes and granulocytes during pregnancy (8, 9). The innate immune response is further characterized by an increased activation monocytes and granulocytes (10, 11) and differences in cytokine production of these cells (9, 12). Moreover, changes in monocyte subsets can be found during pregnancy (13). Similar changes in the immune response have been observed in rodents (7).

The mechanisms that induces these changes in immune responses in pregnancy are only partly understood. Although, the immunological adaptations are necessary in order to tolerate the semiallogeneic fetus, semiallogeneity is only partly responsible for inducing the changes in the maternal immune response, since changes in the immune response do also occur in mice with syngeneic pregnancies (14). Hormonal changes during pregnancy, such as increased progesterone have been shown to be involved in adapting the maternal immune response to pregnancy (15). Physical contact with the placenta during placental circulation may be another mechanism by which innate immune cells are activated during pregnancy (16). However, it has also been shown that the placenta produces many factors into the maternal circulation (17): such as cytokines (18), extracellular vesicles (19), but also fetal DNA (20) may affect immune responses. Other factors suggested to be involved in the adaptation of the immune response to pregnancy are semen (21) or ovarian factors (22).

With the recent knowledge on the role of the gut microbiota in development and maintenance of immune responses (23), we hypothesized that the gut microbiota may be involved in inducing (part of) the immunological changes observed during pregnancy. It has been shown that the gut microbiota may influence the numbers and activation status of various immune cells, such as for instance Treg (24) and Th17 (25) cells. Also, monocyte numbers and their activational state may be influenced by the microbiota (26). As indicated above, these immune cells are also affected by pregnancy. Interestingly, it has been shown that in humans the gut microbiota changes during pregnancy (27), while recently, we have shown that also the mouse gut microbiota changes at the end of pregnancy (14). Moreover, we have shown that the changes in the mouse gut microbiota during pregnancy correlated to differences in expression of immunological changes in the colon (14), suggesting that the gut microbiota affects intestinal immune responses during pregnancy.

In the present study, we aimed in evaluating whether the gut microbiota may also influence the peripheral immune response during pregnancy. Therefore, we first analyzed changes in the gut microbiota during pregnancy in conventional mouse by collecting feces before pregnancy and at days 7, 14, and 18 of pregnancy. We also evaluated the peripheral immune response in these pregnant conventional mice at day 18 of pregnancy. We hypothesized that if some of the peripheral immunological changes would be induced by the microbiota, these changes would not be observed in germfree pregnant mice. Therefore, we also evaluated the peripheral immune response in pregnant germfree mice and compared this to the immune response in the conventional pregnant mice. In the present study we used syngeneic pregnancies, to exclude the effect of semiallogeneity on the immune response to be able to show effects of the microbiota more clearly. Finally, we correlated the gut microbiota to peripheral immunological changes at day 18 of pregnancy in conventional mice.

MATERIALS AND METHODS

Animals

All mice experiment for this study were approved by the Central Committee for Animal experimentation in The Netherlands and experiments were performed according to their guidelines. Male and female wild type conventional C57BL/6JOlaHsd mice were purchased from Envigo (Envigo, Horst, The Netherlands) at an age between 2.5 and 3 months. All conventional mice were cohoused in isolated ventilated cages (5 mice per cage) with a 12-h light,12-h dark cycle. Germfree mice (C57BL/6JOlaHsd) were bred and nurtured at the Central Animal Facility of the UMCG. They were kept in germfree isolators until sacrifice and checked for germfree status regularly by the facility. Germfree mice were cohoused (5 mice per cage) with a 12-h light,12-h dark cycle. All (conventional and germfree) mice were provided with germfree bedding, an irradiated diet, AIN-93M (Research Diet Services, the Netherlands) and sterile water *ad-libitum*. The diet was provided starting at least 3 weeks before pregnancy and

continuing during pregnancy and 3 weeks before sacrifice in non-pregnant mice.

After 3-weeks acclimatization in the facility and on the diet, vaginal smears were taken to check the ovarian cycle of the mice. When in pro-estrus, the female mice were placed with male mice overnight. We used syngeneic pregnancies, to exclude the effect of semiallogeneity on the immune response. The next morning, mice were checked for a plug or sperm in the smear and this day is day 0 of pregnancy. For conventional mice, fecal samples were collected before and during pregnancy (on days 7, 14, and 18; $n = 12$). These conventional mice were terminated at day 18 of pregnancy. Germfree pregnant mice ($n = 9$) were also terminated at day 18. Two groups of non-pregnant mice [1 group of conventional ($n = 12$) and 1 group of germfree mice ($n = 11$)] were terminated for measuring the immune response at di-estrus of the ovarian cycle to ensure low levels of estrogen and progesterone. Termination was done in sterile flow cabinets. At termination, mice were anesthetized with isofluorane/O_2, and blood was collected from the aorta into EDTA tubes (BD-Plymouth, UK). After bleeding, spleens were also collected. For pregnant mice, we counted number of viable fetuses and number of resorptions and weighed individual placentas and fetuses.

Microbiota Measurement

Immediately after collection, fecal samples were snap frozen in screw caps in liquid nitrogen and stored at −80°C until measurement. For DNA isolation 0.25 gr of fecal sample was added to a 2 ml sterile bead beater tube filled with 1 ml of 0.1 mm zirconia/silica beads with 1 ml of lysis buffer (5 M NaCl, 1 M Tris-HCL (pH = 8); 0.5 M EDTA and 10 % SDS). Samples were placed in a bead beater (Percellys 24, Bertin Instruments, Montigny-leBretonneux, France) for 3 min on 5,500 RPM in three cycles of 30–60 s. The samples were heated to 95°C for 15 min while shaking every 5 min and put on ice for 5 min. Hereafter, the tubes were centrifuged for 5 min at maximum speed to pellet debris. The supernatant was transferred into a new sterile 2 ml tube and 300 μl of fresh lysis buffer was added followed by bead beating, heating to 95°C and centrifuged for 5 min at maximum speed to remove any additional debris.

Nucleic acids were cleaned from proteins and cell debris by precipitation using ammonium acetate (260 μl of 10 M ammonium acetate) on ice for 5 min. Tubes were centrifuged for 10 min at full speed at 4°C. Pellet was discarded and supernatant was treated a second time with the same procedure, followed by precipitation of the DNA from the supernatant with isopropanol on ice for 30 min. After centrifugation, the pellets were washed with ethanol 70% and dried. Hereafter, genomic DNA purification was performed according to the protocol of the QIAmp DNA Mini Kit (Qiagen, Benelux, Venlo, the Netherlands). DNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

16S rRNA Gene Sequencing, Quality Control, and Taxonomy Assignment

Subsequently, the DNA was used for the amplification of the V3–V4 region of the 16S rRNA gene using modified 341F (AATGATACGGCGACCACCGAGATCT-ACACTCTTT CCCTACACGACGCTCTTCCGATCT-NNNNCCTAC GGGAGGCAGCAG) and 806R primers (CAAGCAGAA GACGGCATACGAGAT-barcode-GTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT-GGACTACHVGGGTWTCTAAT) containing a 6-nucleotide barcode and flow-cell adaptor on the 806R primer as described elsewhere (28). A 2 × 300 cartridge (Illumina, Eindhoven, the Netherlands) was used to perform both MiSeq library preparation and sequencing. PCR protocol, DNA cleanup and the library preparation were all done as described before (29). Sequence reads with a quality score lower than 0.9 were discarded by PANDAseq to increase the quality. QIIME was used to identify sequence reads until the genus levels, while ARB software (29) was used to identify sequences at the species level.

Isolation and Staining of Spleen Cells

Cells were isolated from the maternal spleen for immune cell staining according to methods described before (14). The spleen was first cut into small pieces, mechanically disrupted between two microscopy slides in 3 ml ice-cold RPMI containing 10% (v/v) heat-inactivated fetal calf serum (FCS). Splenic red blood cells were eliminated by incubation with 4 ml ice-cold ammonium chloride. Falcon tubes with cell strainer caps (Corning, Amsterdam, the Netherlands) (35 μm) were used to remove cell clumps before the cells were counted and used for staining.

All antibodies were diluted and supplemented to a volume of 25 μl with FACS buffer [PBS + 10% FCS (*v/v*)]. Approximately 1×10^6 spleen cells were incubated in Zombie NIR for 30 min. After washing with FACS buffer (2 times), the supernatant was discarded and the cells were incubated for 20 min in FACS buffer [10% FCS (*v/v*)] containing 20% (*v/v*) normal rat serum (Jackson, Newmarket, UK) on ice and in the dark, to prevent non-specific antibody binding. This was followed by incubation in an extracellular antibody mix for 30 min on ice and in the dark (**Table 1**). After 2 washing steps, the cells were fixed in FACS lysing solution (BD Biosciences, Breda, the Netherlands) for 30 min on ice and in the dark. Then cells were washed twice with permeabilization buffer (eBioscience, Vienna, Austria) after which they were incubated with an intracellular blocking medium (20% (v/v) rat serum in permeabilization buffer) for 20 min. After washing, the cells were incubated with the intracellular antibody mix for 30 min on ice and in the dark (**Table 1**). This was followed by 2 washing steps with permeabilization buffer. Finally, the cells were taken up in 200 μl ice-cold 2%(v/v) FACS buffer and stored at 4°C until analysis within 24 hrs. FMO controls were used to set the gates.

Staining of Blood Monocytes

Maternal blood was stained for monocytes subsets and activation status according to an adapted method previously used for rat studies (13). Antibody specifications are shown in **Table 2**. Two hundred microliters of whole blood was diluted with 200 μl RPMI buffer (500 ml RPMI + 50 ml decomplemented FCS), and incubated with 50 μl ice-cold extracellular blocking medium (2% (v/v) Fc-block (purified anti-mouse CD16/32) (Biolegend, San

TABLE 1 | Antibody mix for staining of splenic cells.

Marker	Fluorchrome	Dilution	Supplier	Mix
CD4	PerCp-Cy5.5	75x	Biolegend	Extracellular
CD3	BV605	25x	Biolegend	Extracellular
Tbet	BV421	10x	Biolegend	Intracellular
RORyT	PE	100x	eBioscience	Intracellular
Gata3	AF647	100x	BD Pharmingen	Intracellular
FoxP3	Fitc	50x	eBioscience	Intracellular
Dead/live	Zombie NIR	1000x	Biolegend	

TABLE 2 | Antibody mix for staining of blood monocytes.

Marker	Fluorochrome	Dilution	Supplier
MHC2	PerCp-Cy5.5	200x	Biolegend
Ly6C	AF488	200x	Biolegend
CD43	APC	100x	Biolegend
CD11b	PE	50x	Biolegend
CD80	PB	25x	Biolegend
Ly6G	BV605	25x	BD Horizon

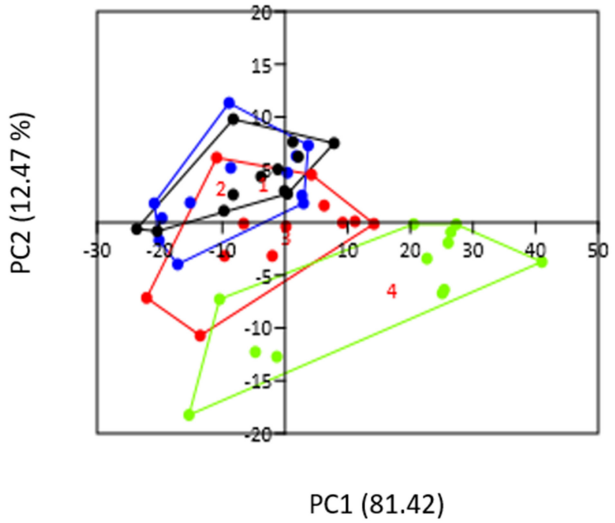

FIGURE 1 | PCA plot representing the gut microbiota of conventional pre-pregnant (black circles), day 7 (blue circles), day 14 (red circles), and day 18 (green circles) mice. The day of pregnancy (PC2) does explain part of the variation. PC1 explains 81.42% of the variation, while PC 2 explains 12.47% of the variation.

Diego, USA) in 10% (v/v) FACS+EDTA buffer) for 10 min on ice in the dark. After centrifugation (1800 RPM, 5 min, 4°C) the supernatant was discarded and the cells were incubated in 25 µl ice-cold antibody mix for 30 min on ice in the dark. After incubation, the cells were incubated with 1 ml FACS lysing buffer for 15 min at room temperature in the dark. After washing for 3 times, the cells were taken up in 500 µl ice-cold 10% (v/v) FACS+EDTA buffer and stored at 4°C until analysis within 24 hrs.

Flow Cytometry

Samples were analyzed by the FACSverse flow cytometer system (BD Biosciences Franklin Lakes, USA), using the FACSsuite software. Analysis was performed by FlowJo version 10 software (FlowJo, LLC, Oregon, USA). Gating strategy was performed as described in **Figures 6A**, **7A**.

Statistics

For the Shannon index, the PCA, the Permanova and the similarity percentage breakdown (Simper) test we used Past3 (30). For statistical analysis (Graphpad Prism) of differences in Shannon index, Firmicutes/Bacteroidetes ratio, bacterial phyla and species between pre-pregnancy and the days of pregnancy, we used the Friedman's test followed by Dunn's post-test. Data were considered significantly different when $p < 0.05$ and considered a trend if p = between 0.05 and 0.1.

Differences in fetal and placental weight and number of (live) fetuses between pregnant conventional and germfree mice were tested using the Mann-Whitney-U test, data were considered significantly different if $p < 0.05$.

For testing the effect of pregnancy (pregnant vs. non-pregnant mice) or the effect of the germfree status (conventional vs. germfree mice) on immune cells, we used the Two-Way ANOVA (TWA). For this, data were first tested for normality using the Kolmogorov-Smirnov test. Data that were not normally distributed were log transformed before the analysis. Post-testing was performed with Fisher's LSD post-test. Since we were mainly interested in the effect of pregnancy, post-tests were only done on the difference between pregnant and non-pregnant mice in both conventional and germfree mice. Data were considered significantly different when $p < 0.05$.

To gain insight into the relationship between the gut microbiota and immune cell changes during pregnancy, we correlated individual microbiota abundances with immune cell data of the same mice. We correlated individual microbiota that were changed during pregnancy to individual immune cells that were affected by pregnancy only in conventional mice using the spearman's correlation coefficient. The correlation coefficients were visualized in clustered heatmaps using Clustvis (31). Cluster analysis using Euclidian distance and Ward's clustering method was performed.

RESULTS

We determined the gut microbiota composition of mice after longitudinal sampling of feces from 12 conventional mice starting before pregnancy and at days 7, 14, and 18 of pregnancy. PCA analysis (**Figure 1**) showed that PC1 explained about 80% of the variation and while PC2, mainly represented by day of pregnancy, explains about 12 % of the variation in our groups. It can be seen that day 18 of pregnancy is significantly different from all other groups (Permanova, $p < 0.05$). Although day 14 of pregnancy also appeared different from pre-pregnancy and

TABLE 3 | The microbiota species explaining the difference in gut microbiome at day 18 of pregnancy vs. pre-pregnancy.

Species	% contribution to day 18 variation	Cumulative contribution	Mean abundance pre-pregnancy	Mean abundance pregnancy day 18	Statistical significance
Allobaculum stercoricanis	29.01	29.01	35.1	52.8	$p < 0.05$
Barnesiella intestinihominis	17.22	46.23	18.4	4.79	$p < 0.05$
Porphyromonas pogonae	3.908	50.13	6.03	3.19	$p < 0.05$
Barnesiella viscericola	3.42	53.55	4.27	1.56	$p < 0.05$
Clostridium leptum	3.295	56.85	1.87	2.95	*ns*
Faecalitalea cylindroides	1.79	58.64	2.7	3.85	$p < 0.05$
Olsenella profusa	1.728	60.37	2.33	1.27	$p < 0.05$
Lactobacillus johnsonii	1.583	61.95	1.3	0.841	*ns*
Clostridium papyrosolvens	1.513	63.46	1.45	0.298	$p < 0.05$
Acetatifactor muris	1.486	64.95	0.975	1.05	*ns*
Flavonifractor plautii	1.444	66.39	0.612	1.29	*ns*
Clostridium fusiformis	1.375	67.77	0.603	1.34	*ns*
Parasutterella excrementihominis	1.309	69.08	1.74	1.01	$p < 0.05$
Blautia coccoides	1.293	70.37	0.594	1.09	*ns*
Romboutsia ilealis	1.245	71.62	0.537	1.18	*ns*
Eisenbergiella tayi	1.245	72.86	0.505	1.2	*ns*
Alloprevotella rava	0.9948	73.86	0.957	0.198	$p < 0.05$
Sutterella parvirubra	0.9198	74.78	1.21	0.689	$p < 0.05$
Mucispirillum schaedleri	0.8793	75.65	0.142	0.706	*ns*
Bifidobacterium animalis	0.8583	76.51	1.16	1.14	*ns*
Alistipes finegoldii	0.8434	77.36	0.922	0.85	*ns*
Bifidobacterium pseudolongum	0.7753	78.13	0.81	0.515	*ns*
Olsenella umbonata	0.7728	78.9	0.897	0.414	*ns*
Desulfovibrio desulfuricans	0.7725	79.68	0.552	0.656	*ns*
Butyrivibrio crossotus	0.6543	80.33	0.298	0.287	*ns*
Bacteroides vulgatus	0.5959	80.93	0.481	0.0983	*ns*
Roseburia faecis	0.5823	81.51	0.229	0.528	$p < 0.05$
Anaerotruncus colihominis	0.5782	82.09	0.279	0.513	*ns*
Clostridium jejuense	0.5744	82.66	0.371	0.392	*ns*
Alistipes senegalensis	0.545	83.21	0.562	0.678	*ns*
Natronoflexus pectinivorans	0.533	83.74	0.518	0.409	*ns*

Significance (last column) was tested using Friedman's tests on all pregnancy days followed by Dunn's posttest comparing pre-pregnancy with day 18.

from day 7 in the PCA plot, this difference was not statistically significant (Permanova, $p > 0.05$).

To identify bacteria that explained the variation between the pre-pregnancy microbiota and day 18 microbiota, we performed a Simper test (**Table 3**). We found that *Allobaculum stercoricanis*, which significantly increased at day 18 vs. pre-pregnancy, explained 29 % of the variation between pre-pregnancy and day 18 of pregnancy. *Barnesiella intestinihominis*, which significantly decreased during pregnancy, explained 17% of the variation between the pre-pregnancy and day 18 microbiota. Various bacterial species (such as *Porphyromonas pogonae, Barnesiella viscericola, Clostridium leptum*) explain between 1 and 3% of the variation between the pre-pregnancy and day 18 microbiota.

Figure 2 shows the Shannon index and the Firmicutes/Bacteroidetes ratio before and during pregnancy. It can be seen that the Shannon index is significantly decreased at day 18 as compared with pre-pregnancy (Friedman test followed by Dunn's posttest). The Firmicutes/Bacteroidetes ratio is significantly increased at day 18 as compared with pre-pregnancy (Friedman's test followed by Dunn's post-test, $p < 0.05$), while a trend toward an increase of the Firmicutes/Bacteroidetes ratio was seen at day 14 of pregnancy (Friedman test followed by Dunn's posttest, $p = 0.08$).

Figure 3 shows differences in abundances of various bacterial phyla occurring during pregnancy in the mouse. Significant changes only occurred at day 18 vs. pre-pregnancy. We found a significantly increased abundance of Firmicutes at day 18 as compared with pre-pregnancy (Friedman's test followed by Dunn's posttest, $p < 0.05$), and a significantly decreased abundance of Bacteroidetes, Actinobacteria, Cyanobacteria and Proteobacteria at day 18 of pregnancy as compared with pre-pregnancy (Friedman's test followed by Dunn's post-test, $p < 0.05$).

Various changes in the microbiota can also be observed at the species level. Examples of changes at the species level can be seen in **Figure 4**. In this figure, examples

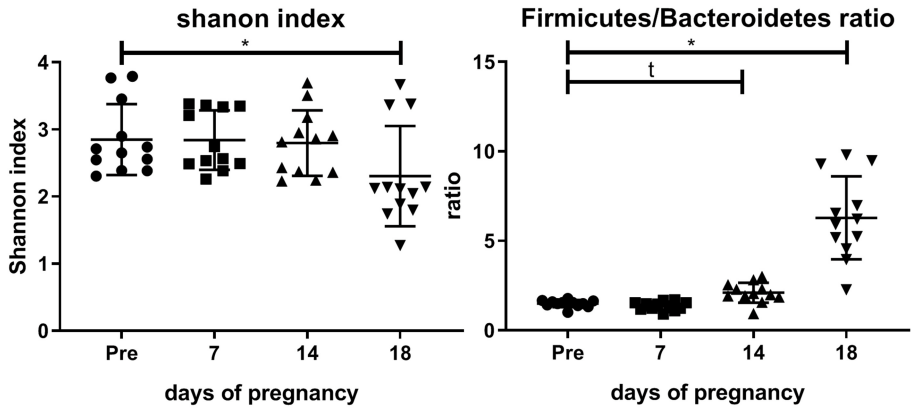

FIGURE 2 | Shannon index (left graph) and Firmicutes/Bacteroidetes ratio (right graph) in the feces of conventional mice measured pre-pregnancy (pre) and at days 7, 14, and 18 of pregnancy ($n = 10$ at each day). *Significantly different from pre-pregnancy (Friedman's test, followed by Dunn's post-test, $p < 0.05$). t, significant trend vs. pre-pregnancy (Friedman's test, followed by Dunn's post-test, $p < 0.1$).

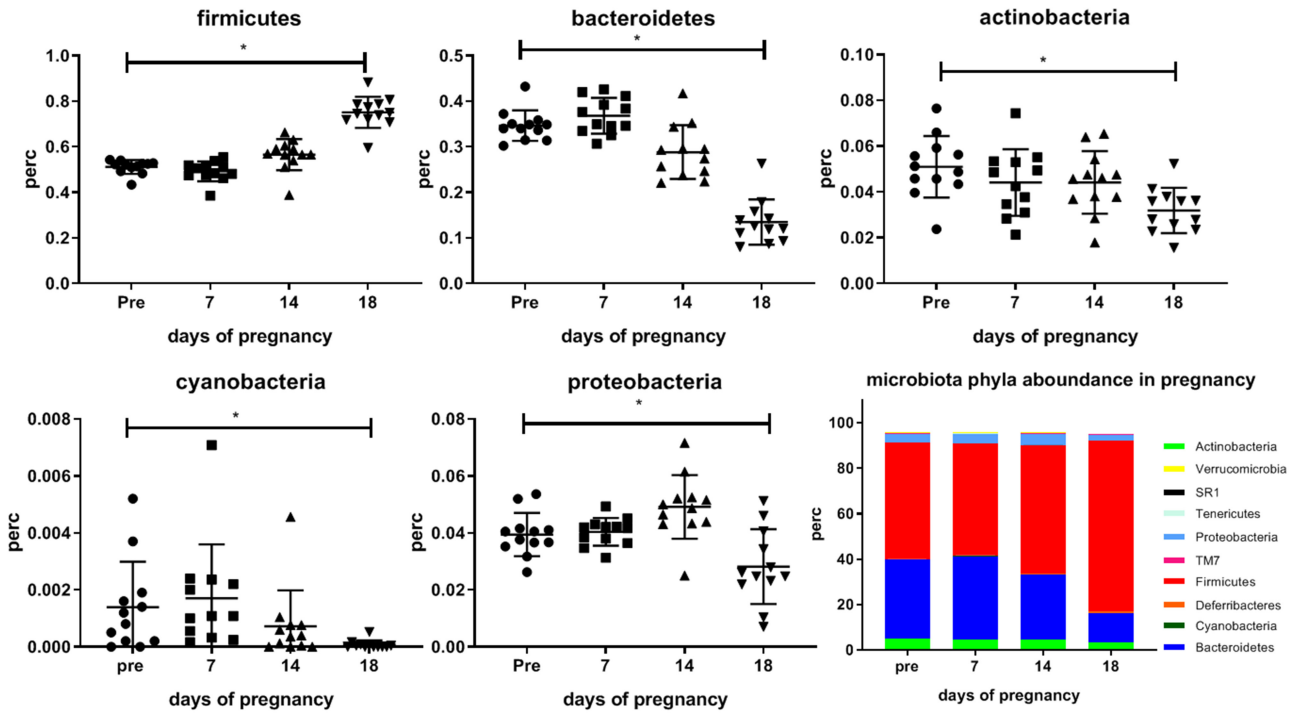

FIGURE 3 | Abundance of the bacterial phyla (Firmicutes, Bacteriodetes, Actinobacteria, Cyanobacteria, and Proteobacteria which significantly differ during pregnancy as compared with pre-pregnancy in the feces of conventional mice measured pre-pregnancy (pre) and during pregnancy at days 7, 14, and 18. Bottom right graph shows stack bars for pre-pregnancy and the 3 days of pregnancy, showing the percentage abundance of the all bacterial phyla present in the feces of these mice. (12 conventional mice were longitudinally studied from pre-pregnancy until the end of pregnancy). *Significantly different from pre-pregnancy (Friedman's test, followed by Dunn's post-test, $p < 0.05$).

of bacterial species showing differences at day 18 of pregnancy as compared with pre-pregnancy (Friedman's test followed by Dunn's posttest, $p < 0.05$) are shown. Various species of the Firmicutes phylum are increased at day 18 of pregnancy vs. pre-pregnancy, such as *Allobaculum sternicoricanis, Faecalitalea cylindroides, Roseburia faecis, Eubacteria plexicaudatum*, while various other bacterial species, such as various Bacteroidetes species are decreased at day 18 of pregnancy vs. pre-pregnancy (*Barnesiella intestinihominis, B viscericola, Alloprevotella rava, and Odoribacter splanchnicus)*. Some bacteria (*B. intestinihominis, B. viscericola, C. papysosolvens, and O. splanchnicus*) already show

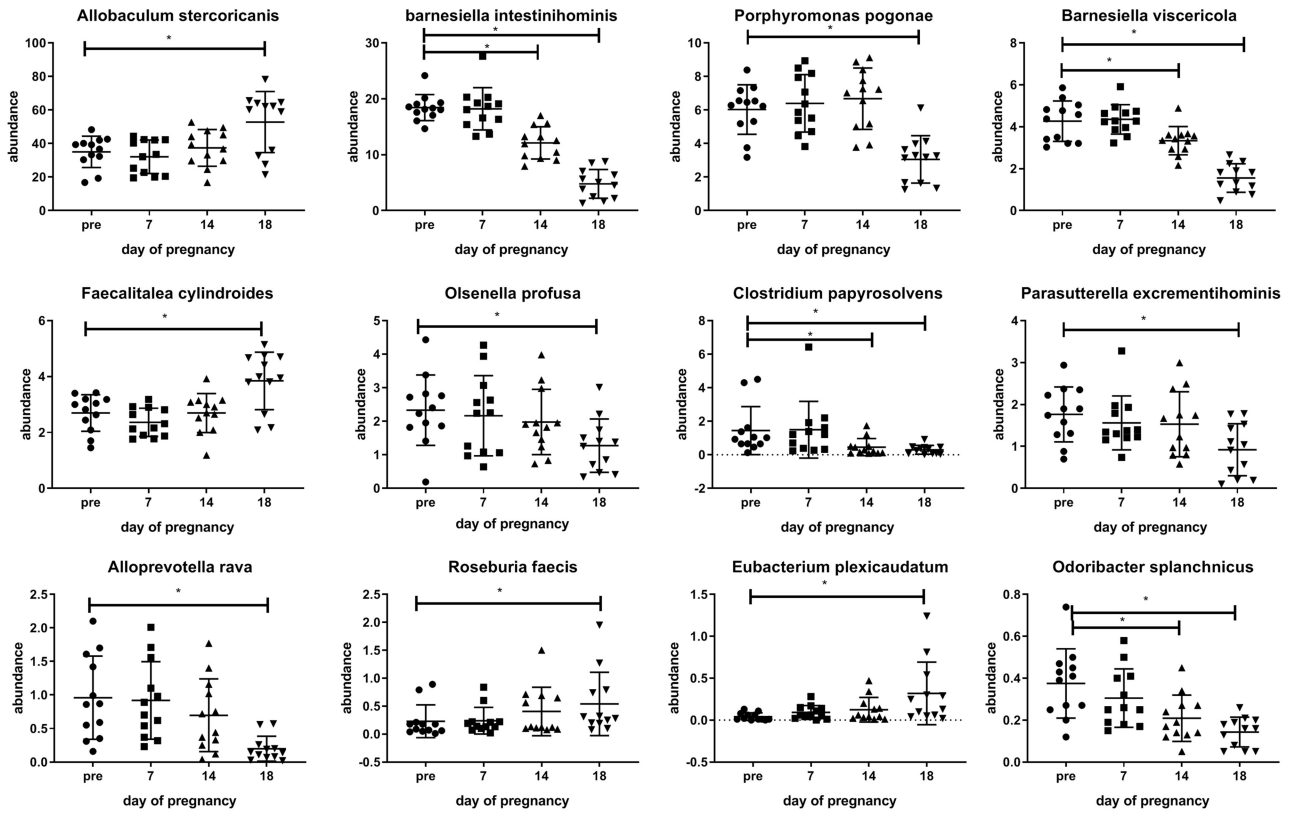

FIGURE 4 | Changes observed in the abundance of various microbiota species in the feces of conventional mice measured pre-pregnancy (pre) and during pregnancy at days 7, 14, and 18. *Significantly different from pre-pregnancy (Friedman's test, followed by Dunn's post-test, $p < 0.05$).

significant changes at day 14 of pregnancy as compared with pre-pregnancy (Friedman's test followed by Dunn's post-test, $p < 0.05$). A complete list of all changes can be found in **Supplementary Table 1.**

Changes in Immune Cells on Pregnancy Day 18 in Conventional and Germfree Mice

To study the effect of the microbiota on immunological changes during pregnancy in mice, we sacrificed conventional mice on day 18 and collected spleens and blood in order to evaluate lymphocyte subsets in the spleen and monocyte subsets in blood. We hypothesized that if the gut microbiota would be involved in the adaptations of the immune response to pregnancy, some of the adaptations observed in conventional pregnant mice would not be observed in germfree mice.

Fetal and Placental Weight in Conventional and Germfree Pregnant Mice

Figure 5 shows that fetal weight is slightly, but significantly decreased in germfree mice, with no significant effect on placental weight. Although the total number of fetuses was similar in conventional and germfree mice, the number of resorptions was increased in germfree mice, resulting in a significantly decreased number of live fetuses in germfree mice.

The Effect of Pregnancy on Thelper Subsets

Figure 6B shows the percentage of Thelper (Th) cells, T-box transcription factor+ (Tbet) cells (Th1 cells), Gata binding protein 3+ (Gata3) cells (Th2 cells), Forkhead box P3+ (FoxP3) cells (Treg cells), RAR-related orphan receptor gamma t+ (RoRgT) cells (Th17 cells) and FoxP3/RoRgT double positive cells. For Th cells, the Two-Way ANOVA ($p < 0.05$) indicated that there was interaction between pregnancy and the germfree status, indicating that the effect of pregnancy on Th cells was different in conventional compared with germfree mice. Post-testing showed an increased percentage of Th cells in pregnant germfree mice vs. non-pregnant germfree mice, but no difference in Th cells in pregnant vs. non-pregnant conventional mice. The percentage of Tbet+ cells is decreased by pregnancy (TWA, $p < 0.05$), but not affected by the germfree status (TWA, $p < 0.05$). Post testing showed that in both conventional and germfree pregnant mice the percentage of Tbet+ cells is decreased as compared with the percentage of Tbet+ cells in non-pregnant mice. The percentage of GATA3+ cells is increased in pregnant conventional mice as compared with non-pregnant conventional mice, but no difference in percentage of GATA3+ cell was found between pregnant and non-pregnant germfree mice (TWA followed by Fisher LSD test, $p < 0.05$). TWA also showed an effect

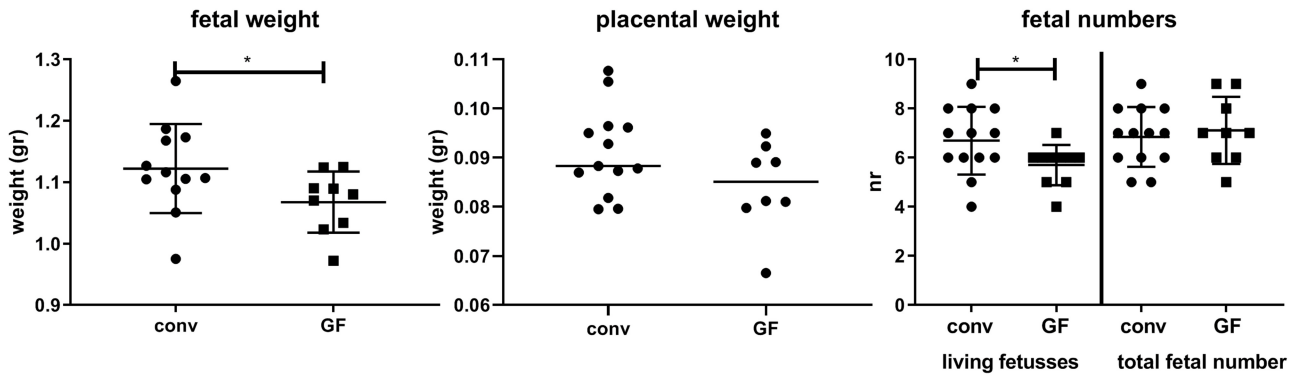

FIGURE 5 | Fetal and placental weight and fetal numbers (living fetuses and total numbers) for conventional day 18 pregnant (conv) and germfree day 18 pregnant (GF) mice. *Significantly different from conventional mice (Mann-Whitney-*U*-test, $p < 0.05$).

of the germfree status, i.e., an increase of GATA3+ cells in germfree mice.

FoxP3+ cells were affected by both pregnancy (increase) and the germfree status (decrease). (TWA, $p < 0.05$). Post testing revealed a significant increase in percentage FoxP3+ cells in conventional pregnant mice vs. conventional non-pregnant mice, but not in pregnant vs. non-pregnant germfree mice. No effect of pregnancy or the germfree status was observed on RoRgT+ or on FoxP3/RoRgT double positive cells.

Effect of Pregnancy on Leucocyte Subsets in Blood

The different leukocyte subsets in the blood are shown in **Figure 7B**. TWA showed an interaction between pregnancy and the germfree status for lymphocytes. Post-testing showed a significantly decreased percentage of lymphocytes in germfree pregnant mice as compared with germfree non-pregnant mice, but no difference in percentage of lymphocytes in pregnant vs. non-pregnant conventional mice (TWA followed by Fisher LSD test, $p < 0.05$). Also, for monocytes and granulocytes, we found an interaction between pregnancy and germfree status (TWA, $p < 0.05$). Only in germfree mice (Fisher LSD test, $p < 0.05$), not in conventional mice, we found a significantly increased percentage of monocytes and granulocytes in pregnant vs. non-pregnant mice.

The Effect of Pregnancy on Monocyte Subsets

It can be observed from **Figure 8A** that the percentage of classical monocytes was affected by pregnancy (TWA, $p < 0.05$); the percentage of classical monocytes is increased in pregnant mice as compared with non-pregnant mice, both in conventional and germfree mice (Fisher LSD test, $p < 0.05$). Germfree status also affected the classical monocytes, i.e., classical monocytes are decreased in germfree mice (TWA, $p < 0.05$) as compared with conventional mice. There was no effect of pregnancy on the percentage of intermediate monocytes, while there was an effect of germfree status, i.e., in germfree mice the percentage of intermediate monocytes was increased as compared with conventional mice (TWA, $p < 0.05$). Non-classical monocytes were affected by pregnancy (TWA, $p < 0.05$); they were decreased in pregnancy in both conventional and germfree mice (TWA, followed by Fisher LSD test, $p < 0.05$). Non-classical monocytes were also increased by the germfree status (TWA, $p < 0.05$).

The Expression of Activation Markers on Monocytes of Pregnant Mice

CD80 is a costimulatory molecule, which is upregulated on antigen presenting cells during activation. For both classical and intermediate monocytes, there was an interaction between pregnancy and germfree status (TWA, $P < 0.05$) (**Figure 8B**). Post testing showed a significantly increased expression of CD80 in pregnant vs. non-pregnant mice in both classical and intermediate monocytes, only in conventional mice (Fisher LSD test, $p < 0.05$), not in germfree mice. No effect of pregnancy or the microbiota status was found on CD80 expression in non-classical monocytes. (TWA, $p < 0.05$). MHCII expression of monocyte subsets was also affected by pregnancy (**Figure 8C**). MHCII in classical monocytes was decreased by pregnancy, both in conventional and germfree mice (TWA, followed by Fisher LSD test, $p < 0.05$) (**Figure 8C**). MHCII expression of intermediate monocytes was affected by pregnancy and the germfree status (TWA, $P < 0.05$). Only in conventional mice, not in germfree mice, MHCII expression was significantly decreased as compared with non-pregnant mice. There was no effect of either pregnancy or the germfree status on MHCII expression on non-classical monocytes.

Correlation Between Microbiota and Immune Cells in Conventional Mice

To gain insight into the relationship between the gut microbiota and immune cell changes during pregnancy in conventional mice, we correlated individual microbiota abundance data with immune cell data of the same mice. We used data from conventional day 18 mice: we used percentages of immune cells and percentage of immune cells expressing activation markers

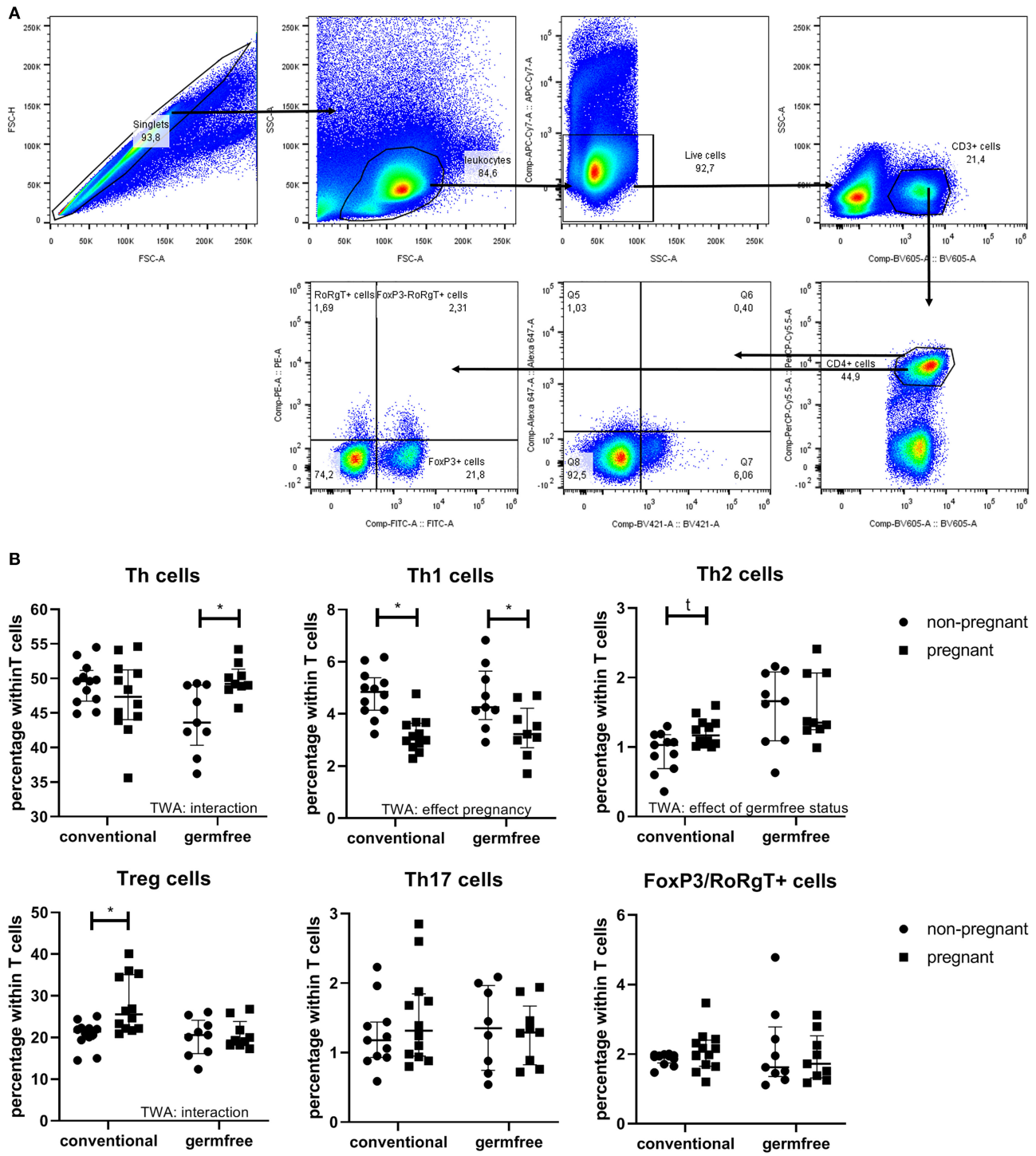

FIGURE 6 | (A) Gating strategy for lymphocyte subsets. First singlets and live cells were selected and copied to the FSC-SSC plot. In this plot, leukocytes were selected and copied to an SSC-BV605 plot, in which the CD3+ cells were selected. The CD3+ cells were copied to a BV605-PerCP-Cy5.5 plot to selected the CD4+ cells. CD4 positive cells were copied to a BV421-Alexa647 plot (to select Tbet+ cells and Gata3+ cells) and to a FITC-PE plot (to select FoxP3+ cells and RoRgT+ cells). For gate setting of the latter two plots we used FMOs. **(B)** Percentage of splenic Th cells, Th1 cells, Th2 cells, Treg cells, Th17 cells, and FOXP3/RORgT double positive cells (within the T cell population) of pregnant and non-pregnant conventional and germfree mice. TWA, Two-way ANOVA. *Significantly different from non-pregnant mice (TWA followed by Fisher's LSD post-test, $p < 0.05$). t: significant trend from non-pregnant mice (TWA followed by Fisher's LSD post-test, $p < 0.1$).

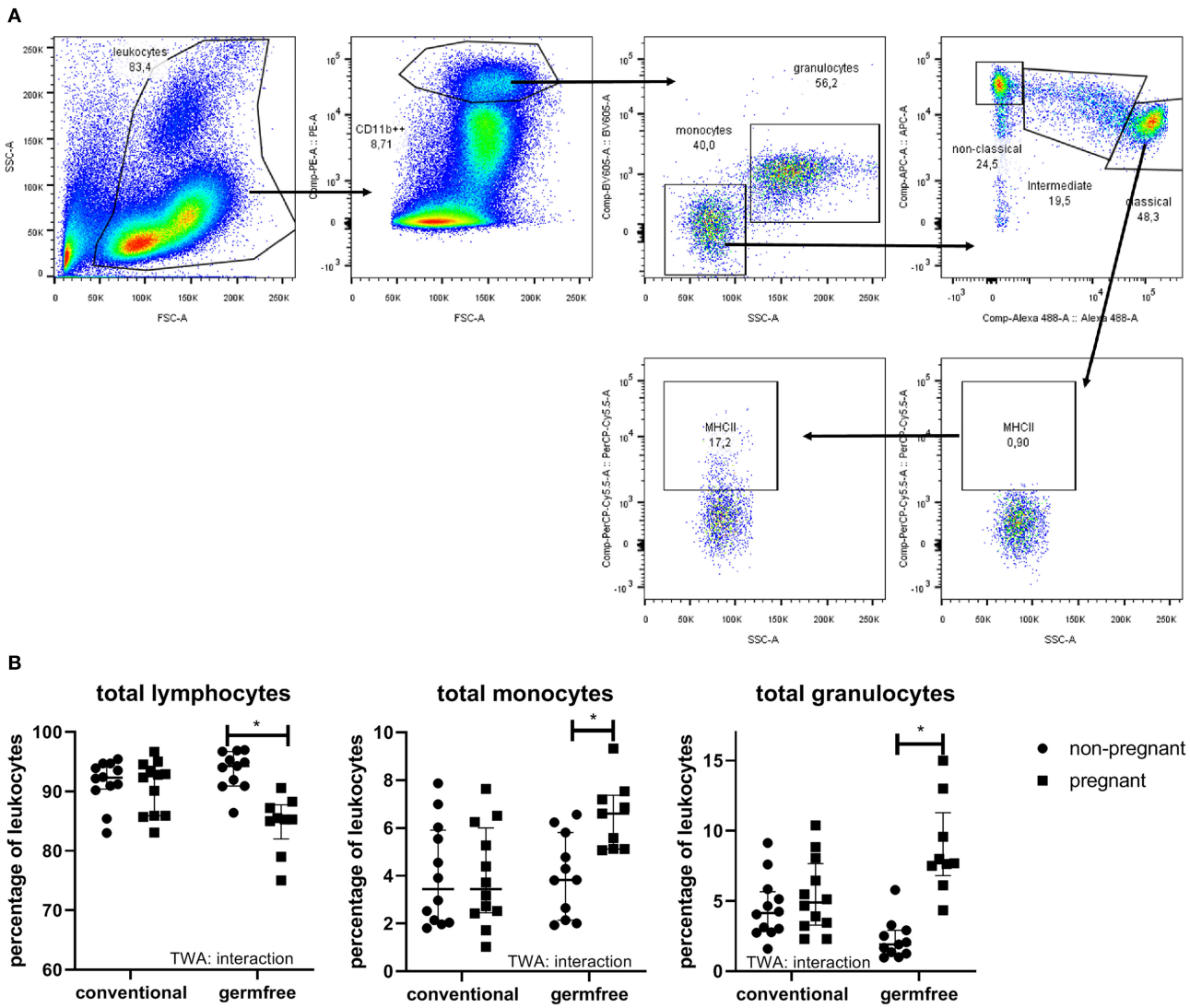

FIGURE 7 | (A) Gating strategy for leukocyte populations and monocytes. In the FSC-SSC plot, we selected the leukocytes, which were copied to the FSC-PE plot to select CD11b++ cells (myeloid cells). These myeloid cells were copied to an SSC-BV605 plot to discriminate monocytes from granulocytes. Percentages of monocytes and granulocytes were derived from this plot. Percentage lymphocytes was 100% of myeloid cells. The monocytes were then copied to an Alexa488-APC plot to discriminate classical, intermediate and non-classical monocytes. Each of these three subsets were then copied to an SSC-PerCPCy5.5, to select MHCII+ cells (example is shown for classical monocytes), using the FMO to set the gates. Each of these monocytes' subsets were also copied to an SSC-Pacific Blue plot to select the CD80+ cells, using FMO to set the gates, similar to MHCII (not shown in the figure). **(B)** Percentage of lymphocytes, monocytes, and granulocytes in whole blood of pregnant (day 18) and non-pregnant conventional and germfree mice. TWA, Two-way ANOVA. *Significantly different from non-pregnant mice (TWA followed by Fisher's LSD post-test, $p < 0.05$).

that were differently regulated by pregnancy in conventional vs. germfree mice. For microbiota data we used bacterial species from conventional mice of which the abundance was different at day 18 of pregnancy as compared with pre-pregnancy. Pearson's correlation coefficients are shown in a heatmap (**Figure 9**). Bacteria in cluster 1, of which most bacteria were upregulated during pregnancy, seem to be positively correlated with immune cell cluster A and B, which contains percentages blood granulocytes and percentages intermediate monocytes expressing MHCII (cluster A) and percentages of splenic FoxP3+ (Treg) and GATA3+ (Th2) cells (cluster B). Bacterial cluster 1 is negatively correlated with immune cell cluster D, which contains splenic percentages of Th cells and percentages of blood monocytes and lymphocytes. Bacterial cluster 2, which contains bacteria that are either upregulated or downregulated during pregnancy, was also negatively correlated to immune cells in cluster D and positively correlated to immune cells in cluster B. Bacteria in cluster 4 are negatively correlated with immune cells in cluster C, most strongly with percentage of CD80 expressing intermediate monocytes. Abundances of

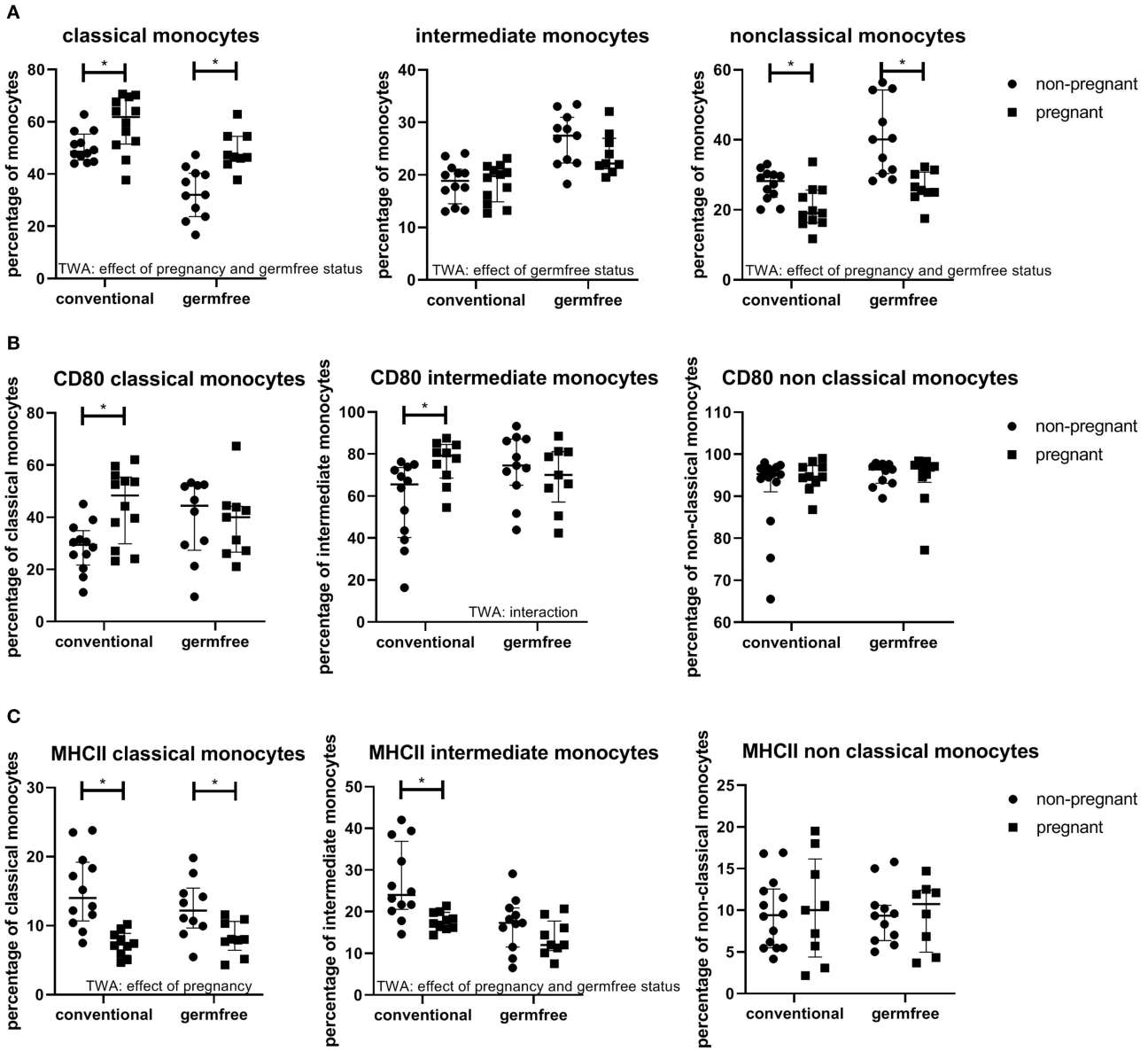

FIGURE 8 | (A) Percentages of monocyte subsets (within the monocyte population) in blood of conventional and germfree pregnant and non-pregnant mice. TWA, Two-way ANOVA. *Significantly different from non-pregnant mice (TWA followed by Fisher's LSD post-test, $p < 0.05$). **(B)** Percentage CD80 positive classical, intermediate and non-classical monocytes in pregnant and non-pregnant conventional and germfree mice. TWA, Two-way ANOVA. *Significantly different from non-pregnant mice (TWA followed by Fisher's LSD post-test, $p < 0.05$). **(C)** Percentage MHCII positive classical, intermediate and non-classical monocytes in pregnant and non-pregnant conventional and germfree mice. TWA, Two-way ANOVA. *Significantly different from non-pregnant mice (TWA followed by Fisher's LSD post-test, $p < 0.05$).

all of the bacteria in cluster 4 are significantly decreased during pregnancy.

DISCUSSION

In the present study we showed that during syngeneic pregnancy in mice, the composition of the gut microbiota changes. This composition has significantly changed at day 18 of pregnancy as compared with pre-pregnancy, with for instance an increased Firmicutes/Bacteroidetes ratio and a decreased Shannon index. Some bacterial changes can already be observed at day 14, for instance a significantly decreased abundance of *B. intestinihominis*. We also showed that in syngeneic conventional pregnant mice, immune responses change during pregnancy. We observed a decreased percentage of Th1 cells in pregnant conventional mice, as well as a trend toward an increased percentage of Th2 cells and an increased percentage

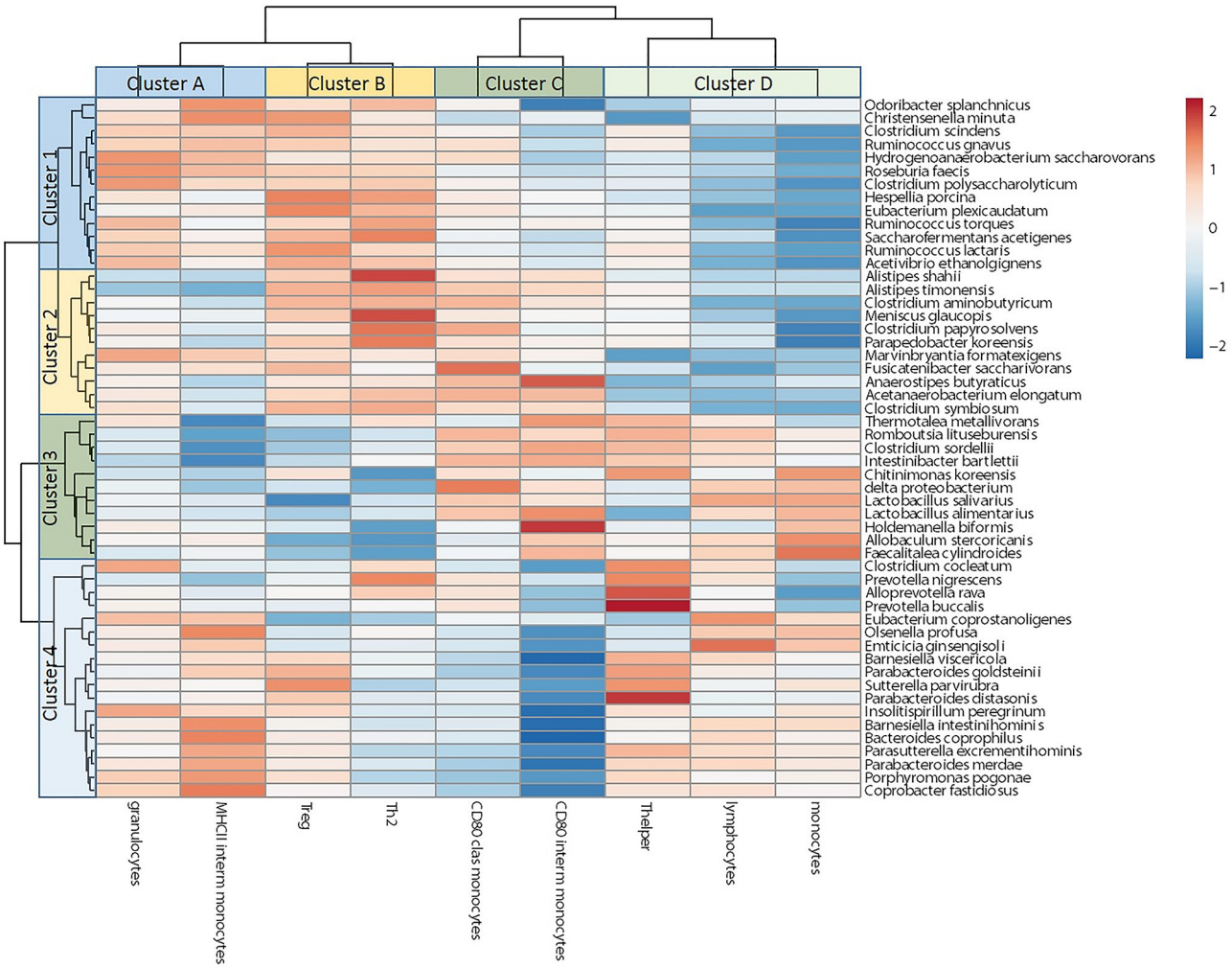

FIGURE 9 | Correlation between microbiota species and immune cell populations in conventional pregnant mice. The figure shows a heatmap of Spearman's correlations coefficients after individual correlation of significantly different microbiota species between day 18 of pregnancy and pre-pregnancy (clusters 1–4) and immune cell populations with different adaptations in conventional vs. germfree mice (clusters A–D).

of Treg cells. Monocyte subsets also changed in conventional syngeneic pregnant mice, with an increased percentage of classical monocytes and a decreased percentage of non-classical monocytes. Moreover, classical and intermediate monocytes showed increased expression of CD80 and decreased expression of MHCII during pregnancy in these mice. Next, we showed that the presence of microbiota may be important in some of these adaptations of the maternal immune response to pregnancy, since we found different adaptations in immune cell numbers of pregnant germfree mice compared with pregnant conventional mice, i.e., the increase in the splenic percentage of FoxP3+ lymphocytes and the trend toward an increased Th2 percentage was only observed in pregnant conventional mice, not in pregnant germfree mice. Moreover, increased CD80 expression on monocytes was only observed in conventional pregnant mice and not in germfree pregnant mice, while decreased MHCII expression was also only observed in conventional pregnant mice and not in germfree pregnant mice. Finally, we correlated the gut bacteria of conventional mice that were significantly changed at day 18 of pregnancy with the immune cells that were significantly changed in pregnant compared with non-pregnant mice in conventional pregnant mice only and showed that clusters of bacteria correlated with clusters of immune cells. Together these data indicate that the maternal gut microbiota may be involved in inducing some of the immunological adaptations to pregnancy.

We found various significant differences in phyla and species abundance at day 18 of pregnancy, with an increased abundance of Firmicutes and a decreased abundance of Bacteriodetes, Actinobacteria, Cyanobacteria, and Proteobacteria as compared with pre-pregnancy. At the species level, we found an increased abundance of for instance *A. stercoricanis, F. cylindroides, R. faecis and E. plexicaudatum, Holdemanella biformis.* These are all butyrate producing Firmicutes (32). Butyrate is an short chain fatty acid, which has immunomodulatory and anti-inflammatory

effects (33). Butyrate can for instance promote differentiation and proliferation of Treg (34). We found decreased abundance of species like *B. Intestinihominis and B viscericola, P. pogonae, A. rava, O. splanchnicus* as well as 2 *Alistipes* species. Most of these bacteria are propionate producers (32). Propionate has been shown to have similar effects as butyrate on the immune response, f.i. in increasing Tregs, however, propionate has been shown to be less effective than butyrate (35). During syngeneic pregnancy in the mouse, there may be an increase in butyrate and a decrease in propionate production by the gut microbiota, which may result in increased plasma butyrate and decrease plasma propionate and this may affect the maternal immune response. This hypothesis needs to be tested in future studies.

The group of Koren et al. (27) was the first to show changes in the gut microbiota during pregnancy in humans. They, however, found an increase in Proteobacteria and Actinobacteria. Since our study have been done in syngeneic pregnancy, differences between our mouse study and the human study may be explained by the presence of the semiallogeneic fetus in the human study, as compared to syngeneic fetuses in our mouse study. Future studies are needed to confirm this hypothesis. We only studied one mouse strain, with one diet and at one location. However, we are not the only group who found differences in gut microbiota during pregnancy. Also previous studies in mice have shown an effect of pregnancy on the abundance of gut microbiota species (14, 36, 37). However, each of these mouse studies found their own specific adaptations of the microbiota to pregnancy. All of these studies have been done in syngeneic pregnancies. Differences between the studies may be due to differences in diet or technical differences in 16S rRNA sequencing (38). Another factor may be genetic differences between the mouse strains, since Elderman et al. has previously shown that changes in the microbiota at day 18 were strain dependent (14). Although in this study various differences in microbiota species were found between pregnant and non-pregnant mice in the from the BALB/c strain, no significant differences in microbiota were found between pregnant and non-pregnant C57BL/6 mice. In the present study, we also used C57BL/6 mice, but a different sub strain (C57BL/6JOlaHsd), and we did find differences in gut microbiota at day 18 of pregnancy. The difference with our previous study may thus be the sub strain used, but also the use of a different diet and the fact that we did a longitudinal study, which may more easily pick up differences in microbiota. The mechanism changing the microbiota during pregnancy are unclear at this time. However, it has been suggested that pregnancy hormones, like progesterone, may affect the gut microbiota (36). Also, diet has been shown to affect the gut microbiota (37).

In view of the known effects of the microbiota on the immune system, we hypothesized that the microbiota during mouse pregnancy may be involved in inducing adaptations of the immune response to pregnancy. Therefore, we sacrificed conventional and germfree syngeneic pregnant (at the end of pregnancy) and non-pregnant conventional and germfree mice and studied their immune response. In line with our previous results and results from others, we found various adaptations in the adaptive immune cells in syngeneic pregnant conventional mice. We showed an increased percentage of FoxP3+ cells and a trend toward an increase in GATA3+ cells, as well as a decrease in the percentage of Tbet+ cells in the spleens of conventional pregnant mice as compared to non-pregnant mice. This is in line with various data showing that the Th1/Th2 balance is decreased in pregnancy (4, 39, 40). Also, the increase in FoxP3+ (Treg cells) is in line with previous data (14). We, for the first time show that also syngeneic germfree mice do adapt their immune response to pregnancy. We found, similar to conventional pregnant mice, a decreased percentage of Tbet+ (Th1 cells) cells in the spleens of pregnant germfree mice as compared with non-pregnant germfree mice. However, in contrast to conventional pregnancy mice, in germfree pregnant mice the splenic percentage of FoxP3+ cells and GATA3+ cells were not different compared with germfree non-pregnant mice. This suggests that the increased splenic percentages of FoxP3+ and (trend to) increased GATA3+ cells in conventional mice at the end of pregnancy may be induced by the gut microbiota. This suggestion is in line with various papers indicating an effect of several bacterial species on Treg cells and Th2 cells (24, 25). Further studies are needed to evaluate whether the microbiota may affect the immune response in semiallogeneic pregnant mice.

In germfree pregnant mice, we found a slightly, but significantly increased number of fetal resorptions and a slightly decreased fetal weight. The mechanism of this increased number of fetal resorptions remains unknown from the present study. Various studies in the 1980s have shown that the housing environment of mice is associated with different numbers of fetal resorptions. Hamilton et al. showed that a cleaner environment (i.e., a specific pathogen free environment vs. a conventional environment) is associated with decreased numbers of resorptions in allogeneic pregnancies (CBA × DBA/2) (41), although other studies found the opposite effect (42) or no effect of these different environments (43). In all these studies, differences were only observed in semiallogeneic pregnancies and not in syngeneic pregnancies, suggesting that the mechanisms are related to rejection of the semiallogeneic fetus. The mechanism of increased fetal resorptions in germfree vs. conventional mice in our study must therefore be different, since we used syngeneic pregnancies. Further studies are therefore necessary to evaluate mechanisms of the increased fetal resorption in germfree mice. We do expect a role of the immune response, especially the immune response in the placenta and mesometrial triangle. Immune cells in the mesometrial triangle are very important for normal placentation. Differences in immune cells in the mesometrial triangle, such as decreased numbers of natural killer cells, may result in defective placentation, and therefore in fetal growth restriction or resorptions (44). We are evaluating the immune cells in the mesometrial triangle in conventional vs. germfree pregnant mice.

Individual mouse data from pregnant conventional mice from blood immune cells and splenic immune cells that were differently affected by pregnancy in germfree vs. conventional mice and individual microbiota species that were different between pregnant and non-pregnant conventional mice were correlated and clustered. The microbiota clustered in 4 clusters.

Cluster 1 contained mainly bacteria that were upregulated during pregnancy, such as *R. faecis*, 2 *Ruminococcus* species and 3 *Clostridium* species. These bacteria positively correlated with percentage of Treg and Th2 cells (cluster B), suggesting that the species in this cluster may upregulate these immune cells. *Roseburia, Rumminococcus* and various commensal *Clostridium* species are known to produce short chain fatty acids (32, 45, 46), which in their turn are known to induce Tregs (47). Various bacterial species of clusters 1, 2, and 3 also correlated positively or negatively with immune cluster D, mainly with monocytes and lymphocytes. This suggests that the microbiota may be involved in hematopoiesis in pregnancy. Indeed, percentages of lymphocytes, monocytes and granulocytes are differently affected by pregnancy in conventional and germfree mice. The effect of the microbiota on hematopoiesis outside pregnancy has been shown before by Josefsdottir et al. (48). Percentages of blood monocyte subsets are affected by pregnancy, with an increase in the percentage of classical monocytes and a decrease in the percentage of non-classical monocytes during pregnancy in both conventional and germfree mice. Since classical monocytes mature in the circulation to non-classical monocytes (49), these data may suggest a decreased maturation of monocytes during pregnancy in these syngeneic mice. Since changes in monocyte subsets are similar in conventional and germfree mice, the microbiota is probably not involved and other pregnancy factors, such as cytokines or exosomes, may be involved in inducing these changes in monocyte subsets.

Bacterial cluster 4 contains only bacteria that were decreased during pregnancy. They were strongly negatively correlated with the expression of CD80 on intermediate monocytes and with the expression of CD80 on classical monocytes. Expression of CD80, which is an activation marker, on these monocyte subsets increased during conventional mouse pregnancy, suggesting that the bacteria in this cluster may normally be involved in downregulating CD80 expression, but due to their decrease in pregnancy, intermediate, and classical monocytes may upregulate CD80 expression. This cluster contains 2 *Barnesiella* species, which have been shown to be anti-inflammatory in mice (50). This suggestion of downregulation of CD80 expression on monocytes by certain bacterial species is in line with the fact that in non-pregnant germfree mice, CD80 expression on intermediate monocytes is increased vs. non-pregnant conventional mice (**Figure 8B**). A similar reasoning would apply to MHC II expression on intermediate monocytes, which is down regulated during pregnancy in conventional mice and positively correlated with the decreased bacteria in cluster 4 suggesting that normally these bacteria upregulate MHC II, but when these bacteria are decreased during pregnancy, MHC II expression is decreased.

In conclusion, our data, together with previous data in mice and humans, indicate that the gut microbiota change during pregnancy. The changes, however, may be species and strain dependent, but may also depend on the diet as well as on the method of 16S RNA sequencing. In the present study, we found an increase in Firmicutes species (such as *A. stercoricanis*) and a decrease in Bacteroidetes species (such as *B. intestihominis, B. viscericola*). The gut microbiota in pregnant mice may be involved in some of the adaptations of the maternal immune response to pregnancy, especially in the increase in Treg and the increased activation of monocytes at the end of pregnancy. Although these data show that the microbiota may be involved in inducing adaptations of the immune response to pregnancy, these data do not show whether the change observed in the microbiota during pregnancy are responsible for these immunological adaptations. However, the fact that we found various correlations between the pregnancy-induced adaptations in immune cells and the pregnancy-induced changes in bacterial species in conventional mice, may suggest a role for the changes in the microbiota at day 18 of pregnancy in the adaptations of the immune response. Further studies, such as studying the immune response after transplantations of fecal material from pregnant and non-pregnant conventional mice into pregnant and non-pregnant germfree mice, are needed to confirm this hypothesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Central Committee on Animal Testing, Netherlands.

AUTHOR CONTRIBUTIONS

The experiments were designed and setup by MF and PV. YL and TB performed experiments. MF, CL-B, HH, and PV wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02976/full#supplementary-material

REFERENCES

1. Veenstra van Nieuwenhoven AL, Heineman MJ, Faas MM. The immunology of successful pregnancy. *Hum Reprod Update*. (2003) 9:347–57. doi: 10.1093/humupd/dmg026
2. Faas MM, de Vos P. Uterine NK cells and macrophages in pregnancy. *Placenta*. (2017) 56:44–52. doi: 10.1016/j.placenta.2017.03.001
3. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is succesful pregnancy a Th2 phenomenon? *Immunol Today*. (1993) 14:353–6. doi: 10.1016/0167-5699(93)90235-D
4. Saito S, Tsukaguchi N, Hasegawa T, Michimata T, Tsuda H, Narita N. Distribution of Th1, Th2, and Th0 and the Th1/Th2 cell ratios in human peripheral and endometrial T cells. *American J Reprod Immunol*. (1999) 42:240–5. doi: 10.1111/j.1600-0897.1999.tb00097.x
5. Ernerudh J, Berg G, Mjösberg J. Regulatory T helper cells in pregnancy and their roles in systemic versus local immune tolerance. *Am J Reprod Immunol*. (2011). 66 (Suppl. 1):31–43. doi: 10.1111/j.1600-0897.2011.01049.x
6. Santner-Nanan B, Peek MJ, Khanam R, Richarts L, Zhu E, Fazekas de St Groth B, et al. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. *J Immunol*. (2009) 183:7023–30. doi: 10.4049/jimmunol.0901154
7. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *AmJ Reprod Immunol*. (2010) 63:601–10. doi: 10.1111/j.1600-0897.2010.00852.x
8. Siegel I, Gleicher N. Changes in peripheral mononuclear cells in pregnancy. *AmJReprodImmunol*. (1981) 1:154–5.
9. Veenstra van Nieuwenhoven AL, Bouman A, Moes H, Heineman MJ, de Leij F, Santema J, et al. Endotoxin-induced cytokine production of monocytes of third trimester pregnant women compared to women in the follicular phase of the menstrual cycle. *Am. J. Obstet. Gynecol.* (2003) 188:1073–7. doi: 10.1067/mob.2003.263
10. Sacks GP, Studena K, Sargent IL, Redman CWG. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *Am J Obstet Gynecol*. (1998) 179:80–6. doi: 10.1016/S0002-9378(98)70254-6
11. Luppi P, Haluszczak C, Betters D, Richard CAH, Trucco M, DeLoia JA. Monocytes are progressively activated in the circulation of pregnant women. *J Leukoc Biol*. (2002) 72:874–84. doi: 10.1034/j.1600-0897.2002.1o041.x
12. Faas MM, Kunnen A, Dekker DC, Harmsen HJ, Aarnoudse JG, Abbas F, et al. Porphyromonas Gingivalis and E-coli induce different cytokine production patterns in pregnant women. *PLoS ONE*. (2014) 9:e86355. doi: 10.1371/journal.pone.0086355
13. Melgert BN, Spaans F, Borghuis T, Klok PA, Groen B, Bolt A, et al. Pregnancy and preeclampsia affect monocyte subsets in humans and rats. *PLoS ONE*. (2012) 7:e45229. doi: 10.1371/journal.pone.0045229
14. Elderman M, Hugenholtz F, Belzer C, Boekschoten M, de Haan B, de Vos P, et al. Changes in intestinal gene expression and microbiota composition during late pregnancy are mouse strain dependent. *Sci Rep*. (2018) 8:10001. doi: 10.1038/s41598-018-28292-2
15. Szekeres-Bartho J, Barakonyi A, Par G, Polgar B, Palkovics T, Szereday L. Progesterone as an immunomodulatory molecule. *Int Immunopharmacol*. (2001) 1:1037–48. doi: 10.1016/S1567-5769(01)00035-2
16. Mellembakken JR, Aukrust P, Olafsen MK, Ueland T, Hestdal K, Videm V. Activation of leukocytes during the uteroplacental passage in preeclampsia. *Hypertension*. (2002) 39:155–60. doi: 10.1161/hy0102.100778
17. Sacks GP, Sargent IL, Redman CWG. An innate view of human pregnancy. *Immunol Today*. (1999) 20:114–8. doi: 10.1016/S0167-5699(98)01393-0
18. Sacks GP, Clover LM, Bainbridge DR, Redman CW, Sargent IL. Flow cytometric measurement of intracellular Th1 and Th2 cytokine production by human villous and extravillous cytotrophoblast. *Placenta*. (2001) 22:550–9. doi: 10.1053/plac.2001.0686
19. Gohner C, Plosch T, Faas MM. Immune-modulatory effects of syncytiotrophoblast extracellular vesicles in pregnancy and preeclampsia. *Placenta*. (2017) 60 (Suppl. 1):S41–51. doi: 10.1016/j.placenta.2017.06.004
20. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci USA*. (1996) 93:705–8. doi: 10.1073/pnas.93.2.705
21. Robertson SA, Prins JR, Sharkey DJ, Moldenhauer LM. Seminal fluid and the generation of regulatory T cells for embryo implantation. *Am J Reprod Immunol*. (2013) 69:315–30. doi: 10.1111/aji.12107
22. Faas MM, van der Schaaf G, Moes H, Heineman MJ, de Vos P. Developing ovarian follicles inhibit the endotoxin-induced glomerular inflammatory reaction in pseudopregnant rats. *Am J Reprod Immunol*. (2004) 51:385–9. doi: 10.1111/j.1600-0897.2004.00173.x
23. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science*. (2012) 336:1268–73. doi: 10.1126/science.1223490
24. Smelt MJ, de Haan BJ, Bron PA, van Swam I, Meijerink M, Wells JM, et al. *L. plantarum*, *L. salivarius*, and *L. lactis* attenuate Th2 responses and increase Treg frequencies in healthy mice in a strain dependent manner. *PLoS ONE*. (2012) 7:e47244. doi: 10.1371/journal.pone.0047244
25. Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe*. (2008) 4:337–49. doi: 10.1016/j.chom.2008.09.009
26. Brandsma E, Kloosterhuis NJ, Koster M, Dekker DC, Gijbels MJJ, van der Velden S, et al. A proinflammatory gut microbiota increases systemic inflammation and accelerates atherosclerosis. *Circ Res*. (2019) 124:94–100. doi: 10.1161/CIRCRESAHA.118.313234
27. Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Backhed HK, et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell*. (2012) 150:470–80. doi: 10.1016/j.cell.2012.07.008
28. Bartram AK, Lynch MD, Stearns JC, Moreno-Hagelsieb G, Neufeld JD. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads. *Appl Environ Microbiol*. (2011) 77:3846–52. doi: 10.1128/AEM.02772-10
29. Heida FH, van Zoonen A, Hulscher JBF, Te Kiefte BJC, Wessels R, Kooi EMW, et al. A necrotizing enterocolitis-associated gut microbiota is present in the meconium: results of a prospective study. *Clin Infect Dis*. (2016) 62:863–70. doi: 10.1093/cid/ciw016
30. Hammer O, Harper DAK, Ryan PD. Past: paleontological statistics software package for educatoin and data analysis. *Paleontol Electron*. (2001). 4:9.
31. Metsalu T, Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using principal component analysis and heatmap. *Nucleic Acids Res*. (2015) 43:W566–70. doi: 10.1093/nar/gkv468
32. Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol*. (2017) 19:29–41. doi: 10.1111/1462-2920.13589
33. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. *Adv. Immunol.* (2014) 121:91–119. doi: 10.1016/B978-0-12-800100-4.00003-9
34. Kosiewicz MM, Dryden GW, Chhabra A, Alard P. Relationship between gut microbiota and development of T cell associated disease. *FEBS Lett*. (2014) 588:4195–206. doi: 10.1016/j.febslet.2014.03.019
35. Asarat M, Apostolopoulos V, Vasiljevic T, Donkor O. Short-chain fatty acids regulate cytokines and Th17/Treg cells in human peripheral blood mononuclear cells *in vitro*. *Immunol Invest*. (2016) 45:205–22. doi: 10.3109/08820139.2015.1122613
36. Nuriel-Ohayon M, Neuman H, Ziv O, Belogolovski A, Barsheshet Y, Bloch N, et al. Progesterone increases bifidobacterium relative abundance during late pregnancy. *Cell Rep*. (2019) 27:730–6 e733. doi: 10.1016/j.celrep.2019.03.075
37. Gohir W, Whelan FJ, Surette MG, Moore C, Schertzer JD, Sloboda DM. Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptional diet. *Gut Microbes*. (2015) 6:310–20. doi: 10.1080/19490976.2015.1086056
38. Sim K, Cox MJ, Wopereis H, Martin R, Knol J, Li MS, et al. Improved detection of bifidobacteria with optimised 16S rRNA-gene based pyrosequencing. *PLoS ONE*. (2012) 7:e32543. doi: 10.1371/journal.pone.0032543
39. Chaouat G, Menu E, Clark DA, Dy M, Minkowshi M, Wegmann TG. Control of fetal survival in CBA x DBA 2 mice by lymphokine therapy. *J Reprod Fertil*. (1990) 89:447–58. doi: 10.1530/jrf.0.0890447

40. Veenstra van Nieuwenhoven AL, Bouman A, Moes H, Heineman MJ, de Leij LF, Santema J, et al. Cytokine production in natural killer cells and lymphocytes in pregnant women compared with women in the follicular phase of the ovarian cycle. *Fertil. Steril.* (2002) 77:1032–7. doi: 10.1016/S0015-0282(02)02976-X
41. Hamilton MS, Hamilton BL. Environmental influences on immunologically associated spontaneous abortion in CBA/J mice. *J Reprod Immunol.* (1987) 11:237–41. doi: 10.1016/0165-0378(87)90060-X
42. Ho HN, Chen SU, Yang YS, Huang SC, Lee TY, Gill TJ. Age, environment, and lymphocyte immunization influence the spontaneous resorption rate in the CBA/J x DBA/2J mouse model. *Am J Reprod Immunol.* (1994) 31:47–51. doi: 10.1111/j.1600-0897.1994.tb00846.x
43. Baines MG, Billingsley KA, De Fougerolles AR, Duclos AJ, Olney HJ, Pomerantz DK, et al. Evaluation of the role of exogenous pathogens on the incidence of embryo loss during early pregnancy in mice. *J Reprod Immunol.* (1994) 26:17–30. doi: 10.1016/0165-0378(93)00863-O
44. Croy BA, Chantakru S, Esadeg S, Ashkar AA, Wei Q. Decidual natural killer cells: key regulators of placental development (a review). *J Reprod Immunol.* (2002) 57:151–68. doi: 10.1016/S0165-0378(02)00005-0
45. Tamanai-Shacoori Z, Smida I, Bousarghin L, Loreal O, Meuric V, Fong SB, et al. Roseburia spp.: a marker of health? *Future Microbiol.* (2017) 12:157–70. doi: 10.2217/fmb-2016-0130
46. Lopetuso LR, Scaldaferri F, Petito V, Gasbarrini A. Commensal Clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathog.* (2013) 5:23. doi: 10.1186/1757-4749-5-23
47. Zeng H, Chi H. Metabolic control of regulatory T cell development and function. *Trends Immunol.* (2015) 36:3–12. doi: 10.1016/j.it.2014.08.003
48. Josefsdottir KS, Baldridge MT, Kadmon CS, King KY. Antibiotics impair murine hematopoiesis by depleting the intestinal microbiota. *Blood.* (2017) 129:729–39. doi: 10.1182/blood-2016-03-708594
49. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood.* (2010) 116:e74–80. doi: 10.1182/blood-2010-02-258558
50. Weiss GA, Chassard C, Hennet T. Selective proliferation of intestinal Barnesiella under fucosyllactose supplementation in mice. *Br J Nutr.* (2014) 111:1602–10. doi: 10.1017/S0007114513004200

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Toxoplasma gondii ROP16$_I$ Deletion: The Exacerbated Impact on Adverse Pregnant Outcomes in Mice

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Imbalance of Th1 and Th2 response at the maternal–fetal interface is considered as a radical event in the pathogenesis of immunity-related pregnant diseases. It has been demonstrated that the ROP16$_I$, a rhoptry protein of *Toxoplasma gondii*, and the viable parasite with ROP16$_I$ may induce M2 macrophage polarization in host innate immunity and may be involved in the adverse pregnant outcomes. However, the mechanisms by which *T. gondii*-derived effectors subvert the immune tolerance in the pathology of pregnancy remain unclear. Here, we constructed the RH strain with ROP16$_I$ deletion (RHΔ*rop16*) to explore the pathogenesis of abnormal pregnancy. We found that C57BL/6 mice infected with RHΔ*rop16* exhibited the increased resorption of fetuses and more severe adverse pathology of placentae at the early phase of gestation, as compared to the mice infected with RH wild type (RH WT) parasite. Additionally, RHΔ*rop16* strain infection significantly promoted M1 macrophage phenotypes of CD80 and CD86, and decreased CD206 expression of M2 macrophages, with upregulation of the iNOS and downregulation of the Arg-1 expression in placental homogenates. Simultaneously, the pro-inflammatory cytokines of IL-12 and TNF-α were elevated whereas the anti-inflammatory cytokine of TGF-β1 was dampened. Moreover, the p38α mitogen-activated protein kinase (p38α MAPK) was notably phosphorylated in placental macrophages infected with both RHΔ*rop16* and RH WT strains compared with the control. Taken together, our findings indicated that ROP16$_I$ deletion of type I RH strain may cause exacerbated adverse pregnant outcomes, which is attributable to subversion of the maternal immune tolerance due to the increased pro-inflammatory cytokines in the pregnant animals. The results also suggest that ROP16$_I$ might be a protective factor and other *T. gondii*-derived molecules might be involved in the M1–Th1 biased pathological process in aberrant pregnancy at the early phase of gestation.

Keywords: *Toxoplasma gondii*, ROP16, macrophage, immune tolerance, adverse pregnant outcome

INTRODUCTION

Toxoplasma gondii is an opportunistic food-borne protozoon with an extraordinarily broad host range of all warm-blooded animals, including humans (Montoya and Liesenfeld, 2004; Leng et al., 2009). Humans and animals are infected by ingesting food that contains cysts or water that is contaminated with oocysts of *T. gondii* (Black and Boothroyd, 2000; Yarovinsky, 2014). It is estimated that over one billion people are chronically infected with this parasite worldwide, although the data of regional investigations vary considerably (Montoya and Liesenfeld, 2004; Dubey, 2009). *T. gondii* infection is usually asymptomatic in immunocompetent individuals but may result in severe consequences in immunocompromised people (e.g., patients with AIDS, organ transplantation, or cancers) (Nissapatorn, 2009; Luma et al., 2013; Schmidt et al., 2013). Importantly, vertical transmission of *T. gondii via* placenta may cause abortion, constituting a serious threat to humans and leading to great loss of livestock production (Montoya and Remington, 2008; Hide et al., 2009). Initial infection of women with *T. gondii* during pregnancy, particularly in the first trimester, may cause miscarriage and preterm birth and increase the susceptibility of fetuses to toxoplasmosis resulting in hydrocephaly, microcephaly, intracranial calcification, and even loss of life (Pfaff et al., 2007; Robbins et al., 2012). The variability of disease severity in infected host is linked to the genetic structure of *T. gondii* strains and to the exposure burden of the parasite (Saeij et al., 2006; Melo et al., 2011; Hunter and Sibley, 2012; Shwab et al., 2014).

Toxoplasma gondii isolates from Europe and North America mostly belong to types I (RH and GT1), II (PRU and ME49), and III (CTG) (Lehmann et al., 2006; Shwab et al., 2014), but those from China present a dramatic difference in genetic structure and virulence, termed as type Chinese 1 (Wang et al., 2013). Studies have revealed that the majority of *T. gondii* isolates causing congenital toxoplasmosis in Europeans possess the feature of type II phenotype whereas type I strains are the most prevalent in Spanish people (Fuentes et al., 2001).

Macrophages can be roughly categorized into two types: classically activated macrophage (M1) and alternatively activated macrophage (M2). As antigen-presenting cells, macrophages have cross-talk between innate immunity and adaptive immunity. *T. gondii* parasite was found to preferentially invade macrophage/DC lineage cells during *in vivo* infection (Courret et al., 2006). Decidual immune cell populations approximately consist of 20% of macrophages that play a critical part in maintaining normal pregnancy. It has been well recognized that M2 macrophages are responsible for sustaining the normal microenvironment of pregnancy at the maternal–fetal interface (Nagamatsu and Schust, 2010). Actually, any subversion of M1/M2 macrophage balance may lead to pregnant disorders, such as pregnant loss, premature birth, or fetal growth restriction (Renaud and Graham, 2008; Brown et al., 2014).

Toxoplasma gondii establishes the long-lasting infection in host and has the developed sophisticated ways to manipulate host immunity. For instance, the parasite delivers effector proteins, which are released from the contents of rhoptries, micronemes, and dense granules of *T. gondii*, into host cells (Jensen et al., 2011; Rosowski et al., 2011; Hunter and Sibley, 2012). The secreted rhoptry proteins (ROPs) and dense granule proteins (GRAs) are characterized to hijack cell-signaling pathways of the host cells, which manipulate host immune response, and therefore determine parasite virulence and infection consequences (Jensen et al., 2011; Hakimi et al., 2017). Interestingly, ROP16 (the member of ROPs) gets injected into the host cell nucleus where it activates signal transducer and activator of transcription Stat3 and Stat6, initiating the signaling cascade of host cells. ROP16$_I$ of type I strains, rather than ROP16$_{II}$ of type II strains, decides ROP16 kinase activity on Stat3 and Stat6 (Yamamoto et al., 2009). Phosphorylation of Stat3 and Stat6 triggers anti-inflammatory response and M2 macrophage activation, which plays a crucial role in modulating host immunity at the early phase and is associated with the consequences of infection at the late phase. Contrarily, the GRA15$_{II}$ directly activates the NF-κB of the host cells, promotes its rapid nuclear transcription, and drives the macrophage to M1 polarization (Rosowski et al., 2011). ROP16$_I$ and GRA15$_{II}$ can work independently of pattern recognition receptors to achieve M1 or M2 polarization (Jensen et al., 2011). Intriguingly, the WH3 strain of type Chinese 1, a predominant genotype in China, carries both key effectors: ROP16$_I$ and GRA15$_{II}$. Our previous study showed that the WH3Δ*rop16* strain with GRA15$_{II}$ background of type Chinese 1 evoked the Th1- and Th17-biased response, leading to subversion of immune tolerance at the maternal–fetus interface and adverse pregnant outcomes (Wang et al., 2018). Recent studies have shown that RHΔ*rop16* infection in mice may cause serious ocular toxoplasmosis in the immune-privileged microenvironment, which is far away from the proliferation sites of the parasite, suggesting that the RH strain, with ROP16$_I$ deficiency, remains a high pathogenesis of severe retinopathy in animal model (Rochet et al., 2019).

It has been known that vertical transmission of viable *T. gondii* parasite is a crucial route in aberrant pregnancy. However, we tried to identify the parasite by *Toxo*-DNA detection and bioassay in the maternal placental tissues with positive antibodies against *T. gondii* and abnormal pregnancies, but failed (data not published). Previous studies revealed that pregnant mice of IL-10 deletion infected with *T. gondii* resulted in a worse pregnancy than the control (Lao et al., 2015). Additionally, adoptive transfer of Treg cells to mice could improve adverse pregnancies of the animals (Liu et al., 2014). All of these investigations strongly suggest that the imbalance of immune tolerance at the maternal–fetal interface, in addition to direct parasite invasion, might be involved in the occurrence of abnormal pregnant consequences. To characterize the role of ROP16$_I$ molecule in the pathogenicity of virulent type I strain, we generated *T. gondii* RHΔ*rop16* utilizing CRISPR/Cas9 technology to decipher the possible influence of ROP16$_I$-deficient parasite infection on and the pathophysiology in the abnormal pregnancies. Our results uncovered that infection of mice with RHΔ*rop16* strain cause exacerbated adverse pregnant outcomes in which type I dominant response and enhanced pro-inflammatory cytokine expression were present in placenta tissues. The data provide further evidence that

ROP16$_I$ plays a potentially protective role in maintaining physiological immune tolerance during pregnancy, and other parasite-derived effectors might be associated with the M1–Th1 biased response at the maternal–fetal interface and the adverse pregnant outcomes.

MATERIALS AND METHODS

Parasite Collection

Toxoplasma gondii RH WT and RHΔ*rop16* tachyzoites were cultured in human foreskin fibroblast (HFF) cells.

T. gondii Infection in Pregnant Mice

The 8- to 10-week-old male and 6- to 8-week-old female C57BL/6 mice were raised for 1 week after purchase to adapt to the animal center environment with normal feeding and drinking at room temperature. The female and male mice were caged overnight at a 2:1 ratio. The female mice were examined for the vaginal plug as gestation day 0 (GD0). All pregnant mice were randomly divided into three groups: control group, RH WT group, and RHΔ*rop16* group, with seven mice in each. All of the experimental procedures were performed in accordance with the Institutional Review Board of AMU Institute of Biomedicine AMU (permit No: AMU26-081108). The studies were performed in licensed Biosafety II Laboratory. The RH WT and RHΔ*rop16* viable tachyzoites were harvested from HFF cells and counted with an Improved Neubauer counting board and diluted to 2×10^3/ml. On gestation day 8 (GD8), mice of RH WT and RHΔ*rop16* groups were intraperitoneally injected with 200 μl of PBS containing 400 wild type and Δ*rop16* tachyzoites, respectively. The control animals were only given equal volume of PBS solution. On GD14, the animals were euthanized, blood was taken, and placentae, fetuses, uterus, and spleens were aseptically removed. The fetuses and placentae were photographed and weighed, and the number of resorbed fetuses was calculated. The resorptivity was determined by the small size, hemorrhagic appearance, and necrotic placenta and embryos. The ratio of the resorption to the total fetuses was recorded as a percentage of fetal loss. The sera were obtained by centrifugation (4,000 rpm, 10 min, 4°C) for ELISA detection.

Pathology of Placental Tissues

Mice were randomly selected into three groups, with seven in each, for placental fixation with 4% paraformaldehyde, paraffin-embedded sections and hematoxylin–eosin staining. The hyperemia of placentae was observed by histopathological analyses. All mice in each group were photographed, with five photos for sections of each animal.

Collection of Macrophages From Uterus and Placental Tissues

The uterus and placental tissues were quickly excised in a culture dish after removal, followed by digestion with 0.5% BSA, 5 mg/ml Collagenase IV, and HEPES (10 mM) (St. Louis, MO, United States) in RPMI 1640 solution (Montreal, QC, Canada), and slightly shaken at 37°C for 30 min at 150 rpm. Subsequently, the suspended cells were filtered through sterile nylon mesh. Macrophages were obtained by density gradient centrifugation at 25/50% Percoll (GE Healthcare Life Sciences).

Isolation of Control Placental Macrophages and Infection of *T. gondii in vitro*

Macrophages in uterus and placental tissues from control pregnant mice were harvested according to the methods previously described and 2×10^6 cells/well were cultured in 12-well plates. Macrophages were infected with 2×10^6 RH WT and RHΔ*rop16* parasites, and the control group remained uninfected. The cells were cultured in an incubator at 37°C for 24 h to obtain macrophages. The macrophages were subjected to detection of the mRNA and protein expression of iNOS and Arg-1 and examination of phosphorylation of p38α mitogen-activated protein kinase (p38α MAPK).

Western Blotting

Placental tissues of all mice and macrophages infected with the parasite were lysed using protein lysate (RIPA: PMSF = 100: 1), followed by centrifugation at 12,000 × *g* for 15 min at 4°C. The denatured proteins were separated by 10% separation gel in SDS-PAGE and transferred to NC membrane followed by blocking in 5% of milk on a shaker for 90 min. The NC membrane was incubated with primary antibodies against β-actin, Arg-1, and iNOS for placental tissues and additional incubation of p-p38α MAPK (Abcam, United Kingdom) for the macrophages infected with the parasite (both had 1:5000 dilution) at 4°C overnight. The anti-β-actin was used as the control. The NC membrane was washed with TBST three times, followed by the horseradish peroxidase-labeled secondary antibody incubation with slight shaking for 90 min. The specific bands were visualized by enhanced chemiluminescence. All experimental data were analyzed by ImageJ 1.46 software.

Measurement of Macrophage Polarization by Flow Cytometry

Macrophages were obtained from the uterus and placental tissues of all mice and 2×10^6 cells with 5 μl of mouse sera were added to each tube and let stand at 4°C for 30 min. The cells were labeled with anti-F4/80-BV421, anti-CD80-PE/cy5, anti-CD86-PE, and anti-CD206-AF647 (New York, BD, United States) at 4°C for 30 min in the dark. The cell surface markers were fixed after washing twice by PBS. All labeled samples were quickly detected by flow cytometry (FCM).

Isolation and Culture of Splenocytes

The spleen tissues were taken out from the pregnant mice of three groups, and half of the tissues were mashed on a sterile nylon mesh to obtain spleen cells. Simultaneously, 2–3 ml of 1 × ACK Lysis buffer was added to the spleen cells in a tube for disrupting erythrocytes. Finally, the lysis was stopped with PBS, and the erythrocyte debris was removed by centrifugation at 2,500 rpm

TABLE 1 | The primers used for qRT-PCR.

Primers	Forward primer (5′–3′)	Reverse primer (5′–3′)
TNF-α	ACGGCATGGATCTCAAAGAC	GTGGGTGAGGAGCACGTAGT
iNOS	CACCTTGGAGTTCACCCAGT	ACCACTCGTACTTGGGATGC
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTATCATTAGGGACATC
IL-12	GATGTCACCTGCCCAACTG	TGGTTTGATGATGTCCCTGA
IL-10	GCTCCTAGAGCTGCGGACT	TGTTGTCCAGCTGGTCCTTT
TGF-β1	CTGGATACCAACTACTGCTTCAG	TTGGTTGTAGAGGGCAAGGACCT
β-actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC

IL, interleukin; iNOS, inducible nitric synthase; Arg-1, arginase-1; TNF, tumor necrosis factor; TGF-β1, transforming growth factor beta 1.

for 5 min, and the spleen cells were isolated and cultured at 37°C for 5 h under stimulation with ionomycin (1 mg/ml) and PMA (20 ng/ml) (St. Louis, MO, United States), and then centrifuged (4,000 rpm × 10 min at 4°C) to obtain supernatants for ELISA detection. The remaining spleen tissues were stored at −80°C for RNA extraction.

Real-Time PCR for Detection of Cytokines

Total RNAs were extracted from spleens, placentae, and macrophages infected with the parasite and then reversed into cDNAs according to the Takara Kit instructions (Takara, Japan). Quantitative detection of IL-12, IL-10, TNF-α, TGF-β1, iNOS, and Arg-1 of placentae was performed with SYBR-Green Premix Ex Taq kit (Takara, Japan). Spleen tissues were only detected for IL-12, IL-10, TNF-α, and TGF-β1. The parasite-infected macrophages were detected for iNOS and Arg-1. The primers used in the qRT-PCR are listed in **Table 1**. The measurements were completed with Roche Applied Science Light Cycler TM 480 instrument. The results were normalized by β-actin and the results were calculated using the $2^{-\Delta\Delta Ct}$ method.

ELISA for Cytokines

The placental tissues were weighed and ultrasonicated in the ratio of 1 mg/10 μl PBS (at 3-s intervals) and centrifuged at 12,000 × *g* at 4°C for 10 min. The supernatants were extracted

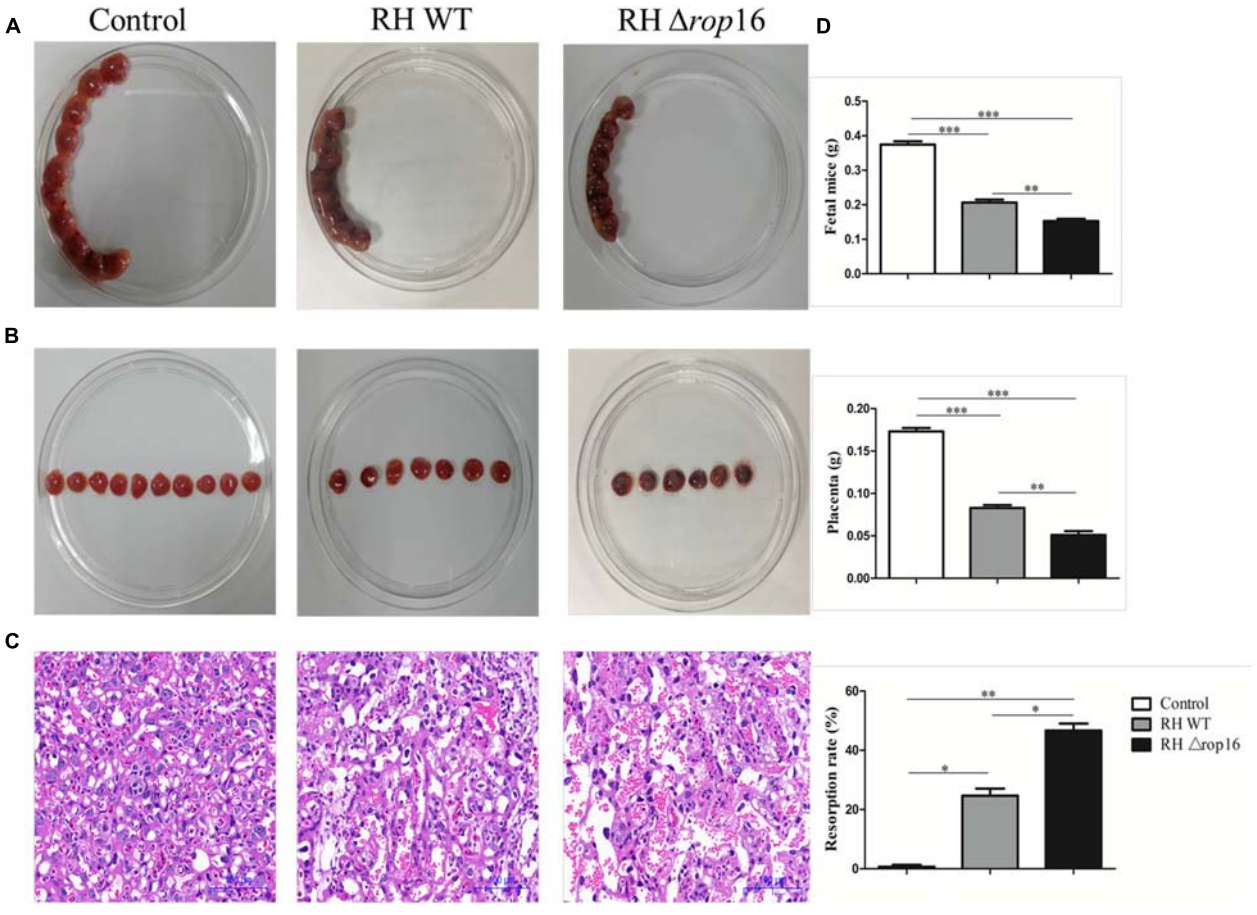

FIGURE 1 | The occurrence of adverse pregnancy outcomes caused by *Toxoplasma gondii* infection. Fetuses and placentae of the control mice developed normally. The less number of fetuses and the more serious hemorrhage of placentae were seen in the mice of the RHΔ*rop16*-infected group when compared with the RH WT group **(A,B)**. H&E staining showed deteriorated hyperemia of placentae caused by RHΔ*rop16* strain infection **(C)**. The weights of fetuses and placentae and fetus resorption of each group were measured after 6 days of infection. The ratio of resorption to total fetuses was calculated as the percentage of fetal loss **(D)**. Each group contained seven mice. The results were presented as mean ± SD and were analyzed by one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

and cytokines of TNF-α (Cusabio, Houston, TX, United States) and IL-12 (Elabscience, Wuhan, China) in the supernatants of placental homogenates, spleen cell culture, and sera were detected by ELISA. The examinations of all specimens were performed three times and the OD values were measured at a wavelength of 450 nm. The concentration of TNF-α and IL-12 was calculated by plotting a standard curve according to the manufacturer's instruction.

Statistical Analysis

The data were presented as the mean ± SD and were statistically analyzed by one-way ANOVA after precheck for normal distribution and homogeneity of variances, and $p < 0.05$ or $p < 0.01$ indicated statistical significance. Analyses of experimental data and graphic production were obtained by GraphPad Prism Software.

RESULTS

RHΔ*rop16* Strain Infection Caused Severe Abnormal Pregnancy

Pregnancy outcomes were examined at GD14 (i.e., 6 days post-infection). The pregnant mice infected with RHΔ*rop16* strain showed a clinical manifestation of wilting and arching, accompanied with aggravated fetal resorption and placental hemorrhage when compared with the RH WT group (**Figures 1A,B**). The H&E staining of placenta tissues exhibited more severe hyperemia in the RHΔ*rop16*-infected group than that in the RH WT group (**Figure 1C**). The weights of placentae and fetuses in mice of the RHΔ*rop16*-infected group were notably reduced compared with the RH WT group. Accordingly, the resorptivity of the fetuses in the RHΔ*rop16*-infected group significantly increased (**Figure 1D**).

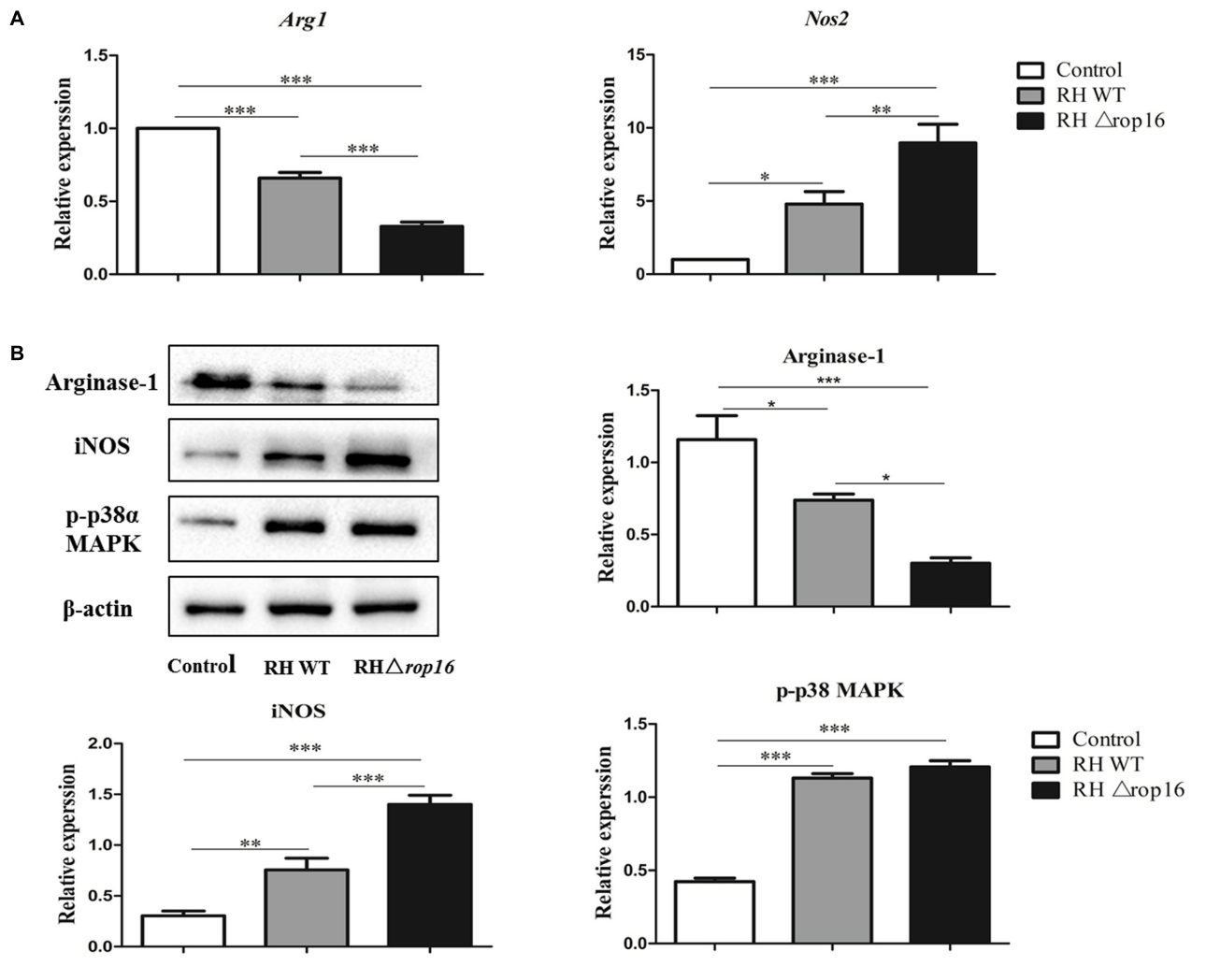

FIGURE 2 | *In vitro* expression of Arg-1, iNOS, and p-p38α MAPK of placental macrophages from control pregnant mice following *T. gondii* infection. Placental macrophages of control pregnant mice were collected and infected with RH WT and RHΔ*rop16 in vitro* for 24 h. The mRNA and protein expressions of Arg-1 and iNOS of macrophages were examined by qRT-PCR **(A)** and Western blotting **(B)**. Phosphorylation of p38α MAPK was detected by Western blotting **(B)**. Each group contained seven mice. The results were given as mean ± SD and were analyzed by one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Expression of iNOS, Arg-1, and p-p38α MAPK Varied in Placental Macrophages Infected With RHΔ*rop16* Strain *in vitro*

To examine the direct impact of *T. gondii* ROP16$_I$ on the bias of macrophages, we isolated primary macrophages from placental and uterine tissues of control pregnant mice. The cells were infected with RH WT and RHΔ*rop16* parasites, and cultured for 24 hr. The data showed that the expression of iNOS was significantly elevated while that of Arg-1 was diminished in the macrophages with RHΔ*rop16* infection when tested by qRT-PCR (**Figure 2A**) and Western blotting (**Figure 2B**). No significant difference of p-p38α MAPK expression, however, was noted between RH WT and RHΔ*rop16*-infected macrophages by Western blotting analysis (**Figure 2B** and **Supplementary Figure S1**).

RHΔ*rop16* Strain Infection Modulated Synthesis of iNOS and Arg-1 in Placental Tissues *in vivo*

Expression of iNOS and Arg-1 in placental tissues in mice of three groups was assayed by qRT-PCR (**Figure 3A**) and Western blotting (**Figure 3B**). As shown in **Figure 3**, the upregulated expression of iNOS mRNAs and downregulated expression of Arg-1 mRNAs were observed in the RHΔ*rop16*-infected group, when compared to the RH WT group. Correspondingly, the protein level of iNOS was remarkably elevated but Arg-1 was reduced when detected by Western blotting in RHΔ*rop16*-inoculated mice.

The Phenotypes of Mouse Placental Macrophages Altered Following RH Δ*rop16* Strain Infection

Density gradient centrifugation was used to isolate placental macrophages for detection of polarization of the macrophages in placental tissues of mice infected with RHΔ*rop16* strain. The surface markers of CD86 and CD80 on M1 macrophages and CD206 on M2 cells were detected by FCM. The results showed that RHΔ*rop16* infection induced higher expression of CD86 (**Figure 4A**) and CD80 (**Figure 4B**) but lower expression of CD206 (**Figure 4C**) on the surface of placental macrophages, when compared to the RH WT infection group.

RHΔ*rop16* Infection Evoked Th1-Biased Response in Spleens, Placentae, and Sera

Tissues of placentae and spleens were collected from three groups, and the mRNA expression of IL-12, TNF-α, IL-10, and TGF-β1 was measured by qRT-PCR. The results showed that the mRNA expression of IL-12 and TNF-α in the spleens (**Figure 5A**) and placentae (**Figure 5D**) were highly transcribed,

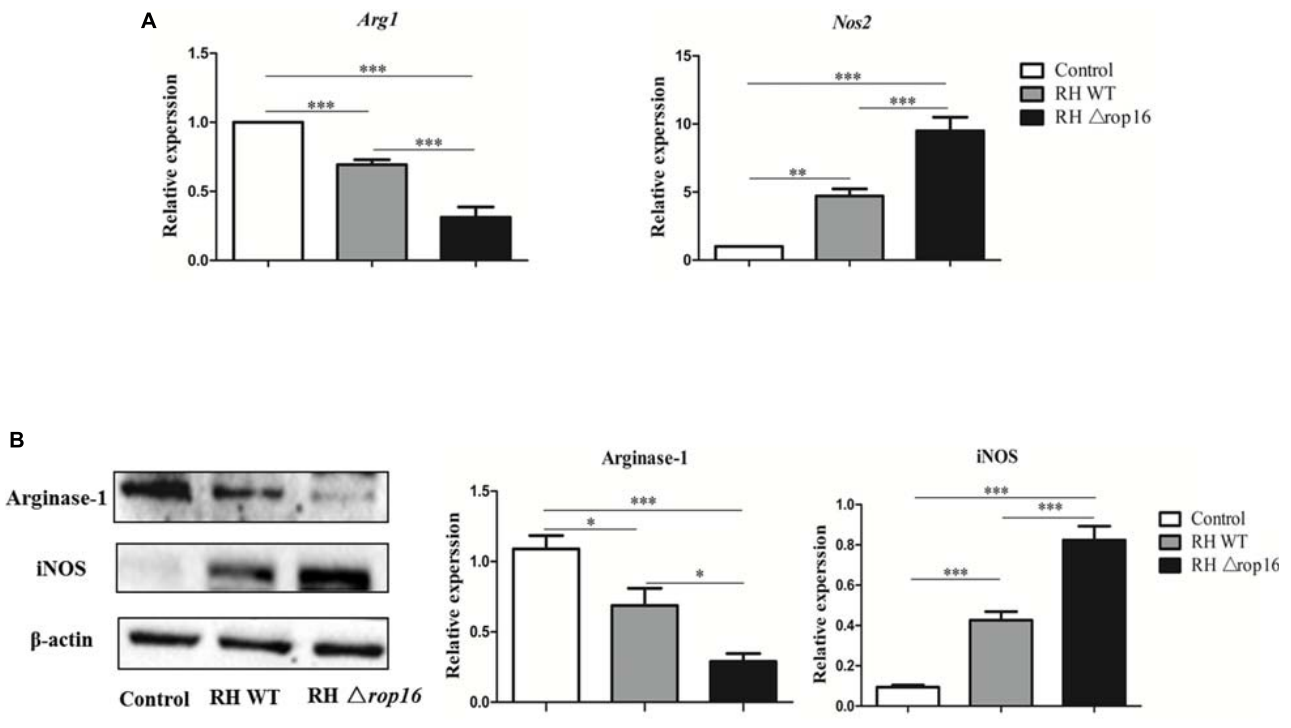

FIGURE 3 | *In vivo* expression of Arg-1 and iNOS in placental tissues. Placental tissues were removed from three experimental groups. The mRNA and protein expressions of Arg-1 and iNOS of placental tissues were examined. The relative mRNA expressions of Arg-1 and iNOS were detected in placental tissues of mice infected with RHΔ*rop16* strain **(A)**. The corresponding results of protein expressions were also noted with Western blotting **(B)**. The results were presented as mean ± SD obtained from seven mice in each group and were analyzed by one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

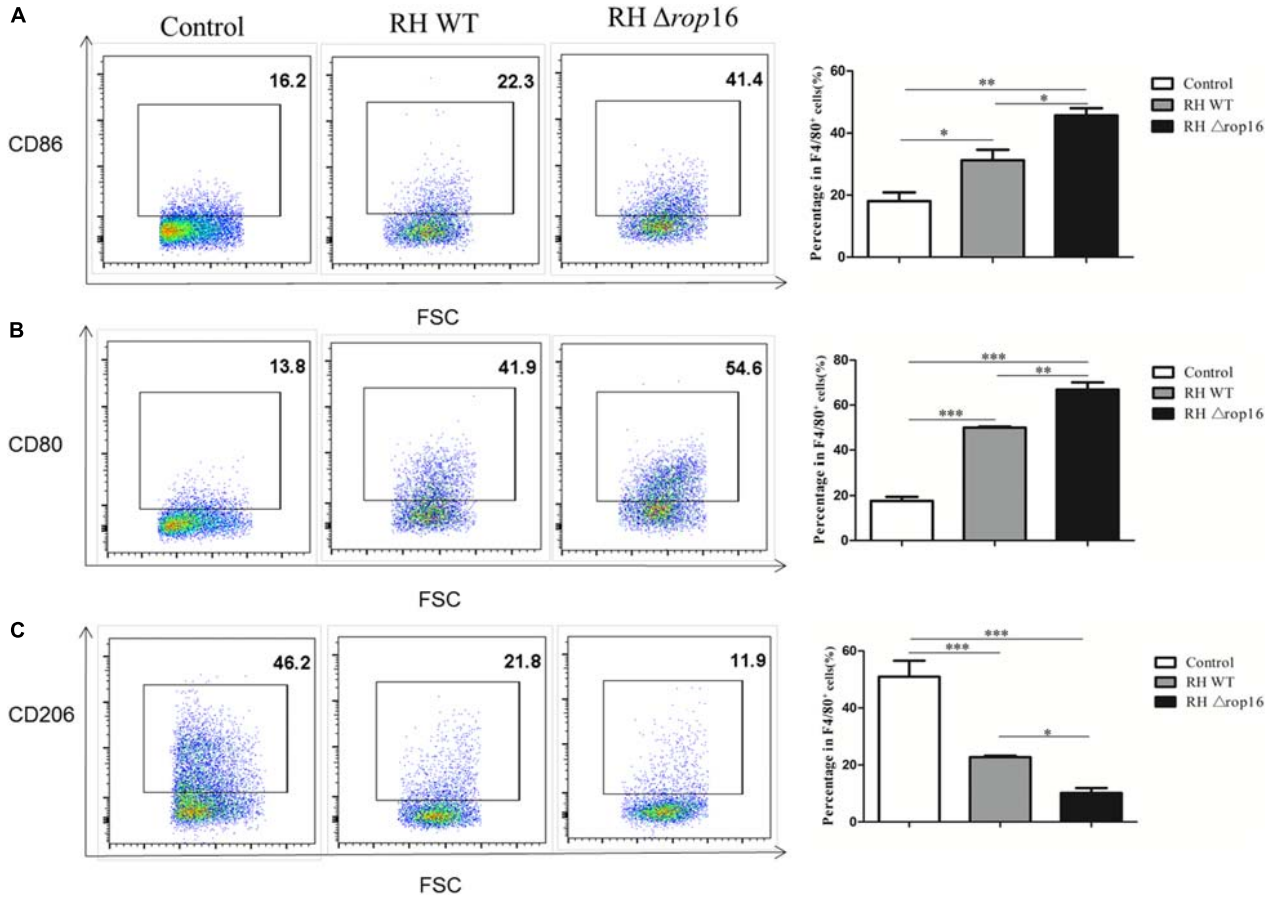

FIGURE 4 | Expression of CD86, CD80, and CD206 markers at the maternal–fetal interface. Placental macrophages were removed from three experimental groups. A high expression of CD206 but low expression of CD80 and CD86 of macrophages were noted in control pregnant mice by flow cytometry, while RHΔ*rop16* parasite infection stimulated high-level expressions of CD86 **(A)** and CD80 **(B),** and low level of CD206 **(C)** in the placental macrophages of pregnant mice. Experimental and control group each contain seven mice. The results were given as mean ± SD and were analyzed by one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

but expression of TGF-β1 mRNA was dramatically dampened (**Figures 5B,E**) in the animals of RHΔ*rop16*-inoculated mice, when compared to the RH WT infection group. Unexpectedly, the level of IL-10 expression in the spleens (**Figure 5B**) and placentae (**Figure 5E**) was significantly elevated in either of the two infected groups compared to the control. However, the ratio of IL-10/IL-12 as well as IL-10/TNF-α in the spleens (**Figure 5C**) and placentae (**Figure 5F**) of the RHΔ*rop16*-infected mice remained low in comparison to the control mice. Additionally, the ELISA assay revealed the significantly increased levels of pro-inflammatory cytokines of IL-12 and TNF-α in the supernatants of splenocytes and placental homogenates, and sera as well, in RHΔ*rop16*-infected mice (**Figures 6A–C**).

DISCUSSION

It has been known that numerous immune cells contribute to maintaining the equilibrium state of the maternal–fetal immune interface. Macrophages are the first line of defense in innate immunity. During normal pregnancy, macrophages also perform functions such as angiogenesis, tissue repair, and immune tolerance (Mor and Abrahams, 2003). Alternatively activated macrophages (M2 macrophages), uterine/decidual NK cells, as well as Tregs are crucial cell populations that contribute to the immune tolerance at the maternal–fetal interface and keep the fundamental physiological processes of pregnancy by secreting large amounts of cytokines and chemokines (Lidstrom et al., 2003; Kalkunte et al., 2008; Co et al., 2013; Gaynor and Colucci, 2017). A growing body of evidence indicated that pregnancy failure induced by *T. gondii* infection is associated with the altered maternal immunity (Zhang et al., 2012; Zhao et al., 2017). It has been reported that the LILRB4 expression of decidual macrophages decreased after infection with *T. gondii*, which induced polarization of classically activated macrophages (M1 macrophages) and inhibited the immune bias of M2 direction, leading to adverse pregnancy outcomes (Li et al., 2017). In addition, trophoblast cells are the only cells that are in direct contact with the maternal immune system and play an important part in embryo implantation and maternal–fetal

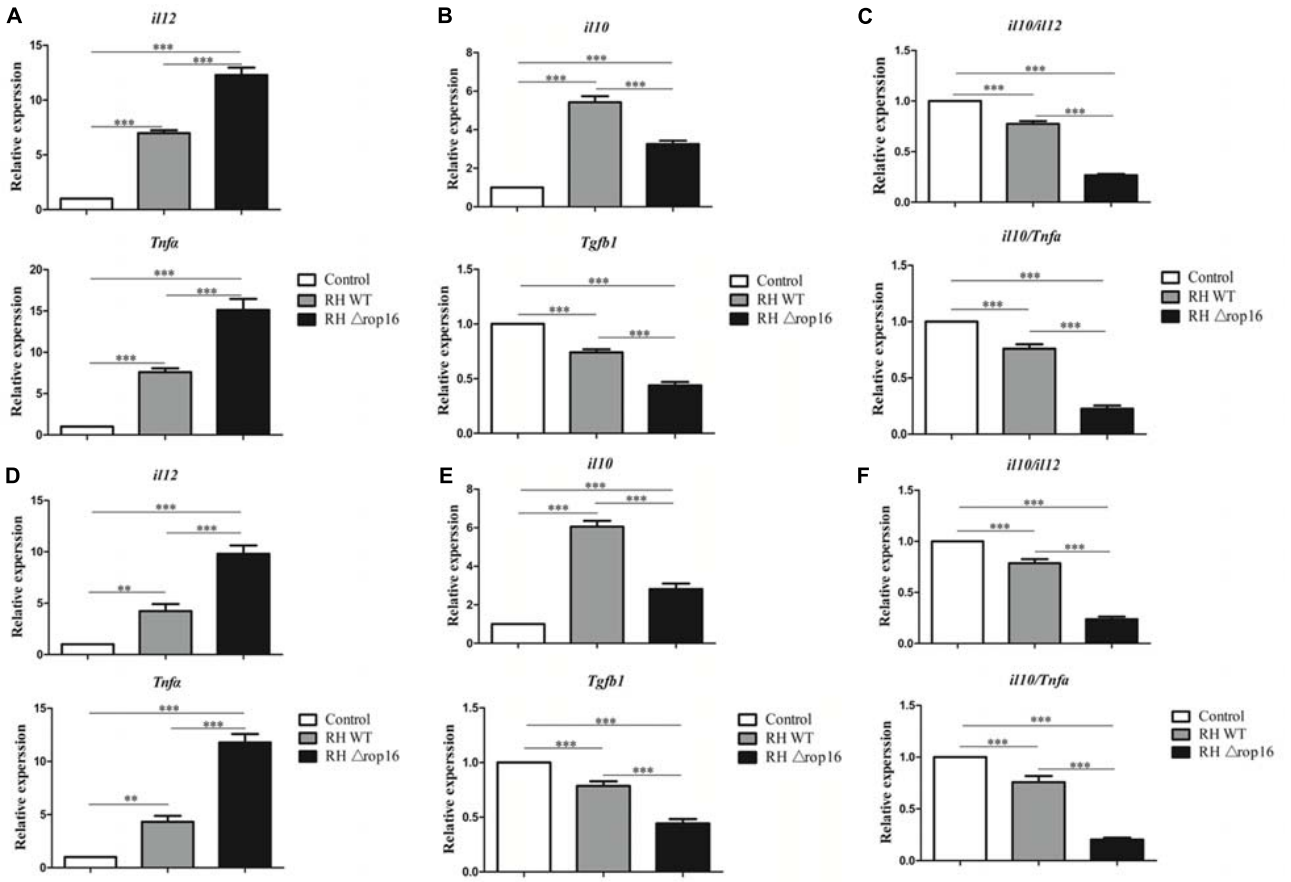

FIGURE 5 | Expression of IL-12, TNF-α, IL-10, and TGF-β1 in spleens and placentae of mice with *T. gondii* infection. Spleen tissues and placentae were taken from three groups of experimental mice. The mRNA transcriptions of pro-inflammatory cytokines of IL-12 and TNF-α and anti-inflammatory cytokine of IL-10 and TGF-β1 were examined in spleen tissues **(A,B)** and placentae **(D,E)**. The ratio of mRNA transcriptions of IL-10 to IL-12 and IL-10 to TNF-α was calculated in spleen tissues **(C)** and placentae **(F)**. Each sample was performed in triplicate. The results were presented as mean ± SD from seven mice of each group (**$p < 0.01$, ***$p < 0.001$).

immune tolerance. Apoptosis of trophoblast cells induced by inflammation may directly impact the microenvironment of maternal–fetal interface, resulting in abnormal pregnant outcomes such as miscarriage (Reister et al., 2001). Additional investigations showed that *T. gondii* infection induced the activation of macrophages and their secretion of a variety of pro-inflammatory factors through oxidative stress-mediated apoptosis of placental trophoblasts, more likely leading to adverse pregnancy events (Aschkenazi et al., 2002; Neale et al., 2003; Liu et al., 2013; Xu et al., 2015). Overall, any peripheral or local infection in the maternal side during the first trimester is prone to cause serious adverse pregnancy outcomes.

Toxoplasma gondii effectors of ROP16$_{I}$ and GRA15$_{II}$ are responsible for inducing macrophage polarizations and play a pivotal role in host innate immunity and directly affect the consequences of infection or disease. During invasion into host cells, *T. gondii* injects the rhoptry protein ROP16 into the host cytoplasm, which subsequently localizes to the parasitophorous vacuole membrane (PVM). We previously found that deletion of ROP16$_{I}$ of either RH strain of type I or WH3 strain of type Chinese 1 did not affect the virulence of the parasite to mice (Wang et al., 2018), although ROP16 is believed as one of polymorphic and strain-dependent virulence factors (Saeij et al., 2006). Studies have shown that ROP16$_{I}$, rather than ROP16$_{II}$, serves as a central regulator of parasite replication and transmission at early phase of infection and drives the macrophages to M2 polarization. Contrarily, GRA15$_{II}$, one of the key molecules secreted by dense granules of type II strains, evokes strong M1 macrophage polarization by directly activating NF-κB and facilitating a high expression of IL-12 and iNOS that are involved in the subsequent Th1 predominant immune response and the pro-inflammatory reaction (Jensen et al., 2011; Rosowski et al., 2011). We previously demonstrated that *T. gondii* strains of type Chinese 1, distinct from the strains of archetypal I, II, and III strains prevalent in the other continents of the world, carry both ROP16$_{I}$ and GRA15$_{II}$ alleles. Deletion of ROP16$_{I}$ of the Chinese 1 WH3Δ*rop16*, with GRA15$_{II}$ genetic background, resulted in remarkably adverse pregnancy outcomes in mice (Wang et al., 2018). However, the pathogenic feature of the RHΔ*rop16* strain of type I, with neither ROP16$_{I}$ nor GRA15$_{II}$

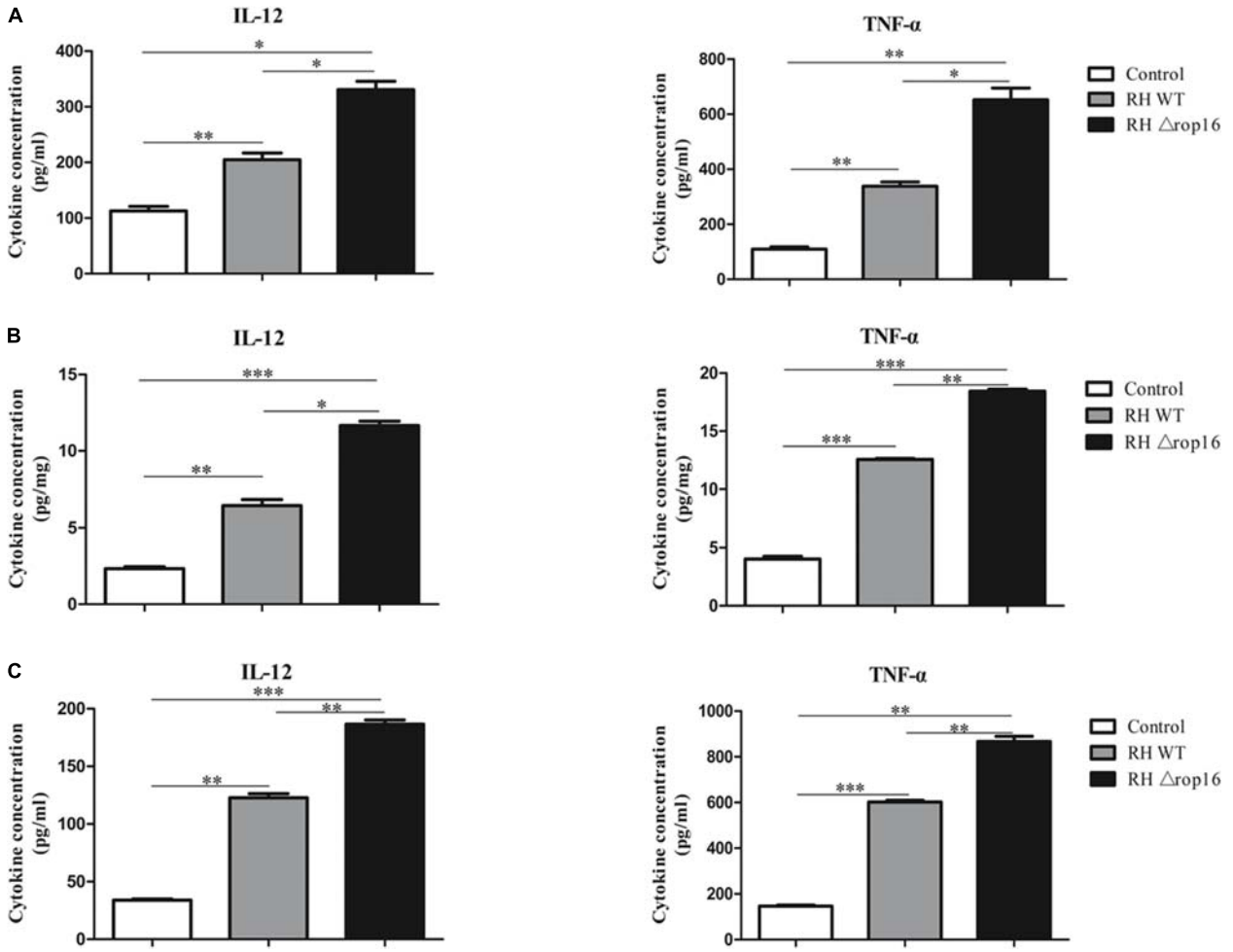

FIGURE 6 | IL-12 and TNF-α expression in splenocytes, placental tissues, and sera. The supernatants of splenocytes and placental homogenates were taken from three experimental groups. The sera were obtained by centrifugation (4,000 rpm, 10 min, 4°C) from the three groups. Expressions of IL-12 and TNF-α in the supernatants of splenocytes (pg/ml) **(A)**, placental homogenates (pg/mg) **(B)**, and sera (pg/ml) of pregnant mice **(C)** were evaluated with ELISA. Each sample was performed in triplicate. Each group contained seven animals. The results were presented as mean ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

background, in *T. gondii*-induced aberrant pregnancy remains unknown. Thus, we examined the pathology of adverse pregnant outcomes caused by ROP16$_I$-deficient *T. gondii* (RHΔ*rop16*) and the mechanisms by which the parasite infection subverts the immune tolerance at the maternal–fetal interface in the murine model. We noted that all of the mice infected with *T. gondii* had clinical manifestations of wilting and arching; however, the RHΔ*rop16* strain caused the exacerbated abnormal pregnancy outcomes when compared with the RH WT strain, as evidenced by the lower number and reduced weights of placentae and fetuses, hemorrhage of placentae, and a high rate of fetal resorption. A previous study showed that deficiency of ROP16$_I$ of the RH strain did not affect proliferation of the tachyzoites (Butcher et al., 2011), but no parasites were seen in the damaged placenta tissues of RHΔ*rop16*-infected mice (data not shown), suggesting that the pathology of placentae might be attributable to the biased M1–Th1 immune response following infection. Additionally, our data indicated that IL-12 and TNF-α were significantly increased whereas TGF-β1 was decreased in the RHΔ*rop16*-infected animals; the ratios of IL-10/IL-12 and IL-10/TNF-α of the RHΔ*rop16*-infected mice declined significantly compared to those of the RH WT group, indicative of the Th1-polarized response in RHΔ*rop16* infection. Moreover, placental macrophages tended to be driven to M1 skewing after 6 days of *T. gondii* infection. The results suggest that the pro-inflammatory factors in the placentae induced by RHΔ*rop16* infection might be involved in the aberrant pregnancy of mice, which is attributable to the deletion of ROP16$_I$ of the RH strain.

Theoretically, any negative impact of infections on immune tolerance at the maternal–fetal microenvironment may lead to abnormal pregnancy. It has been demonstrated that *T. gondii* type II strains are frequently found in human infection of Europeans (Wujcicka et al., 2014) and are responsible for pregnant failure (Liu et al., 2018), which might be related to GRA15$_{II}$-induced M1–Th1 dominant response. Genetic structures of *T. gondii* in

South America are complex, and these non-archetypal strains may cause serious consequences of pregnancies (Rajendran et al., 2012). It is speculated that multiple parasite-derived molecules, in addition to the direct invasion of parasites, may contribute to the adverse pregnant consequences. We here noted that mice infected with the RH strain with *rop16* deletion caused deteriorated pregnant outcomes, suggesting a protective influence of ROP16$_I$ on normal pregnancy. Similar reports were given by Jensen et al. (2011) that ROP16$_I$ might have an ameliorative effect on *T. gondii*-induced ileitis. Additionally, our studies strongly suggested that other *T. gondii* effectors, besides GRA15$_{II}$, might be involved in the pathology process of pregnancy. This speculation is supported by the previous investigations that demonstrated that GRA24, a member of GRAs family with 542 amino acids similar to GRA15$_{II}$ of *T. gondii* in function, can bypass the classical MKK3/MKK6 and MKK4 pathways to directly bind to the p38α MAPK, leading to activation and nuclear translocation of the host kinase and increasing IL-12 secretion and M1 macrophages polarization (Kim et al., 2005; Braun et al., 2013). Further approaches are needed to identify the effectors of *T. gondii* that induce IL-12 production and M1 polarization in the occurrence of abnormal pregnancy.

Taken together, the RH strain of type I *T. gondii* with ROP16$_I$ deletion may cause severe abnormal pregnant outcomes in mice, with the feature of M1 macrophage phenotype and Th1 dominant response in system and placental tissues, suggesting that ROP16$_I$ might be a protective factor and other parasite-derived molecules may be involved in the pathology process of pregnancy failure.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical permission was obtained from the Institutional Review Board of AMU Institute of Biomedicine AMU (Permit No. AMU26–081108).

AUTHOR CONTRIBUTIONS

JS, WW, and WC elaborated and designed the study, and drafted the manuscript. WC, CW, and TX performed the experiments. QL analyzed the data. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.03151/full#supplementary-material

FIGURE S1 | The expression of β-actin (40KD), Arg-1(36KD), and p-p38 MAPK (40KD) of placental macrophages infected with the parasite were examined by Western blotting.

REFERENCES

Aschkenazi, S., Straszewski, S., Verwer, K. M., Foellmer, H., Rutherford, T., and Mor, G. (2002). Differential regulation and function of the Fas/Fas ligand system in human trophoblast cells. *Biol. Reprod.* 66, 1853–1861. doi: 10.1095/biolreprod66.6.1853

Black, M. W., and Boothroyd, J. C. (2000). Lytic cycle of *Toxoplasma gondii*. *Microbiol. Mol. Biol. Rev.* 64, 607–623. doi: 10.1128/mmbr.64.3.607-623.2000

Braun, L., Brenier-Pinchart, M. P., Yogavel, M., Curt-Varesano, A., Curt-Bertini, R. L., Hussain, T., et al. (2013). A *Toxoplasma* dense granule protein, GRA24, modulates the early immune response to infection by promoting a direct and sustained host p38 MAPK activation. *J. Exp. Med.* 210, 2071–2086. doi: 10.1084/jem.20130103

Brown, M. B., von Chamier, M., Allam, A. B., and Reyes, L. (2014). M1/M2 macrophage polarity in normal and complicated pregnancy. *Front. Immunol.* 5:606. doi: 10.3389/fimmu.2014.00606

Butcher, B. A., Fox, B. A., Rommereim, L. M., Kim, S. G., Maurer, K. J., Yarovinsky, F., et al. (2011). *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. *PLoS Pathog.* 7:e1002236. doi: 10.1371/journal.ppat.1002236

Co, E. C., Gormley, M., Kapidzic, M., Rosen, D. B., Scott, M. A., Stolp, H. A., et al. (2013). Maternal decidual macrophages inhibit NK cell killing of invasive cytotrophoblasts during human pregnancy. *Biol. Reprod.* 88:155. doi: 10.1095/biolreprod.112.099465

Courret, N., Darche, S., Sonigo, P., Milon, G., Buzoni-Gatel, D., and Tardieux, I. (2006). CD11c- and CD11b-expressing mouse leukocytes transport single *Toxoplasma gondii* tachyzoites to the brain. *Blood* 107, 309–316. doi: 10.1182/blood-2005-02-0666

Dubey, J. P. (2009). Toxoplasmosis in sheep–the last 20 years. *Vet. Parasitol.* 163, 1–14. doi: 10.1016/j.vetpar.2009.02.026

Fuentes, I., Rubio, J. M., Ramirez, C., and Alvar, J. (2001). Genotypic characterization of *Toxoplasma gondii* strains associated with human toxoplasmosis in Spain: direct analysis from clinical samples. *J. Clin. Microbiol.* 39, 1566–1570. doi: 10.1128/JCM.39.4.1566-1570.2001

Gaynor, L. M., and Colucci, F. (2017). Uterine natural killer cells: functional distinctions and influence on pregnancy in humans and mice. *Front. Immunol.* 8:467. doi: 10.3389/fimmu.2017.00467

Hakimi, M. A., Olias, P., and Sibley, L. D. (2017). *Toxoplasma* effectors targeting host signaling and transcription. *Clin. Microbiol. Rev.* 30, 615–645. doi: 10.1128/CMR.00005-17

Hide, G., Morley, E. K., Hughes, J. M., Gerwash, O., Elmahaishi, M. S., Elmahaishi, K. H., et al. (2009). Evidence for high levels of vertical transmission in *Toxoplasma gondii*. *Parasitology* 136, 1877–1885. doi: 10.1017/S0031182009990941

Hunter, C. A., and Sibley, L. D. (2012). Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. *Nat. Rev. Microbiol.* 10, 766–778. doi: 10.1038/nrmicro2858

Jensen, K. D., Wang, Y., Wojno, E. D., Shastri, A. J., Hu, K., Cornel, L., et al. (2011). *Toxoplasma* polymorphic effectors determine macrophage polarization

and intestinal inflammation. *Cell Host Microbe* 9, 472–483. doi: 10.1016/j.chom.2011.04.015

Kalkunte, S., Chichester, C. O., Gotsch, F., Sentman, C. L., Romero, R., and Sharma, S. (2008). Evolution of non-cytotoxic uterine natural killer cells. *Am. J. Reprod. Immunol.* 59, 425–432. doi: 10.1111/j.1600-0897.2008.00595.x

Kim, L., Del Rio, L., Butcher, B. A., Mogensen, T. H., Paludan, S. R., Flavell, R. A., et al. (2005). p38 MAPK autophosphorylation drives macrophage IL-12 production during intracellular infection. *J. Immunol.* 174, 4178–4184. doi: 10.4049/jimmunol.174.7.4178

Lao, K., Zhao, M., Li, Z., Liu, X., Zhang, H., Jiang, Y., et al. (2015). IL-10 regulate decidual Tregs apoptosis contributing to the abnormal pregnancy with *Toxoplasma gondii* infection. *Microb. Pathog.* 89, 210–216. doi: 10.1016/j.micpath.2015.10.002

Lehmann, T., Marcet, P. L., Graham, D. H., Dahl, E. R., and Dubey, J. P. (2006). Globalization and the population structure of *Toxoplasma gondii*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11423–11428. doi: 10.1073/pnas.0601438103

Leng, J., Butcher, B. A., and Denkers, E. Y. (2009). Dysregulation of macrophage signal transduction by *Toxoplasma gondii*: past progress and recent advances. *Parasite Immunol.* 31, 717–728. doi: 10.1111/j.1365-3024.2009.01122.x

Li, Z., Zhao, M., Li, T., Zheng, J., Liu, X., Jiang, Y., et al. (2017). Decidual macrophage functional polarization during abnormal pregnancy due to *Toxoplasma gondii*: role for LILRB4. *Front. Immunol.* 8:1013. doi: 10.3389/fimmu.2017.01013

Lidstrom, C., Matthiesen, L., Berg, G., Sharma, S., Ernerudh, J., and Ekerfelt, C. (2003). Cytokine secretion patterns of NK cells and macrophages in early human pregnancy decidua and blood: implications for suppressor macrophages in decidua. *Am. J. Reprod. Immunol.* 50, 444–452. doi: 10.1046/j.8755-8920.2003.00112.x

Liu, T., Zhang, Q., Liu, L., Xu, X., Chen, H., Wang, H., et al. (2013). Trophoblast apoptosis through polarization of macrophages induced by Chinese *Toxoplasma gondii* isolates with different virulence in pregnant mice. *Parasitol Res.* 112, 3019–3027. doi: 10.1007/s00436-013-3475-3

Liu, X., Jiang, M., Ren, L., Zhang, A., Zhao, M., Zhang, H., et al. (2018). Decidual macrophage M1 polarization contributes to adverse pregnancy induced by *Toxoplasma gondii* PRU strain infection. *Microb. Pathog.* 124, 183–190. doi: 10.1016/j.micpath.2018.08.043

Liu, Y., Zhao, M., Xu, X., Liu, X., Zhang, H., Jiang, Y., et al. (2014). Adoptive transfer of Treg cells counters adverse effects of *Toxoplasma gondii* infection on pregnancy. *J. Infect. Dis.* 210, 1435–1443. doi: 10.1093/infdis/jiu265

Luma, H. N., Tchaleu, B. C., Temfack, E., Doualla, M. S., Ndenga, D. P., Mapoure, Y. N., et al. (2013). HIV-associated central nervous system disease in patients admitted at the Douala General Hospital between 2004 and 2009: a retrospective study. *AIDS Res. Treat.* 2013:709810. doi: 10.1155/2013/709810

Melo, M. B., Jensen, K. D., and Saeij, J. P. (2011). *Toxoplasma gondii* effectors are master regulators of the inflammatory response. *Trends Parasitol.* 27, 487–495. doi: 10.1016/j.pt.2011.08.001

Montoya, J. G., and Liesenfeld, O. (2004). Toxoplasmosis. *Lancet* 363, 1965–1976. doi: 10.1016/S0140-6736(04)16412-X

Montoya, J. G., and Remington, J. S. (2008). Management of *Toxoplasma gondii* infection during pregnancy. *Clin. Infect. Dis.* 47, 554–566. doi: 10.1086/590149

Mor, G., and Abrahams, V. M. (2003). Potential role of macrophages as immunoregulators of pregnancy. *Reprod. Biol. Endocrinol.* 1:119. doi: 10.1186/1477-7827-1-119

Nagamatsu, T., and Schust, D. J. (2010). The contribution of macrophages to normal and pathological pregnancies. *Am. J. Reprod. Immunol.* 63, 460–471. doi: 10.1111/j.1600-0897.2010.00813.x

Neale, D., Demasio, K., Illuzi, J., Chaiworapongsa, T., Romero, R., and Mor, G. (2003). Maternal serum of women with pre-eclampsia reduces trophoblast cell viability: evidence for an increased sensitivity to Fas-mediated apoptosis. *J. Matern. Fetal Neonatal Med.* 13, 39–44. doi: 10.1080/jmf.13.1.39.44

Nissapatorn, V. (2009). Toxoplasmosis in HIV/AIDS: a living legacy. *Southeast Asian J. Trop. Med. Public Health* 40, 1158–1178.

Pfaff, A. W., Abou-Bacar, A., Letscher-Bru, V., Villard, O., Senegas, A., Mousli, M., et al. (2007). Cellular and molecular physiopathology of congenital toxoplasmosis: the dual role of IFN-gamma. *Parasitology* 134, 1895–1902. doi: 10.1017/S0031182007000200

Rajendran, C., Su, C., and Dubey, J. P. (2012). Molecular genotyping of *Toxoplasma gondii* from Central and South America revealed high diversity within and between populations. *Infect. Genet. Evol.* 12, 359–368. doi: 10.1016/j.meegid.2011.12.010

Reister, F., Frank, H. G., Kingdom, J. C., Heyl, W., Kaufmann, P., Rath, W., et al. (2001). Macrophage-induced apoptosis limits endovascular trophoblast invasion in the uterine wall of preeclamptic women. *Lab. Invest.* 81, 1143–1152. doi: 10.1038/labinvest.3780326

Renaud, S. J., and Graham, C. H. (2008). The role of macrophages in utero-placental interactions during normal and pathological pregnancy. *Immunol. Invest.* 37, 535–564. doi: 10.1080/08820130802191375

Robbins, J. R., Zeldovich, V. B., Poukchanski, A., Boothroyd, J. C., and Bakardjiev, A. I. (2012). Tissue barriers of the human placenta to infection with *Toxoplasma gondii*. *Infect. Immun.* 80, 418–428. doi: 10.1128/IAI.05899-11

Rochet, E., Argy, N., Greigert, V., Brunet, J., Sabou, M., Marcellin, L., et al. (2019). Type I ROP16 regulates retinal inflammatory responses during ocular toxoplasmosis. *PLoS One* 14:e0214310. doi: 10.1371/journal.pone.0214310

Rosowski, E. E., Lu, D., Julien, L., Rodda, L., Gaiser, R. A., Jensen, K. D., et al. (2011). Strain-specific activation of the NF-kappaB pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein. *J. Exp. Med.* 208, 195–212. doi: 10.1084/jem.20100717

Saeij, J. P., Boyle, J. P., Coller, S., Taylor, S., Sibley, L. D., Brooke-Powell, E. T., et al. (2006). Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science* 314, 1780–1783. doi: 10.1126/science.1133690

Schmidt, M., Sonneville, R., Schnell, D., Bige, N., Hamidfar, R., Mongardon, N., et al. (2013). Clinical features and outcomes in patients with disseminated toxoplasmosis admitted to intensive care: a multicenter study. *Clin. Infect. Dis.* 57, 1535–1541. doi: 10.1093/cid/cit557

Shwab, E. K., Zhu, X. Q., Majumdar, D., Pena, H. F., Gennari, S. M., Dubey, J. P., et al. (2014). Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. *Parasitology* 141, 453–461. doi: 10.1017/S0031182013001844

Wang, C., Cheng, W., Yu, Q., Xing, T., Chen, S., Liu, L., et al. (2018). *Toxoplasma* Chinese 1 strain of WH3Δrop16I/III/ gra15II genetic background contributes to abnormal pregnant outcomes in murine model. *Front. Immunol.* 9:1222. doi: 10.3389/fimmu.2018.01222

Wang, L., Chen, H., Liu, D., Huo, X., Gao, J., Song, X., et al. (2013). Genotypes andmouse virulence of *Toxoplasma gondii* isolates from animals and humans in China. *PLoS One* 8:e53483. doi: 10.1371/journal.pone.0053483

Wujcicka, W., Wilczyński, J., and Nowakowska, D. (2014). Do the placental barrier, parasite genotype and Toll-like receptor polymorphisms contribute to the course of primary infection with various *Toxoplasma gondii* genotypes in pregnant women? *Eur. J. Clin. Microbiol. Infect. Dis*. 33, 703–709. doi: 10.1007/s10096-013-2017-3

Xu, X., He, L., Zhang, A., Li, Q., Hu, W., Chen, H., et al. (2015). *Toxoplasma gondii* isolate with genotype Chinese 1 triggers trophoblast apoptosis through oxidative stress and mitochondrial dysfunction in mice. *Exp. Parasitol.* 154, 51–61. doi: 10.1016/j.exppara.2015.04.008

Yamamoto, M., Standley, D. M., Takashima, S., Saiga, H., Okuyama, M., Kayama, H., et al. (2009). A single polymorphic amino acid on *Toxoplasma gondii* kinase ROP16 determines the direct and strain-specific activation of Stat3. *J. Exp. Med.* 206, 2747–2760. doi: 10.1084/jem.20091703

Yarovinsky, F. (2014). Innate immunity to *Toxoplasma gondii* infection. *Nat. Rev. Immunol.* 14, 109–121. doi: 10.1038/nri3598

Zhang, H., Hu, X., Liu, X., Zhang, R., Fu, Q., and Xu, X. (2012). The Treg/Th17 imbalance in *Toxoplasma gondii*-infected pregnant mice. *Am. J. Reprod. Immunol.* 67, 112–121. doi: 10.1111/j.1600-0897.2011.01065.x

Zhao, M., Zhang, H., Liu, X., Jiang, Y., Ren, L., and Hu, X. (2017). The effect of TGF-beta on Treg Cells in adverse pregnancy outcome upon *Toxoplasma gondii* infection. *Front. Microbiol.* 8:901. doi: 10.3389/fmicb.2017.00901

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frontiers in Immunology

REVIEW
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The Interplay Between Reproductive Tract Microbiota and Immunological System in Human Reproduction

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In the last decade, the microbiota, i.e., combined populations of microorganisms living inside and on the surface of the human body, has increasingly attracted attention of researchers in the medical field. Indeed, since the completion of the Human Microbiome Project, insight and interest in the role of microbiota in health and disease, also through study of its combined genomes, the microbiome, has been steadily expanding. One less explored field of microbiome research has been the female reproductive tract. Research mainly from the past decade suggests that microbial communities residing in the reproductive tract represent a large proportion of the female microbial network and appear to be involved in reproductive failure and pregnancy complications. Microbiome research is facing technological and methodological challenges, as detection techniques and analysis methods are far from being standardized. A further hurdle is understanding the complex host-microbiota interaction and the confounding effect of a multitude of constitutional and environmental factors. A key regulator of this interaction is the maternal immune system that, during the peri-conceptional stage and even more so during pregnancy, undergoes considerable modulation. This review aims to summarize the current literature on reproductive tract microbiota describing the composition of microbiota in different anatomical locations (vagina, cervix, endometrium, and placenta). We also discuss putative mechanisms of interaction between such microbial communities and various aspects of the immune system, with a focus on the characteristic immunological changes during normal pregnancy. Furthermore, we discuss how abnormal microbiota composition, "dysbiosis," is linked to a spectrum of clinical disorders related to the female reproductive system and how the maternal immune system is involved. Finally, based on the data presented in this review, the future perspectives in diagnostic approaches, research directions and therapeutic opportunities are explored.

Keywords: microbiota, immunology, female reproductive tract, vaginal, endometrial, placental, preterm birth, pregnancy complications

INTRODUCTION

Impact of Microbiome Research on Health Care

The human microbiome represents the collection of microbes living inside and on the surface of human body. Research efforts by the scientific community worldwide are increasingly focused on understanding the role of microbiota in health and disease. Since the completion of the Human Microbiome Project (HMP), and publication of the characteristics and functions of human microbiota located in different body habitats, this field of science has gained momentum (1). Current microbiome research tries to fill in the missing details in the pathophysiology and explain the seemingly random variation in disease severity and phenotype of confounding factors such as ethnicity, geographical location and societal habits. Due to advances in microbiome research, scientists have obtained valuable insight in many complex disorders such as obesity, cancer and inflammatory bowel disease, and a similar trend is observed in female reproductive tract in both physiological and pathological states (2).

Global Burden of Pregnancy Complications

Because of the invasive nature of sampling methods, microorganisms populating the female reproductive tract remain less explored compared to microbiota populating the intestines; nonetheless, they represent an appreciable proportion (around 9%) of the female microbial network (3). Furthermore, disrupted female reproductive tract microbial communities have been implicated in reproductive and pregnancy complications, as reviewed in (4, 5). Reproductive and pregnancy complications are of global health interest, and comprise diverse health problems that occur prior to conception and during gestation. Notably, these involve the mother's health, the baby's health or both. Such problems may entail difficulties to conceive, or arise throughout gestation and span from the inability to maintain pregnancy in the first weeks of gestation, to its early termination in the third trimester. An increasing body of evidence associates microorganisms (including mutualistic) to the onset of reproductive health and maternal-fetal conditions, and to some of the major obstetrical syndromes, including premature birth, premature rupture of the membranes, premature labor, intrauterine growth restriction, and stillbirth (6).

The inability to conceive is an often neglected health concern affecting individuals around the globe. In 2010, an estimated 48.5 million couples worldwide were infertile, with little changes over the previous two decades (7). Early pregnancy loss (frequently before 13 weeks of gestation) is estimated to occur in 15–20% of recognized pregnancies, without major geographical differences (8–10). Of note, morbidity and pregnancy complications at more advanced stages of gestation (e.g., preeclampsia, preterm labor and stillbirth) often have a higher burden in low-resources settings, especially in south Asia and sub-Saharan Africa (11). Among such late complications, preterm birth remains the leading cause of worldwide neonatal morbidity and mortality: approximately 10.6% of all live births in 2014 were preterm, 80% of which occurred in Asian and sub-Saharan African countries (12).

Current Gaps in Knowledge

Over recent years technological advancements, in particular sequencing-based methods for bacterial detection (metagenomics and 16S rRNA gene amplicon sequencing), have greatly expanded the literature on diverse microbiota colonizing the female reproductive tract both in healthy and disease states (5, 13). However, the wide spread application of sequencing-based methods incorporates a potential for false-positive results (i.e., low specificity) due to contamination as in case of the placenta (14). Our current knowledge is limited on how these microbiota interact with host cells, including local immune mediators, and whether this interaction is causally involved in the pathogenesis of pregnancy complications. It is well known that the mechanisms pivotal in regulating the establishment and maintenance of pregnancy, including epigenetic regulation and immune adaptation, are directly affected by local microorganisms (5, 13). Furthermore, the impact of maternal factors such as BMI, pre-existing disorders or life style habits (e.g., diet and nutrition) on this host-microbiota interaction need to be integrated and thoroughly studied in future studies (15). Understanding the interplay at the fetomaternal interface is essential for developing predictive human biomarkers for implantation and placentation and is a key step toward designing novel therapeutic approaches.

Aims

This review aims to present an account of currently available literature on reproductive tract microbiota, describing the composition and function of microbiota and their links to pregnancy complications. The potential mechanisms of interaction between microbes and the immune system are discussed with respect to specific locations, focusing on the unique changes that characterize physiological pregnancy. Furthermore, the clinical impact of abnormal microbiota composition, i.e., "dysbiosis," on female reproductive biology, from the pre-conceptional stage throughout early and late pregnancy. Common methodological challenges confronting research in the field related to study design and technical issues of sample collection and assay standardization are highlighted. Finally, we present options with respect to translation of various aspects and insights to future therapeutic approaches in reproductive medicine.

COMPOSITION OF REPRODUCTIVE TRACT MICROBIOME IN RELATION TO PREGNANCY

Vaginal Microbiota

The community of microbes in the female lower genital tract plays a fundamental role in the promotion of homeostasis and in the prevention of colonization by pathogenic microorganisms. Compared to other sites, the vagina appears to harbor particularly simple microbial communities of low diversity (1). Although

relatively simple at the genus-level, the diversity of *Lactobacilli* in the vaginal space is nonetheless higher than at other body sites (1). Many studies among non-pregnant women of reproductive age report that *Lactobacillus* spp. is the predominant species in the vagina, although the possibility of normal vaginal microbiota dominated by bacteria other than Lactobacilli seems to be plausible [as reviewed in (16)]. Current evidence suggests that the vaginal bacterial community is in a state of dynamic equilibrium (17). The composition of the vaginal microbiota correlates with the most dominant bacterial community composition (i.e., community state type; CST) across time (17). This notion appears to be in line with the concept of community resilience. Community resilience suggests that the ability of a microbial ecosystem to mitigate composition changes depends on the presence of beneficial species with stabilizing roles. For instance, *Lactobacillus crispatus*-dominated communities are less likely to transition to non-beneficial CST, than communities dominated by other *Lactobacillus* spp., like *L. iners* (17, 18).

In non-pregnant women of reproductive age, transient variations in the vaginal microbiota's dynamic equilibrium are the results of physiological changes in response to hormones during the menstrual cycle, or human activities (e.g., sexual intercourse and hygienic practices) (18). In addition, other constitutional and environmental factors, including age, ethnicity, geographical variation and sexual habits influence the bacterial communities detected in the lower urogenital tract (19–21). For example, Lactobacilli-dominated vaginal microbiota has been shown to be less prevalent among non-Caucasian women in several studies (22–24), though, when present, their beneficial role seems maintained. A well-known study performed in North America characterized the vaginal microbiota of women of reproductive age from four ethnic groups (Caucasian, African-American, Hispanic and Asian): five groups of microbial communities, called Community State Types (CSTs) were identified. Whereas CST-IV was the most diverse CST and was also associated with a higher local pH, the remaining four (CST-I, CST-II, CST-III, and CST-V) were dominated by Lactobacilli (20). Lactobacilli thrive in anaerobic environments and produce lactic acid, therefore contributing to the acidic vaginal environment. Several studies have reported how depletion of these microorganisms often leads to vaginal dysbiosis, which is occasionally symptomatic, and at times associated with several important reproductive complications (25, 26).

The microbiota residing in the cervix have been sparsely studied as an independent entity. Current evidence suggests a strong similarity between bacterial communities in the cervix and in the vaginal area, suggesting ascending bacterial colonization from the vagina to the cervix (27). Such microbial communities are sometimes jointly referred to as cervicovaginal microbiota (28).

While in non-pregnant reproductive age women the vaginal microbiota is relatively dynamic, in healthy pregnancies it is characterized by an increase in stability (29). This is one of the physiological changes taking place in response to gestation. Other changes in this microbial community located in the vaginal area are an overall decrease in richness (number of different species present) and diversity (of the microbial ecosystem, i.e., the relative abundance of species). Overall, abundance of *Lactobacillus* spp. appears to be higher in healthy pregnant women, than in women with complicated pregnancies; whereas *Mycoplasma* and *Ureaplasma* appear to be lower (30). Most of the studies in the field seem to agree that Lactobacilli, in particular *L. crispatus*, *L. iners*, *L. jenseii*, and *L. gasseri* are the dominant bacteria detected in the vaginal microbiota of pregnant women (29, 31, 32).

Some of the factors influencing vaginal microbiota's diversity during pregnancy are: gestational age (32), previous pregnancy history (33) and ethnicity (31, 34). In particular, *L. crispatus* was the most dominant species in a Caucasian cohort (35), *L. iners* in an African American one (29) and, surprisingly, *L. acidophilus* in a mostly Hispanic population where *L. crispatus* was not detected, even though tested for (36). Although ethnic and geographical differences in vaginal microbiota are not yet understood, nor consistently observed (32), a combination of colonization by gut microbes, hygienic and sexual practices, and host genetics may contribute to underlying mechanisms (37).

As pregnancy progresses, the vaginal microbiota changes with an increase in the relative abundance of Lactobacilli and decrease in anaerobe or strict-anaerobe species, until around 36 weeks of gestation, when the number of species increases significantly (31). Such composition has been reported to resemble that of the vaginal microbiota in the non-pregnant state (34). Throughout pregnancy, some species of Lactobacilli associate with "normal" (i.e., healthy) vaginal microbiota, whereas the presence of other species reflect an abnormal, less beneficial microbial community. The former has been reported for *L. crispatus*, whereas the latter for *L. gasseri* and *L. iners* (35). Finally, reported changes in the vaginal microbiota after delivery include a decrease in *Lactobacillus* species and an increase in anaerobe ones (38), irrespective of the vaginal communities during pregnancy and independent of ethnicity (39).

It is becoming increasingly apparent from studies in the field that, besides investigation of the vaginal microbiota as an ecological community (defined by its most abundant species), individual (even low abundant) species with less beneficial roles should be considered, as their mere presence might be sufficiently detrimental to a healthy microbiotal equilibrium.

Endometrial Microbiota

Unlike the vagina, the endometrium has not been extensively studied as a site of commensal bacterial colonization, in part likely due to the relatively limited access to uncontaminated samples. Historically, the uterus has been considered sterile (40), and presence of bacteria in endometrial, placental or amniotic fluid samples was viewed as pathological. However, with the advent of culture-independent techniques, recently compiled data support both the prevalence and variation of bacterial communities in the endometrium and their possible role in reproductive health (41) but is still under debate (14). Together, much of our knowledge comes from the interpretation and comparison of the role of microbiome in anatomically related sites.

In healthy non-pregnant women, the endometrium appears to harbor a unique, low-biomass microbiota, dominated by a

few bacterial species including *Bacteroidetes* (*Flavobacterium* spp.) and *Firmicutes* (*Lactobacillus* spp.) (5, 41, 42). Compared to the vaginal microbiome, the endometrium harbors a significantly lower quantity of microbes, suggesting that the cervix and/or uterine environment serve as a barrier for ascending microorganisms (42). Since Lactobacilli and Streptococci represent the most dominant bacteria in the vagina and in the cervix, respectively, the occurrence of these species in the uterine cavity may indicate contamination during sample collection. Therefore, the use of paired samples from vagina, cervix and endometrial fluid has been proposed in order to facilitate interpretation (43).

A recent review reported that, in studies using culture-dependent techniques, no bacterial family was reported more than once in uterine or endometrial samples, indicating one of the challenges in determining the complexity of "normal" reference microbiota of the uterine cavity in this manner. However, in culture-independent analyses, *Lactobacilli*, *Bifidobacteriaceae*, *Comamonadaceae*, and *Streptococcaceae* were reported more than once in the uterine cavity, suggesting that the culture-independent technology approximate microbiotal complexity more truthfully (41).

Placental Microbiota

The notion that the uterus represents a sterile environment during pregnancy was first challenged with the advent of the Human Microbiome Project (HMP) (1) and other studies (44). The detection of unique bacterial DNA profiles in human placenta supported the idea that a rich microbial community normally exists *in utero*. Surprisingly, these early data suggested that the placental microbiome resembles the oral cavity microbiome rather than that of adjacent sites, e.g., the vagina. Since these early reports, several reservations on this notion and its implications on pregnancy complications have been voiced, noting that the low-biomass that bacterial DNA in the placenta represents, is sensitive to capturing background contamination (from DNA extraction kits, polymerase chain reaction reagents, and laboratory environments) (45, 46). Recent meticulous analyses of metagenomic DNA, consistently found no significant differences in the abundance and/or presence of a microbiota between placental tissue (term women without labor) and background technical controls (14, 47). The proposed link between oral dysbiosis and pregnancy complications puts the debate about placental microbiota in the focus: clinical studies on the association between gingivitis and PTB have reported bacteria in the very old structures of the placenta (48). In the context of placental microbiome analysis, the pitfalls associated with technology of choice, in addition to the methodological problems, needs careful consideration (see section "Discussion," diagnostic challenges).

MATERNAL IMMUNE RESPONSE IN PREGNANCY

During normal pregnancy, the immune system of the female reproductive tract is uniquely challenged by the fact that it has to protect against invading pathogens while simultaneously tolerating and supporting implantation and growth of the semi-allogenic fetus in a tightly regulated process (49). The implantation phase is characterized by low grade pro-inflammatory immune reactivity, including the production of major cytokines IL-6, IL-8, and tumor necrosis factor (TNF)-α. This response is believed to support local repair of endometrial injury and removal of cellular debris during trophoblast invasion and implantation. The placentation phase is predominantly an anti-inflammatory state, which is needed to ascertain tolerance of maternal immune cells to paternal antigens expressed by placental trophoblasts for the fetus and, at later stages, *vice versa*. The final parturition phase again requires controlled pro-inflammatory immune reactivation to trigger labor, delivery and placental rejection (50). Dysregulation of this tight immunological balance, based either in the (immuno)genetic constitution of the parents or the (local) uterine environment, may underlie several pregnancy complications. The homeostasis of the female reproductive tract during pregnancy depends on the interactive protective roles of epithelial defenses and immune cells. A broad variety of innate-(predominantly macrophages, dendritic cells and innate lymphoid cells) and adaptive immune cells (especially T cells) can be found each in different anatomic compartments with their own unique and specialized role. This section summarizes key immune players involved in immuno-modulatory events that support normal pregnancy, both the genetic and environmental aspects will be discussed in successive paragraphs. The maternal immune regulation in pregnancy has been extensively described (51) and is beyond the scope of this review.

Epithelial Defenses

Like in many other mucosal tissues, the female reproductive tract's first line of defense against pathogens is a physical barrier that, among other things, consists of a mucous layer, IgA antibodies and commensal bacteria limiting colonization by pathogenic bacteria. In addition, epithelial cells lining the female reproductive tract physically block pathogen invasion and produce protective molecules like antimicrobial peptides (AMPs). AMPs are multifunctional molecules with important roles in direct microbial killing, protection against proteolytic enzymes from various pathogens including bacteria, fungi, and some viruses, and modulation of both innate and adaptive immune responses (52, 53). AMPs are produced either constitutively or after induction by inflammatory stimuli, and are present on the mucosal surface, in decidual stroma, in endometrial fluid and even in amniotic fluid (54). In humans, AMPs have been linked to key regulatory processes in implantation and are implicated in the pathogenesis of various pregnancy complications (52, 55). An example of an AMP found in the uterus is the secretory leukocyte protease inhibitor (SLPI), which has antiviral and antifungal properties, and acts as a bactericidal against gram negative as well as gram-positive bacteria such as *Escherichia coli* and *Staphylococcus aureus* (56).

Uterine Natural Killer Cells

Natural Killer (NK) cells are a critical component of the innate immune system and comprise up to 70% of all endometrial leukocytes during the secretory phase of the menstrual cycle and in early pregnancy, but decline in numbers by mid-gestation (49). The main function NK cells in the vagina is to provide protection against a broad variety of viruses. Like their blood counterparts, vaginal NK cells recognize virus- or stress-associated molecules and they destroy infected cells through the release of toxic granules containing granzymes and perforins or though interaction with death receptors (57). The exact function of uterine NK cells (uNK) is not completely clear as they clearly differ from NK cells in peripheral blood in surface markers and cytokine repertoire. uNK cells are poorly cytotoxic, but are a potent source of cytokines such as Interferon (IFN)γ, TNF-α, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Interleukin (IL)-10 as well as proangiogenic factors like vascular endothelial growth factor (VEGF) (58–60). Studies using genetic mouse models lacking uNK cells provided histological evidence of failed trophoblast invasion and defective spiral artery remodeling and highlighted the critical role for uNK in for normal placentation (61, 62).

Macrophages and Dendritic Cells

Myeloid cells (macrophages and dendritic cells) represent the second most abundant immune cell subset in the endometrium and account for 10–20% of the decidual leukocyte population (49). They are responsible for the surveillance and scavenging of bacteria present on mucosal surfaces and act as antigen presenting cells (APCs) (58, 63, 64). APCs are equipped with pattern recognition receptors (PRR), such as Toll-like receptors (TLRs), allowing them to recognize the so-called pathogen-associated molecular patterns (PAMPs). PAMPs are species-specific and include molecules from the microbial cell wall (e.g., peptidoglycan) and cell membranes (e.g., LPS) or virus-derived single stranded DNA or double stranded RNA. PAMPs promote the production of cytokines and cell adhesion molecules that lead to the recruitment other immune cells such as neutrophils and NK cells. In addition, APCs have a major function in instructing the adaptive immune response by virtue of expressing major histocompatibility complex (MHC) class II receptors. In contrast to uNK cells, the numbers of decidual macrophages remain relatively constant throughout gestation, however, the diverse repertoire of macrophages in cytokines production, in regulation of T cell responses and in tissue repair suggest an important role in decidualization (49, 59). Together with the uNK cells, uterine macrophages are also postulated to facilitate angiogenesis during placentation and more importantly, spiral artery remodeling by production of growth factors and clearance of cell debris (60, 65).

Uterine dendritic cells (uDC) have a tolerogenic phenotype and both uDC and uterine macrophages produce IL-10, TGF-β, and indolomine2,3 (IDO) contributing to a tolerogenic and receptive micro-environment (66). In addition, uDC have been shown to interact in a bidirectional manner with uNK cells in mouse models, as well as *in vitro* human studies (67, 68). As a subclass of APC, uDC promote uNK differentiation and activation in a contact-dependent manner and via the production of IL-15 (69).

T Lymphocytes

Adaptive immune cells (B- and T lymphocytes) provide highly specific and long-lasting cellular and humoral immunity against pathogens. While B cells are relatively infrequent in the female reproductive tract, T cells can be consistently found in both the vaginal compartment as well as in the uterus, albeit, in the uterus, their number and phenotype highly vary depending on stage of the menstrual cycle and pregnancy. CD8+ (cytotoxic) T cells represent the most abundant adaptive lymphocyte subset in the pregnant uterus (70). CD4+ (helper) T cells are less abundant, however, important in production of cytokines and interaction with other immune cells upon activation by APC (71). Traditionally, pregnancy was considered an immune privileged state by the different cytokines produced from a dominant T-cell phenotype (Th2) involved in immune tolerance, over another phenotype (Th1) involved in immune rejection. The dominance of Th1 polarized T cells was considered detrimental to embryo implantation and was associated with obstetric complications mainly preeclampsia (72). More recent studies show that the Th1/Th2 paradigm is not inclusive enough and that fetal tolerance is a complex process involving more specialized T cell subtypes, such as Th17 and regulatory T (Treg) cells (51). In humans, Treg cells have been shown to migrate from peripheral blood to the decidua (73), and their levels peak during the second trimester of pregnancy (74). They produce IL-10, leukemia inhibitory factor (LIF), transforming growth factor (TGF)-β, and heme oxygenase 1 (HO-1) contributing to fetal-maternal immune tolerance (75). It is clear that tight regulation of T cell activation and polarization is essential to balance the protection from pathogens, mediated by Th1/Th17 CD4+ T cells and CD8+ (cytotoxic) T cells *versus* tolerance to paternal antigens expressed by fetal cells, mediated by Th2 and Treg cells (76).

The induction of regulatory T cells (suppressor T cells/Treg) is favored over pro-inflammatory Th17 cells through interaction of uDC and uNK cells (77), corroborating the importance of intricate immunological instruction in acquisition of tolerance during implantation. Both macrophages and uDCs can be activated by encountering pathogens in the endometrium and start the process of phagocytosis, internalization, and degradation of the components of the antigen. They subsequently present these bacterial peptides to T-cells via MHC receptors, which activate the T-cells to initiate a cell-mediated and/or humoral immune response via MHC class II molecules (78).

Even though the intricate role of the immune system in pregnancy is not completely understood, collaborative action of innate- and adaptive immune cells appear to be critical for orchestration of the immunological changes required for successful fertilization, implantation and pregnancy.

INTERACTION BETWEEN MATERNAL IMMUNE SYSTEM AND REPRODUCTIVE TRACT MICROBIOTA

The interaction between microbiota and the immune system is a complex process that is crucial for maintaining normal homeostasis in organs, albeit under the influence of several constitutional and environmental factors. We hypothesize that under normal circumstances, a healthy lifestyle (including diet, physical and psychological aspects) would result in a normal reproductive tract microbiota "eubiosis," kept in check by a well-balanced immune regulation (**Figure 1**). Disturbance of this delicate balance could lead to either to inappropriate immune response and an exaggerated inflammatory reaction, or to downregulated immune response and dominance of pathogenic bacteria over normal commensals "dysbiosis". Evidence on the existence of such balance during in pregnancy is limited and hence, the data on host-microbiota interaction discussed in this section are largely derived from the non-pregnant population.

Host-Microbiota Interaction in the Vagina

Vaginal Protection by Lactobacilli

The vaginal mucosa is a barrier that provides protection against invading pathogens, as a result of the interaction between its epithelial cells, the immune system, and symbiotic microorganisms (79). The microbiota residing in the vaginal space are an active critical component in such defense system against infections. In particular, *Lactobacillus* spp. are thought to protect the upper genital tract from ascending infection, such as sexually transmitted ones (80). The main mechanism associated with the protective effect *Lactobacillus* spp. is the ability to produce lactic acid, thus maintaining a local pH of <4.5, deleterious to pathogens (81). Another defense mechanism is the Lactobacilli's production of bacteriocins, which directly inhibit or kill bacterial and viral pathogens (81). The ability to form micro-colonies that adhere to epithelial cells and prevent adhesion of pathogens is additional means of defense by vaginal microbiota, as is their ability to trigger the host's defense (81). *In vitro* studies have shown that certain *Lactobacillus* species are able to temper inflammation by, for example, a reduction of IL-6, IL-8, and TNF-α secretion after bacterial stimulation of toll-like receptors (TLRs) (82). The association between Lactobacilli-poor vaginal ecosystems and an increased risk of sexually transmitted infection is strong [as reviewed in (80, 83)]. Nonetheless, non-Lactobacilli-dominated vaginal microbiota occur in 25% of asymptomatic women, which challenges the notion of Lactobacilli as the sole microbial defense mediator (17, 84). Possible other explanations may be that maintenance of low pH in non-Lactobacilli-dominated vaginal microbiota is achieved in a different manner (18), or that not all suboptimal microbiota are manifested as symptomatic, whereas such CSTs may nevertheless correlate with increased risk for adverse reproductive health outcomes (85). Furthermore, as discussed before, distinct *Lactobacillus* species appear to differentially affect microbiota, e.g., with *L. iners* being more conductive to pathogen invasion than *L. crispatus* (25).

Hormonal Regulation of Host-Vaginal Microbiota Interactions

Most evidence and knowledge on the interactions between vaginal microbiota and host immune system comes from studies in non-pregnant women and should be extrapolated with caution to infer host-vaginal microbiota interactions in pregnancy. Key regulators of this interaction are sex hormones, which regulate the release of pro-inflammatory cytokines, chemokines and antimicrobial peptides, and contribute to the selection of vaginal microbial species [as reviewed in (86)]. In particular, estradiol has been implicated in the shift from a *Lactobacillus*-poor to a *Lactobacillus*-rich vaginal microbiota during puberty, as well as a reverse shift after menopause (86). Estrogen-induced glycogen synthesis in epithelial cells and production of glycogen-metabolites (maltose, maltotriose, α-dextrines) provides substrates for conversion to lactic acid by Lactobacilli (87–89). At reproductive age, a healthy vaginal microbiota was found to amplify the fluctuation in local immune responses in synchrony with hormonal changes during the menstrual cycle (90). In particular, women with a *Lactobacillus*-poor vaginal microbiota altered hormone-associated immune change may correlate with an increase susceptibility to infections (90). The close interplay between immune status and vaginal microbiota composition is further supported by the correlation of a less beneficial vaginal immune signature with ongoing HIV infection status in post-menopausal women (91).

Vaginal Immuno-Microbiotal Interactions

As an integral part of the defense mechanisms of the vaginal space, the local microbiota is required to interact with the host immune system. The ability of the host to protect against pathogenic microorganisms, but not react against the symbiotic microbes residing in the vagina, relies on the bi-directional relationship between immune system and microbiota (92). Such interplay helps maintain an immune-tolerant environment, more so during pregnancy. As a result of this symbiotic tolerance, bacterial communities thrive in the vaginal environment, contribute to local immune defense. Conversely, dysbiosis of the vaginal communities has been implicated in the disruption of the mucosal layer, decreasing the ability of the mucus and vaginal secretions to trap and inactivate pathogens. This might also facilitate the formation of epithelial entry portals for the same pathogens (93).

A possible mechanism for vaginal dysbiosis is the increased production of pro-inflammatory cytokines and chemokines, associated with the increase in pathogenic microbial diversity, which contributes to further recruitment of immune cells and amplification of the inflammatory response [reviewed in (86)]. Clinical studies performed on vaginal samples from sub-Saharan Africa, have shown a correlation between the presence of a non-*Lactobacillus* dominant microbiota and a rise in inflammatory cytokines and chemokines in the vagina (94, 95). Selected non-beneficial bacteria found in the vaginal tract induce pro-inflammatory cytokines and chemokines in *in vitro* co-cultures with vaginal epithelial cells. Relevantly, *L. crispatus*, the most well-known beneficial vaginal microorganism, does not induce inflammatory cytokine release in such settings [reviewed in

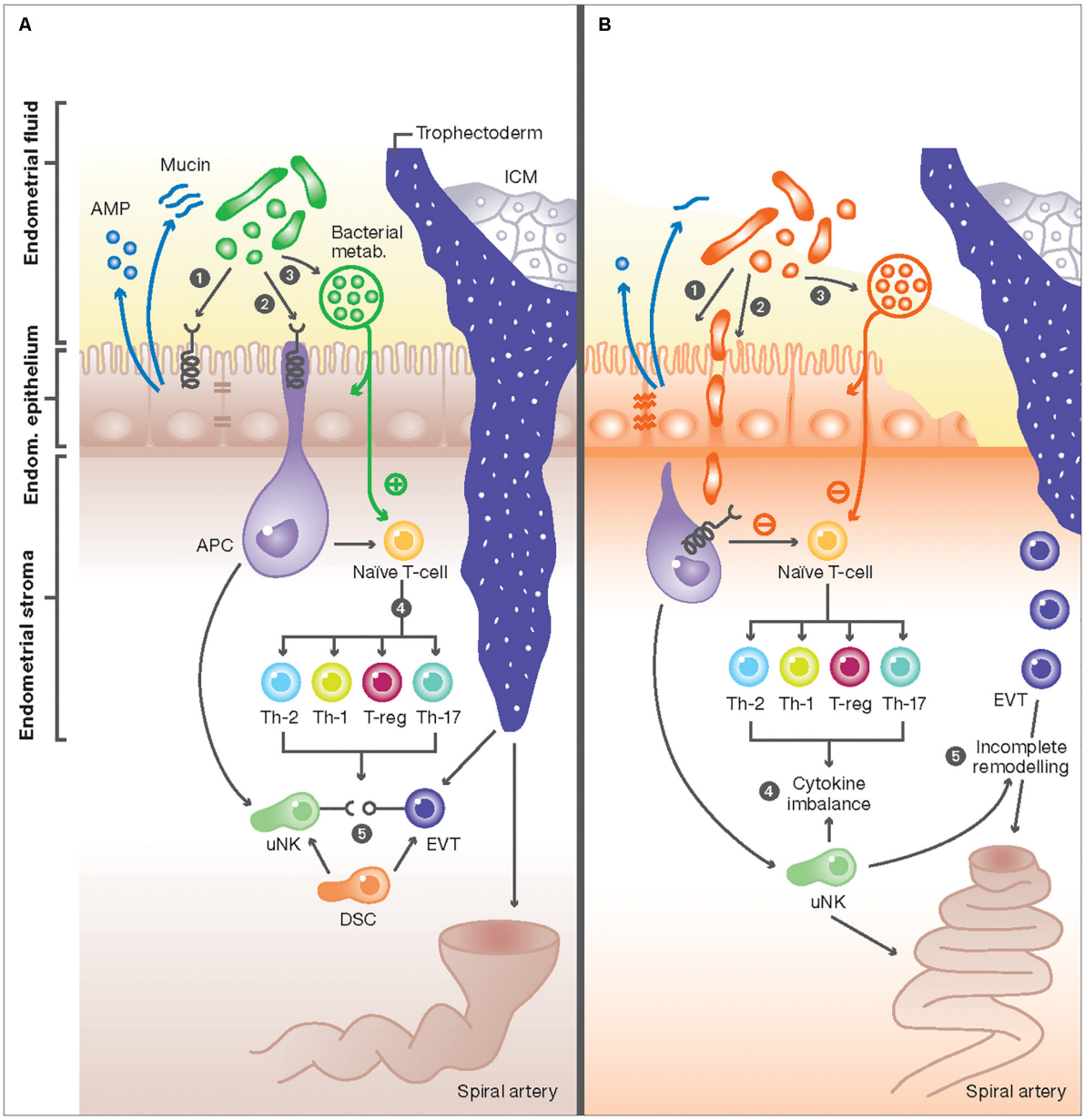

FIGURE 1 | The interaction between the endometrial microbiome and local immune mediators. **(A)** In healthy women, commensal bacterial communities interact with immune cells with at the feto-maternal interface through three potential mechanisms: (1) Commensal bacteria (green) maintain a healthy physical barrier by stimulating the production of different antimicrobial peptides (AMP) from endometrial cells and preserving epithelial tight junctions and stable mucus production. (2) Once encountered by immune cells in the endometrium, e.g., antigen presenting cells (APC), commensal bacteria trigger signal transduction via pattern recognition receptors (PRR) through their pathogen-associated molecular patterns (PAMPs). (3) Commensal bacteria can also produce metabolites, such as polysaccharides and short-chain fatty acids (SCFAs), that potentially affect immune responses in endometrial epithelial cells and T-cells, or alter the endometrial fluid pH to produce a competitive niche microenvironment against pathogenic bacteria. These mechanisms result in activation of uterine NK (uNK) cells and the development of specific subsets of T-cells, characterized by high number of regulatory T-cells (Treg), low number of Th-17, and a switch from the Th1 to Th2 cytokine production (4). The interaction of activated uNK cells (KIR receptors) with HLA-C and -G from extravillous trophoblasts (EVT) from the implanting embryo will also promote EVT invasion, stromal matrix degradation, angiogenesis and ultimately the remodeling of maternal spiral arteries (5). These adaptive changes ensure an immunotolerant milieu for the semi-allogenic fetus and are essential steps essential step in normal placentation. **(B)** Disturbance of the normal endometrial microbiome can negatively impact the implantation process: (1) First, the dominance of non-commensal bacterial communities could weaken the integrity of the endometrial mucosal barrier by affecting the epithelial tight junctions and reducing AMP and mucin secretion. (2) This in turn will further weaken host defense mechanisms and allow pathogens to enter the endometrial stroma and elicit a profound immune reaction from APC and other immune cells harboring pattern recognition receptors (PRR). (3) Aberrant stimulation of T-cells, either directly from invading pathogens breaching the mucosal barrier or indirectly from absorbed bacterial products results in disbalance in cytokine production in favor of the pro-inflammatory Th-1 types, predominated by TNF-a, IFN, IL-2, and IL-10 (4). Aberration in uNK cell maturation, either primary or secondary to shallow EVT invasion, is a possible link between disturbed endometrial microbiome and incomplete remodeling of maternal spiral arteries, characteristic of the great obstetric syndromes (5).

(82, 96)]. In accordance with this, another study had shown how the possible contribution of vaginal dysbiosis to infections of the urinary tract was mediated by defects of the immune response (97).

Healthy vaginal microbiota was associated, both *in vitro* and *in vivo,* with increased expression (mRNA and protein) of defensins, specific types of vaginal antimicrobial peptides (AMP) that prevent binding of pathogen-specific proteins to human cells. AMP levels were significantly lower in bacterial vaginosis conditions, *in vitro* and *in vivo* (98). The expression of other types of antimicrobial peptides, the secretory leukocyte protease inhibitor and the human epididymis protein 4, correlates with the presence of less beneficial vaginal microbes (96). The complement system was proposed as a key player in adverse pregnancy outcome, as female microbiota composition regulates complement function in the maternal vasculature (99). Complement dysregulation in the intrauterine space, promotes inflammation and triggers a cascade of physiological changes (cervical changes, degradation of collagen, uterine decidua activation and uterine contractility), which in turn increases risk for preterm delivery (97).

Such combined evidence suggests that the vaginal microbiota modulates the local immune system and inflammatory response at least in part through interaction with epithelial cells, thereby influencing the susceptibility to infection. Although interactions between the maternal immune system and vaginal microbiota appear to be complex and far from completely understood, based on the above outlined observations it was suggested that the overall microbiota composition, rather than any individual microbial population, underlies adverse interactions with the host-immune system in the female reproductive system (100).

Host-Microbiota Interaction During Implantation and Placentation

Successful implantation and subsequent formation of the placenta (placentation) encompass several steps ensuring tissue adhesion between fetal trophoblasts and maternal tissues and the adaptation of their blood vessels and to facilitate nutrient supply (101). These steps involve mechanisms such as angiogenesis (101), decidualization (102) and immune response adaptation (60). This immune adaptation is essential in pregnancy and is required in order to avoid a graft *vs.* host disease between the semi-allogenic trophoblast and maternal tissues, including immune cells, decidual microbiota and other decidual components such as epithelial and stromal cells and blood vessels. Not surprisingly, this complex interaction is postulated to affect subsequent stages of pregnancy, and is implicated in many pregnancy complications (103).

Published literature points to the existence of a diverse and metabolically active endometrial microbiota and predicts an important physiologically modulatory role of the main function of its host tissue: harboring and nurturing the developing embryo. In healthy women, the presence of commensal bacterial communities in the cycling endometrium mediate physiological responses from various cells at the feto-maternal interface, including epithelial and stromal cells in the endometrium, immune cells and trophoblasts from the implanting embryo. Although the exact molecular nature and extent of these interactions are not fully understood, evidence from *in vitro* experiments and animal models have provided important insights (13, 55, 104, 105). Based on currently available knowledge, we postulate the following mechanisms:

1) Commensal bacteria interact with endometrial epithelial cells to maintain a healthy physical and antimicrobial barrier against pathogens. The binding of commensal bacteria to epithelial cells triggers the release of various antimicrobial peptides (AMPs) into the uterine cavity, which constitute part of key defense mechanisms of epithelial tissues against a proteolytic enzymes from various pathogens including bacteria, fungi, and some viruses (52, 53). In addition to the production of AMPs, commensal bacteria induce a biochemically neutral and biophysically stable mucus production by endometrial cells and stabilize the adherens junctions and tight junctions (55, 106). The maintenance of an intact and stable epithelial barrier is an integral part of the natural defense strategies in preventing the colonization and penetration protecting the endometrium from opportunistic microbial infections.

2) Commensal bacteria can alter the immune response at the cellular level through numerous components of the innate and adaptive immune system in the endometrium. The key sensors of bacterial presence in tissues are antigen presenting cells (APCs), in the endometrium they are represented by macrophages and dendritic cells (uDCs) (see section "Maternal Immune Response in Pregnancy"). Both cell types play an important role in maintaining tolerance against the commensal microbiota by modulating the immune response of other components of the innate and adaptive systems (63, 107). Macrophage-derived IL-10 is critical for Foxp3 + Treg cell development, maintenance, and expansion (77). uDCs also are a major source of IL-23 which, in combination with other cytokines, influences the differentiation of Th17 (77). Although both macrophages and DCs can process and present bacterial antigens, differences in their physiology and function suggest they have complementary roles in the immune response against bacteria.

3) The downstream effects of triggering the immune system at the feto-maternal interface by bacteria is the activation of uterine NK (uNK) cells and the development of specific subsets of T-cells, characterized by high number of regulatory T (Treg) cells, low number of Th-17, and a switch from the Th1 to Th2 cytokine production. These adaptive changes ensure an immunotolerant milieu for the semi-allogenic fetus and are essential steps in normal placentation. The interaction of activated uNK cells via specific receptors (KIR) with HLA-C and -G from extravillous trophoblasts (EVT) from the implanting embryo will also promote EVT invasion, stromal matrix degradation, angiogenesis and ultimately the remodeling of maternal spiral arteries.

How the host-microbiota interactions affect the maternal immune response during implantation is not clearly understood. One of the factors, which govern immune modulation and maternal tolerance is the Pre-Implantation Factor (PIF) (108). PIF is a 9–15 amino acid peptide secreted by viable placenta with high concentrations in the maternal circulation in the first trimester (108, 109). PIF shows local effects on the endometrium and trophoblast promoting implantation and invasion of the trophoblast and has a direct impact on immune cell function and targets neutrophils and macrophages, as well as CD4+ and CD8+ T-cells (109). PIF reduces the activation of the NALP3 inflammasome complex (mainly TLR-4 mediated) resulting in reduction of pro-inflammatory cytokines such as IL-1β, IL-18, and IL-33 (110, 111). In addition, PIF creates an anti-inflammatory milieu by reducing IFNγ and stimulating IL-10 secretion, as well as enhancing Th2 cytokines. In context of the macrophages responding to bacterial stimuli, PIF blocks the release of nitric oxide induced by lipopolysaccharides (LPS) (108). This effect is present in case of excessive stimulus only. Thus, PIF operates as an immune modulator, rather than an immune suppressor with minimal impact on the innate, but firm effect on the adaptive immune response (108). Additional protective effects on the embryo protection and development include targeting the protein-disulfide isomerase (PDI) and heat shock proteins (HSP), which impact oxidative stress and protein misfolding (112, 113). Overall PIF is an example of embryonal factor shaping maternal immune response and therefore promoting successful implantation by generating an anti-inflammatory milieu and facilitates immune tolerance. The interaction of microbiome and PIF on pregnancy complications are currently under investigation.

CLINICAL IMPLICATIONS OF DYSBIOSIS AND DISTURBED IMMUNE SYSTEM

Abnormal composition and/or function of the of reproductive tract dysbiosis is implicated in various gynecological disorders and pregnancy complications (4, 114, 115) (**Figure 2**). Although many gynecological disorders have been linked to dysbiosis and can indirectly affect reproductive outcomes [e.g., endometriosis and ectopic pregnancy (55)], discussion of these specific complications is outside the scope of this review. Depending on the anatomical site within the female reproductive tract, dysbiosis is associated with various clinical disorders The clinical implications of dysbiosis of the female reproductive tract in relation to pregnancy ranging from infections: BV, bacterial vaginosis; PID, pelvic inflammatory disease; STI's, sexually transmitted infections; early pregnancy complications: RM, recurrent miscarriage; RIF, recurrent implantation failure; and late pregnancy complications: pPROM, premature pre-labor rupture of membranes and placental dysfunction.

Preconceptional Period (Genital Tract Infections)

As outlined in preceding sections, the immune system and microbiota play an interactive and collaborative role in maintaining a physiological healthy state in the reproductive tract. Disturbance of this physiological interaction has been implicated in the onset of diverse complications related to female reproductive health.

One of the largest longitudinal cohort studies that assessed vaginal microbiota vs. risk of STI is the Longitudinal Study of Vaginal Flora (LSVF) based in the United States (116). Women diagnosed with BV (by Nugent score assessment) had a nearly 2-fold increased risk of STI, like trichomonal, gonococcal, and/or chlamydial infection (of note: microbiota-testing preceded detection of STI by 3 months). This evidence is in agreement with previous reports that define vaginal dysbiosis as a predictor for gonorrhea and chlamydial infection (117). More recently, individuals with chlamydial infection were found more likely to have a cervicovaginal microbiota dominated by *L. iners*, or non-*L. crispatus* anaerobic bacteria (118), although not all associations were statistically significant and may depend on ethnicity (119, 120).

Human Papilloma Virus (HPV) and the Human Immunodeficiency Virus (HIV) represent sexually transmitted viral infections increasingly studied in association with reproductive tract microbiota. Women with detected or persistent HPV infections showed a more diverse vaginal microbiota, in studies conducted among African/Caribbean and Italian women and suggested an association with *Atopobium* spp. and *G. vaginalis* (121, 122). Among Nigerian women, the prevalent high-risk HPV (hrHPV) infection was associated with a decrease in Lactobacilli and abundance of anaerobes, particularly of the general *Prevotella* and *Leptotrichia* (123). In Asia an association between increased vaginal bacterial diversity and presence of HPV were reported, with a Korean study suggesting Fusobacteria, in particular *Sneathia* spp. to be particularly implicated (124). A Chinese study identified several microbial genera in hrHPV-infected women (*Bifidobacterium*, *Bacillus*, *Megasphaera*, *Sneathia*, *Prevotella*, *Gardnerella*, *Fastidiosipila,* and *Dialister*), while another set (*Bifidobacterium*, *Megasphaera*, *Bacillus*, *Acidovorax*, *Oceanobacillus,* and *Lactococcus*) in hrHPV-infected pregnant women. In pregnancy, this study showed an association between a more diverse cervical microbiota and HPV (125). In addition, several genera and species were associated to HPV positivity (*Ureaplasma parvum*), HPV negativity (*Brochothrix*, *Diplorickettsia*, *Ezakiella*, *Faecalibacterium*, and *Fusobacterium*), likelihood of reinfection (*Actinomyces*) or persistence (*Prevotella*, *Dialister*, and *Lachnospiraceae*) (126). Recent data also suggested altered microbiota in placenta, cervix and mouth in the presence of HPV infection (119).

Two decades ago, the absence of Lactobacilli was already associated with an increased risk of acquiring HIV infection in a cohort of Kenyan women (120). Similarly, vaginal dysbiosis was suggested as a contributor to the acquisition of HIV in Ugandan and Zimbabwean women (127). Conversely, Rwandan women with a *Lactobacillus*-dominated (particularly *L. crispatus*) cervicovaginal microbiota were less likely to be infected with HIV, hrHPV, as well as Herpes Simplex Virus 2 (HRV 2) (128). Low pH, due to lactic acid production by Lactobacilli, was suggested as a main strategy to prevent HIV infection, either by means of inactivating the virus, or inactivating T

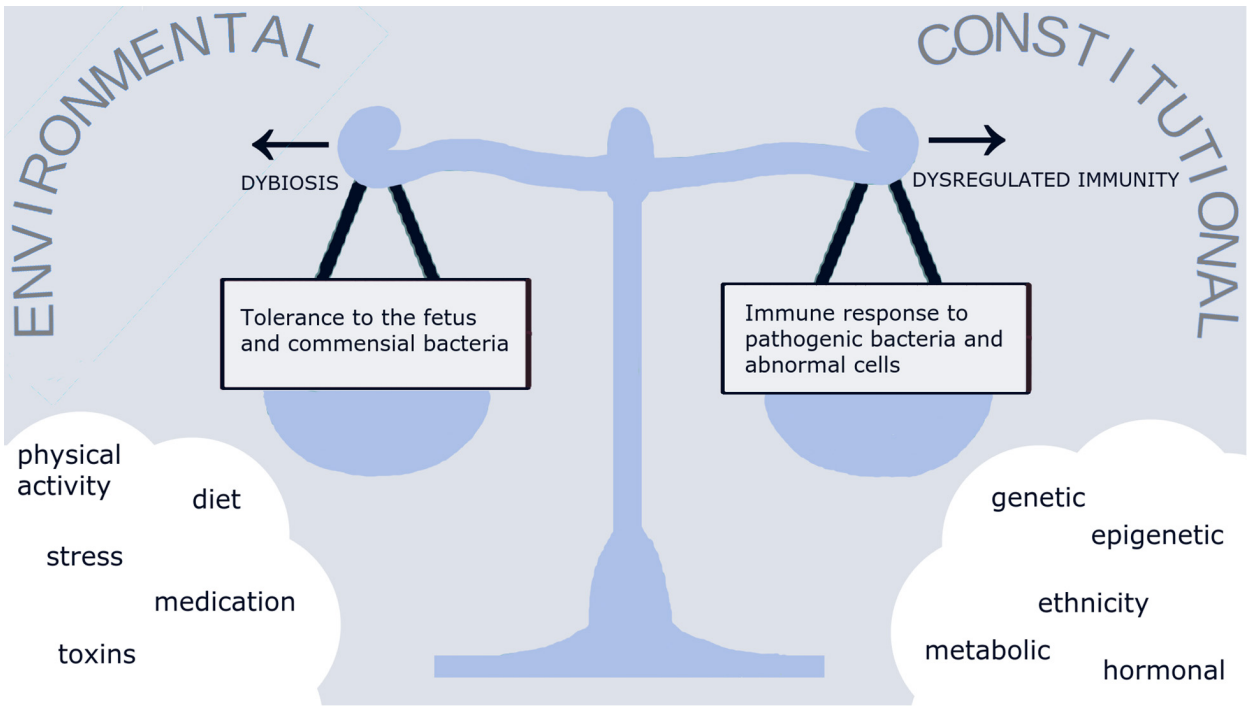

FIGURE 2 | The conceptual relationship of reproductive tract microbiome and immune response. A multitude of environmental and constitutional factors affect the balance between tolerance to the fetus and commensal bacteria on one hand, and the immune response to pathogens and abnormal cells on the other hand. Loss of balance between the microbiome and immune responses would lead to either abnormal commensal microbiome and pathogenic infection "dysbiosis" or excessive immune reaction and sterile inflammation "dysregulated immunity."

lymphocytes, thus decreasing their susceptibility to HIV infection (129). Other possible defense mechanisms of vaginal microbiota to HIV include the production of peroxide or bacteriocins by Lactobacilli, although their effect on viral biology is not fully understood (129). These findings are seemingly congruous with the protective role of Lactobacilli in urinary tract infections [as reviewed in (130)].

The above mentioned sexually transmitted infections, among others, have been extensively implicated in diverse reproductive tract complications, from infertility, to adverse pregnancy outcomes. A growing body of evidence collectively supports the association between vaginal dysbiosis and different genital tract infections and corroborates an important physiological role for microbiota in protection from, or susceptibility to pathogenic infections. Interaction and cross-regulation between the vaginal microbiota and the immune responses in the lower genital tract are therefore crucial in creating a protective environment against external pathogens, thus ensuring the right condition for the initiation of pregnancy.

Early Pregnancy (Infertility and Recurrent Miscarriage)

In recent years, the availability of culture-independent sequence techniques has led to a rise in the number of studies investigating the association of disturbed vaginal and endometrial microbiome composition and reproductive failure, focusing mainly on implantation failure. In women undergoing *in vitro* fertilization (IVF), the percentage of vaginal and endometrial Lactobacilli were significantly lower than non-IVF patients and healthy volunteers (131). In addition, studies have shown that presence of various bacterial contaminants, such as Enterobacteriaceae, Streptococcus, Staphylococcus, and Gram-negative bacteria, from catheter tips at the time of embryo transfer had a negative impact on pregnancy outcome, as reviewed in (13). Using 16S ribosomal RNA sequencing of paired endometrial and vaginal samples from 13 fertile women and 35 infertile patients undergoing IVF, Moreno et al. showed that the presence of a non-*Lactobacillus*-dominated microbiota (defined as <90% *Lactobacillus* spp.) was associated with significant decreases in implantation (60% vs. 23%), pregnancy (70% vs. 33%), ongoing pregnancy (59% vs. 13%) and live birth (59% vs. 7%) rates (43). Using a similar approach in a cohort of 31 women, Bernabeu et al. showed that women achieving pregnancy after IVF (cryotransfer of a single embryo) showed a greater presence of *Lactobacillus* spp., while a trend toward higher alpha diversity in vaginal samples was found in patients who did not achieve pregnancy and no difference in beta diversity (132). In a recent large prospective cohort study, Koedooder et al., used a new technique "IS-PRO" (see section "Diagnostic Challenges") to examine microbial profiles of vaginal microbiota in 192 women undergoing IVF. Women with a low percentage of Lactobacillus in their vaginal sample were less likely to have a successful embryo implantation (133). This failure was correctly predicted in 32

out of 34 women based on the vaginal microbiota composition, resulting in a predictive accuracy of 94% (sensitivity, 26%; specificity, 97%). Additionally, the degree of dominance of *Lactobacillus crispatus* was an important factor in predicting pregnancy: none of the women who had a negative prediction (low chance of pregnancy) became pregnant. Taken together, these data suggest that a balanced, less diverse vaginal microbiota, dominated by *Lactobacillus* species increased the chances of a successful outcome.

In women with recurrent reproductive failure, ascribing a causative or correlative connection to aberrant microbiota is controversial. This group is composed of women with recurrent miscarriage (RM) (defined as loss of two or more clinically or biochemically established pregnancies) (134) and women with recurrent implantation failure (RIF), defined as loss of two or more pregnancy losses after transfer of good-quality embryos (135). RM and RIF both have heterogeneous etiology, with diverse risk factors being implicated covering genetic, metabolic, hormonal, immune maternal aspects. Although several groups have studied microbiota disturbances in women with recurrent reproductive failure, the complex pathogenesis has hampered any meaningful conclusion on the role of reproductive tract dysbiosis in this early pregnancy disorder. Analysis of endometrial samples from women investigated for recurrent reproductive failure showed that the uterine microbiota was dominated by *Bacteroides* species in >90% of the women (35). However, dissimilarities in dominance of *Prevotella* spp. or *L. crispatus* due to possible contamination from the vagina limits the interpretation of these data.

Chronic endometritis (CE) is an inflammatory condition typified by dysregulated interactions between endometrial pathogens and the endometrium. Chronic endometritis is a persistent inflammation of the endometrium, characterized by the presence of plasma cells syndecan-1 (CD138) on immunohistochemical staining of endometrial biopsies [reviewed in (136)]. Although various pathogens have been implicated in causing CE, the most commonly reported species were common bacteria (*Escherichia coli, Enterococcus faecalis*, and *Streptococcus agalactiae*) in 77.5%, followed by *Mycoplasma*/*Ureaplasma* (25%) and *Chlamydia* (13%) (137).

Recently, research has focused on the role of CE in reproductive failure, with various studies reporting a wide range prevalence depending on the clinical characteristics of the studied group and reflecting heterogeneity of diagnostic methodology and definitions. Studies have found an increased prevalence of CE in women with recurrent pregnancy loss (13%) (138) and RIF (30%) (139), while the rate of CE in the general infertility population was suggested to be much lower, with a prevalence of 2.8% among 606 infertility patients (140). A recent meta-analysis of five studies (total of 796 patients) concluded that women receiving antibiotic therapy for CE did not show any reproductive advantage in comparison with untreated controls (141). However, patients with cured CE, confirmed by a repeat biopsy, showed higher clinical pregnancy rate (OR 4), ongoing pregnancy rate/live birth rate (OR 6.8) and implantation rate (OR 3.2) (141). The exact mechanism of how CE affects implantation is yet unknown, but a negative effect on endometrial receptivity by abnormal infiltration of plasma cells (B lymphocytes) and antibody production is suggested (142). Similarly, as the causal connection between CE and dysbiosis is unknown, it is unclear whether a status of dysregulated immune response in the endometrium is responsible for the higher prevalence of dysbiosis, or whether dysbiosis is the cause of CE.

How the disturbance of the endometrial microbial ecosystem can have a negative impact implantation process is not fully understood. Parallel to the proposed mechanism of interaction between the microbiota and endometrial cells, a disturbed balance can act upon the following mechanisms (**Figure 3**). (1) First, the dominance of non-commensal bacterial communities could weaken the integrity of the endometrial mucosal barrier by affecting the epithelial tight junctions and reducing AMP and mucin secretion; (2) This in turn could further weaken host defense mechanisms and allow pathogens to enter the endometrial stroma and elicit a profound immune reaction from APC and other immune cells harboring pattern recognition receptors (PRR); (3) Aberrant stimulation of T-cells, either directly from invading pathogens breaching the mucosal barrier, or indirectly from absorbed bacterial products, results in disbalance in cytokine production in favor of the pro-inflammatory Th-1 types, predominated by TNF-a, IFN, IL-2, and IL-10. Abnormal uNK cell maturation, either primary or secondary to shallow extravillous trophoblast (EVT) invasion, is a possible link between a disturbed endometrial microbiome and incomplete remodeling of maternal spiral arteries, characteristic of the great obstetric syndromes (143).

Late Pregnancy (Premature Delivery, Premature Rupture of Membranes, Chorioamnionitis and Placental Dysfunction)

Alteration of the ecology of the female reproductive tract has been linked to maternal and fetal health, and to adverse pregnancy outcomes (27, 79, 144, 145). The most widely studied pregnancy complication in relation to the vaginal microbiota is preterm birth (PTB) (146–148). The prevalence of a *Lactobacillus*-poor microbiota was inversely correlated with gestational age at delivery in some studies (38), not in others (149). Distinctive species of Lactobacilli were reported to be associated with differential pregnancy outcomes. Indeed, in two ethnically distinct cohorts, *L. crispatus* was associated with a low PTB risk, whereas *L. iners* was not (42). Also, *Gardnerella* was also associated with PTB and coexisted with *L. iners*, but not with *L. crispatus* (42). Based on these findings, a model was suggested to help describe the interplay between key vaginal bacterial species: the presence of *G. vaginalis* correlates with adverse outcomes, such as PTB and symptomatic Bacterial Vaginosis (BV); *G. vaginalis* and *L. crispatus* are strongly mutually exclusive, while this is not the case for *L. iners* and *Gardnerella* (150). Comparison of vaginal samples of 90 women who delivered at term and 45 women with preterm birth suggested that a specific signature: the presence of BV-associated bacterium (BVAB) 1, *Prevotella* cluster 2, *Sneathia amnii* and BVAB-TM7 in early

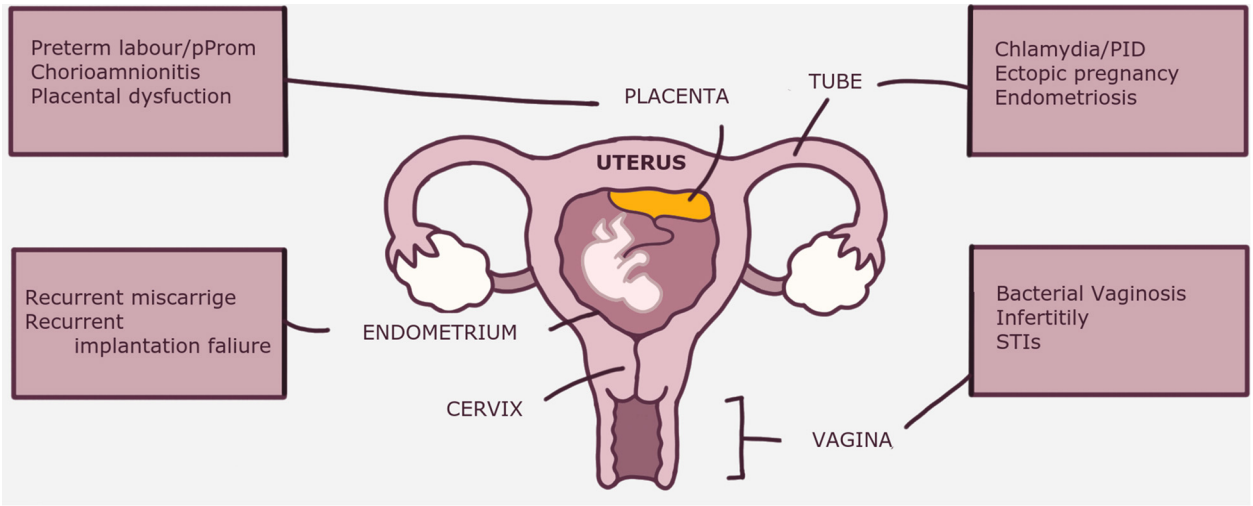

FIGURE 3 | The clinical implications of dysbiosis (abnormal microbiota) of the female reproductive tract in relation to pregnancy. Some disorders, e.g., endometriosis are indirectly related to pregnancy disorders through their negative impact on fertility. BV, bacterial vaginosis; PID, pelvic inflammatory disease; PPROM, premature prelabor rupture of membranes; RM, recurrent miscarriage; RIF, recurrent implantation failure; STI's, sexually transmitted infections.

pregnancy, may be useful for prediction of PTB risk, particularly in high-risk populations of African ancestry (147).

Abnormal vaginal colonization in the second trimester was also associated with an increased PTB risk (151). Similarly, in a predominantly African-American population, an increased vaginal microbial community richness and diversity between the first and second trimester was associated with PTB (152). In addition to decreased *Lactobacillus* spp., specific pathogens have been linked to PTB in different populations, such as *Klebsiella pneumonia*, *Gardnerella, Ureaplasma* and other genera, including *Prevotella, Atopobium*, *Sneathia*, *Gemella*, *Megasphaera*, *Dorea*, *Streptococcus*, and *Escherichia/Shigella* (38).

At this point, although one out of four preterm births appears to be associated with intra-amniotic infection, and some associations between microbial states or abundance of individual species with PTB have been reported, it does not appear clear whether changes in the bacterial communities of the lower genital tract allow for a clear identification of women at risk (144). A recent overview summarizing studies on the association between vaginal microbiota and PTB emphasized the methodological heterogeneity, and scarcity of, studies in the field (146). Overall, research on the topic has produced conflicting outcome, often related to ethnical background of the women included and the associated risk degree of PTB. Nonetheless, more recent studies more consistently report an association between vaginal dysbiosis and PTB, possibly resulting from the improved understanding of the contribution of *L. iners* to this association (146).

Multiple studies provide evidence of placental and amniotic fluid microorganisms affecting miscarriage, chorioamnionitis, premature rupture of membranes (PROM), stillbirth, preeclampsia (PE), and intra uterine growth restriction (IUGR) rates (143, 153). In some cases of spontaneous preterm delivery, microorganisms have been found to invade the amniotic cavity, leading to increased maternal/neonatal morbidity and mortality (154). Infectious bacteria gain access, typically via ascending route and/or perturbations of the vaginal microbiota and can have an adverse impact on pregnancy outcomes (146). Chorioamnionitis is an inflammatory disease of the extraembryonic membranes, placenta and amniotic fluid due to microbial invasion mostly commonly *Ureaplasma* and *Mycoplasma* spp. infections (155). Culture-dependent studies identified members of the genera *Prevotella*, *Bacteroides*, *Peptostreptococcus*, *Gardnerella*, *Mobiluncus* and genital mycoplasmas in the placentae of women delivering preterm with or without PE, suggestion the involvement of multiple bacterial strains (15). In line with this, antibiotic treatments have not reduced the rates of preterm birth, suggesting that a single inflammatory/infectious pathway may not fully explain the problem (144). In contrast, DNA-based investigations of the placental microbiota in PTB showed increased enrichment of *Burkholderia* spp. and an increased relative abundance of Alphaprotoebacteria and Actinomycetales and mixed non-cultivable anaerobes (15). However, in case of chorioamnionitis a higher abundance of *Streptococcus agalactiae*, *Fusobacterium nucleatum* and *Ureaplasma parvum* was reported (15). These results fuel the debate whether a prenatal bacterial microbiota really exist (45). In physiological pregnancies or in the presence and absence of active labor, many "causal" microorganisms or their DNA are detectable in the placenta and amniotic fluid (156, 157). Since microbiotal ratios of specific microbial species change throughout gestation, the placental response to such environmental cues possibly does as well (158). This is supported by the observation that PIF is differently expressed in the placenta throughout gestation or in response to an inflammatory insult (109). Since, as of yet, the amniotic fluid and the membranes are not available for non-invasive sampling, the time of onset of

chorioamnionitis is not available for clinical stratification and decision making. Besides placental inflammation, systemic inflammation in concert with oxidative stress and endothelial dysfunction plays a role in pregnancy complications such as IUGR or PE (159).

The interaction of vaginal and endometrial microbiota with local maternal components modulates the maternal immune system. Although the contribution of the fetal immune system to this interplay is currently unknown, it is conceivable that when the fetus initiates an inflammatory response, premature labor may impose a risk to fetal wellbeing. Conversely, there is an inherent risk that the fetus may be injured by a longer stay *in utero* either directly through microbial toxins or through proinflammatory cytokines. This delicate balance between maternal and fetal needs possibly dictates the course and outcome of pregnancy and may contribute to long-term health of the offspring. This may be another example how future health is primed by the intrauterine environment according to the principles of the DOHAD (Developmental Origins of Health and Disease) hypothesis (160).

FUTURE PERSPECTIVE

Diagnostic Challenges

As discussed throughout this review, one of the major limitations in microbiome research is the diversity of the techniques used and the databases linked to those techniques to identify individual species and determine the composition of microbiota. Such differences potentially introduce significant variations in analysis and constitute a major source of difference in interpretation of data. Although currently no compelling evidence points to the existence of a universal mammalian placental or fetal microbiota, consensus on the importance of uniform technological and analytical approaches warrants further investigation into standardization of microbiota research and incorporating geographical, ethnic and societal data (45) (**Table 1**). These recommendations are general for all microbial community profiles, including the female reproductive tract microbiota, and are expected to reduce variation and inconsistency between studies on microbiomes.

Moreover, most of the techniques described have not undergone rigorous validation steps and certification by regulatory organs, such as the European commission *in vitro* diagnostic (CE-IVD) label or the American Food and Drug Administration (FDA) approval, limiting their commercial availability. The availability of CE-IVD-certified microbiome analysis tools would meet part of the recommendations (see: **Table 1**) and allow more reliable comparisons between studies in different countries and settings. Besides the widespread Next-Generation Sequencing (NGS) approaches, our group developed another detection technique called *IS-pro*, first described in 2009 (161). The *IS-pro* test is already CE-IVD certified and is currently being used in many different disease profiling activities and applications including analyses of the vaginal microbiome as a predictor for outcome of *in vitro* fertilization (133). A wider application of this technique to study the role of reproductive tract microbiome in various pregnancy complications is expected to be available in the near future.

Fundamental Research Developments and Directions

The general concept of interactions between commensal microbiota and human cells has recently been embraced as a principal of human physiology. The realization that a dysbalanced and/or harmful microbiotal composition frequently correlates with specific clinical conditions, has led to a sharp rise in studies on host-microbiota interaction over the last decade (25, 55, 114, 162, 163). Although it is becoming increasingly clear that the interplay between host and microbiota also affects human reproductive biology, the exact molecular mechanisms underpinning these interactions are far from understood.

All long-lasting physiological adaptations of cells and tissues in response to altered environmental conditions have their basis in altered epigenomic programming. Environmental changes are detected by a myriad of cellular sensing mechanisms and, via signal transduction routes, ultimately reach the nucleus where environmental cues are translated to epigenetic regulation and chromatin remodeling. This epigenetic regulation of genetic input and potential environmental cues underlies maternal physiological plasticity and embryonal development during gestation. Programming of immune cells during immunological responses is mediated by cellular stress responses and is typically accompanied by metabolic changes in the cell. Prolonged disturbance of these interactions is associated with the development of immunological disorders, such as chronic inflammatory conditions (164). The close link between cellular metabolism and epigenetic responses has its origin in early evolution, has enabled multicellularity and is closely connected to organismal survival (165). Hence, fine-tuning of epigenetic control occurs in conjunction with cellular metabolic status, as available energy ultimately directs and limits cell responses. Recent advances in this field have identified oxygen, numerous metabolic intermediates, cellular reduction (NADH, $FADH_2$) and energy equivalents (ATP, GTP), as direct molecular effectors of epigenetic regulatory activity (165). Conversely, cellular metabolic adaptation is controlled by epigenetic regulation, substantiating the reciprocal nature of the physiological interaction between metabolism and epigenetics (166). Sex hormones, metabolic profiles, nutrition, maternal stress, drugs, smoking and air pollution represent obvious examples of environmental cues that, via active modulation of epigenetic regulatory mechanism.

Interestingly, microbial metabolites, among which Short-Chain Fatty Acids (SCFA), like acetate, butyrate and propionate, are known to harbor the ability to alter epigenetic status of numerous cell types; studied examples thereof include the effect of such compounds on immune cells (78). In the context of human reproduction, it is conceivable that microbial metabolites, directly (e.g., via SCFA) or indirectly (e.g., acidification, alkalization, inflammatory responses), can either support (healthy microbiota) or upset (unbalanced/harmful microbiota) local cell-cell communication and tissue physiology

TABLE 1 | Steps to obtain reliable microbiota*.

1. Larger sample sizes
2. Simultaneous use of different detection methods
3. The elimination of extracellular DNA prior to molecular microbial profiling
4. rigorous controls for reagents and equipment at all steps during sample processing and analysis
5. The determination of the relative abundance of bacterial groups should be preceded by an absolute quantification of the bacterial load in samples and controls
6. Prespecified and quantified bacterial mock communities to the examined samples will help to reveal biases and identify batch effects
7. Demonstration of metabolically active and proliferating diverse bacteria within the placental or fetal tissue will be required to prove the existence of a viable, diverse and unique bacterial community that merits the term microbiota.
8. Taking into consideration geographical, ethnic and societal habits of the population

This table is adapted from the text and advices provided in Hornef M, Penders J. Does a prenatal bacterial microbiota exist? Mucosal Immunol. 2017 May;10(3):598–601.

and adaptation. As such, all relevant reproductive processes, including fertilization, implantation, placentation, immune tolerance, embryonal development, infant and adult health and may be harmfully altered by dysbiosis (167). Such gene-environment interactions are yet to be examined in detail in the context of reproductive health and disease.

Therapeutic Opportunities

Given the association between pregnancy complications and microbiota, the question of modulation strategies is valid. Postnatal dietary strategies, including human colostrum/milk or prebiotics/probiotics, reduce morbidities in preterm infants (163). Evidence of prenatal strategies to support reproductive success and fetal health is slowly emerging: modulation of early microbiota in pregnancy shows promising effects (168). Maternal bacteria were shown to enter the gastrointestinal tract of the fetus (169) and microbiota alteration in the neonate and placenta is detectable in pregnant women receiving probiotics (170) but still under debate (14).

Recently our group published a meta-analysis on the relation between vaginal microbiota and early pregnancy development after IVF and the effect of probiotics thereon (71). It provides an overview of published studies describing long term modulation of the vaginal microbiome using *Lactobacillus*-based probiotics. Patients were often treated by Metronidazole, and followed up with a *Lactobacillus*-based probiotic. Besides lactobacillus-based probiotics, mixtures of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* strains were also used in different studies (71). Further studies assessing the potential to modulate pregnancy outcomes are needed.

The notion that the neonatal immune system can be shaped by early fetal microbial colonization by inference implies that reciprocal interactions between the host and microbiota exist (171). This hypothesis is in line with the evidence that factors like PIF mediate maternal immune tolerance during pregnancy (108). Fine tuning of immune modulation is not only relevant for embryo implantation but also for defense against potentially harmful microbes. An exaggerated maternal immune response is putatively linked to preterm birth and fetal loss (172). Therefore, a novel strategy could be the maternal immune system modulation by synthetic PIF (110). Inflammatory challenge during pregnancy results in endogenous PIF expression and additional administration of synthetic PIF could prevent fetal loss.

The neonatal microbiota, mainly that of the gut, skin and oral cavity, has long been postulated to be acquired postnatally known as "postnatal microbiota seeding". Alternatively, the hypothesis of perinatal microbiota transfer and its potential relevance to infant and adult general health is gaining attention. Given the importance of transfer of maternal microbiota to the child via colonization, the significance of birth mode is of high interest (173). Vaginal birth exposes the baby to maternal vaginal and intestinal microbiota, whereas cesarean section limits exposure of the newborn to parental dermal microbiota and any microbes present in the surgical theater. The "prophylactic" antibiotic treatment of the mother during labor or cesarean section may aggravate any effect of non-natural birth on colonization and may also negatively affect intestinal microbiota of the offspring (174). Hence, birth mode may have long-lasting effects on the composition of the newborn gut microbiome, and predispose for adverse health outcomes (174). The notion that cesarean delivery deprives the infant of exposure to vaginal microbiota and consequently leads to neonatal dysbiosis has led to the popularized, yet unsubstantiated and potentially hazardous practice of "vaginal seeding". In a pilot study of 18 mother-infant pairs, there was partial restoration of microbiome (mainly of the skin and oral cavity and less so of the gut) in infants exposed to vaginal fluids from a vaginally placed gauze after cesarean delivery (n = 4), compared to non-exposed infants (n = 7), and resembling the microbiome of vaginally delivered infants (n = 7) (175). This widely cited trial was criticized for the small sample size and the potential bias from confounders such as intrapartum antibiotic prophylaxis and maternal BMI (173, 174). Although postnatal vaginal seeding altered newborn intestinal microbiota composition over several months, this intervention is believed to introduce an inherent risk of transferring pathogenic microbes (e.g., HPV, GBS) onto the newborn (176). For this reason, perinatal seeding is currently not encouraged as standard practice by the American College of Gynecology and Obstetrics (177). Whether and how vaginal seeding has any long-term beneficial health effects for the offspring requires large properly conducted studies with robust microbiome analysis.

As microbiota represents an environmental factor which affects host-microbe interactions at the epigenetic level, detailed understanding of the molecular workings of the close functional interplay between host cell systems and microbiota holds the promise that therapeutic intervention strategies can be

designed for the benefit of general and reproductive health. These include the use of probiotics, beneficial microbial metabolites, rational diets and/or (ant)agonists of specific biological response pathways (162). Combined the research cited in his review define opportunities for modulation of the female reproductive tract microbiota, while harnessing its protective and immuno-regulating role during pregnancy. Such opportunities should take into consideration individual differences in microbial communities, and tailor therapeutics to different anatomical and gestational factors in an attempt to provide precision tools for reproductive health.

CONCLUSION

The composition and interaction of the female reproductive tract microbiome with the host not only shape the mothers' physiology and health during pregnancy, but also that of the fetus in accordance with the developmental origins of health and disease principles. Scientific knowledge on and insight into the properties and workings of human microbiota has increased over the last decade. However, the definition and possible implications of beneficial versus harmful microbes in physiological and pathological pregnancies is just beginning to emerge. A growing body of evidence associates stability of reproductive tract microbiota to reproductive health and maternal-fetal status during gestation, in which the interplay between microbiota and the maternal immune response takes up a prominent position. The important question of whether and how reproductive tract microbiota can be modified during and beyond pregnancy is under debate and awaits solid confirmation. The challenge for future research is to deliver standardized and validated reference methods for comparative analysis and interpretation of reproductive tract microbiomes, in order to understand their role in various clinical disorders and test the implementation of individualized therapies in large prospective trials.

AUTHOR CONTRIBUTIONS

SA-N and EA designed and wrote the manuscript and illustrations. LW, MSc, MSp, and JV wrote the sections of the manuscript and contributed to the interpretation of the results. SM, MM, and BK conceived the original idea, supervised the project, and edited the manuscript. All authors provided the critical feedback and contributed to the final version of the manuscript.

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REFERENCES

1. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature.* (2012) 486:207–14. doi: 10.1038/nature11234
2. NIH Human Microbiome Portfolio Analysis Team. A review of 10 years of human microbiome research activities at the US national institutes of health, fiscal years 2007-2016. *Microbiome.* (2019) 7:31. doi: 10.1186/s40168-019-0620-y
3. Sirota I, Zarek S, Segars J. Potential influence of the microbiome on infertility and assisted reproductive technology. *Semin Reprod Med.* (2014) 32:35–42. doi: 10.1055/s-0033-1361821
4. Power ML, Quaglieri C, Schulkin J. Reproductive microbiomes: a new thread in the microbial network. *Reproduct Sci.* (2017) 24:1482–92. doi: 10.1177/1933719117698577
5. Younes JA, Lievens E, Hummelen R, van der Westen R, Reid G, Petrova MI. Women and their microbes: the unexpected friendship. *Trends Microbiol.* (2018) 26:16–32. doi: 10.1016/j.tim.2017.07.008
6. Romero R. Prenatal medicine: the child is the father of the man*. *J Matern Fetal Neonatal Med.* (2009) 22:636–9. doi: 10.1080/14767050902784171
7. Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA. National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. *PLoS Med.* (2012) 9:e1001356. doi: 10.1371/journal.pmed.1001356
8. Rossen LM, Ahrens KA, Branum AM. Trends in risk of pregnancy loss among US women, 1990-2011. *Paediatr Perina Epidemiol.* (2017) 32:19–29. doi: 10.1111/ppe.12417
9. Dhaded SM, Somannavar MS, Jacob JP, McClure EM, Vernekar SS, Yogesh Kumar S, et al. Early pregnancy loss in Belagavi, Karnataka, India 2014–2017: a prospective population-based observational study in a low-resource setting. *Reprod Health.* (2018) 15:2683–8. doi: 10.1186/s12978-018-0525-4
10. Dellicour S, Aol G, Ouma P, Yan N, Bigogo G, Hamel MJ, et al. Weekly miscarriage rates in a community-based prospective cohort study in rural western Kenya. *BMJ Open.* (2016) 6:e011088. doi: 10.1136/bmjopen-2016-011088
11. Lozano R, Wang H, Foreman KJ, Rajaratnam JK, Naghavi M, Marcus JR, et al. Progress towards millennium development goals 4 and 5 on maternal and child mortality: an updated systematic analysis. *Lancet.* (2011) 378:1139–65. doi: 10.1016/S0140-6736(11)61337-8
12. Chawanpaiboon S, Vogel JP, Moller A-B, Lumbiganon P, Petzold M, Hogan D, et al. Global, regional, and national estimates of levels of preterm birth in 2014: a systematic review and modelling analysis. *Lancet Glob Health.* (2019) 7:e37–46. doi: 10.1016/S2214-109X(18)30451-0
13. Franasiak JM, Scott RT. Endometrial microbiome. *Curr Opin Obstet Gynecol.* (2017) 29:146–52. doi: 10.1097/GCO.0000000000000357
14. de Goffau MC, Lager S, Sovio U, Gaccioli F, Cook E, Peacock SJ, et al. Human placenta has no microbiome but can contain potential pathogens. *Nature.* (2019) 572:1–21. doi: 10.1038/s41586-019-1451-5
15. Pelzer E, Gomez-Arango LF, Barrett HL, Nitert MD. Review: maternal health and the placental microbiome. *Placenta.* (2017) 54:30–7. doi: 10.1016/j.placenta.2016.12.003
16. Redelinghuys MJ, Ehlers MM, Dreyer AW, Kock MM. Normal flora and bacterial vaginosis in pregnancy: an overview. *Critic Rev Microbiol.* (2016) 1:1–12. doi: 10.3109/1040841X.2014.954522
17. 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:ra52–132. doi: 10.1126/scitranslmed.3003605
18. Hickey RJ, Zhou X, Pierson JD, Ravel J, Forney LJ. Understanding vaginal microbiome complexity from an ecological perspective. *Trans Res.* (2012) 160:267–82. doi: 10.1016/j.trsl.2012.02.008
19. Gupta VK, Paul S, Dutta C. Geography, ethnicity or subsistence-specific variations in human microbiome composition and diversity. *Front Microbiol.* (2017) 8:1162. doi: 10.3389/fmicb.2017.01162
20. Noyes N, Cho K-C, Ravel J, Forney LJ, Abdo Z. Associations between sexual habits, menstrual hygiene practices, demographics and the vaginal microbiome as revealed by Bayesian network analysis. *PLoS One* (2018) 13:e0191625. doi: 10.1371/journal.pone.0191625

21. Jespers V, van de Wijgert J, Cools P, Verhelst R, Verstraelen H, Delany-Moretlwe S, et al. The significance of *Lactobacillus crispatus* and *L. vaginalis* for vaginal health and the negative effect of recent sex: a cross-sectional descriptive study across groups of African women. *BMC Infect Dis.* (2015) 15:115. doi: 10.1186/s12879-015-0825-z
22. Zhou X, Hansmann MA, Davis CC, Suzuki H, Brown CJ, Schütte U, et al. The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. *FEMS Immunol Med Microbiol.* (2010) 58:169–81. doi: 10.1111/j.1574-695X.2009.00618.x
23. Bayigga L, Kateete DP, Anderson DJ, Sekikubo M, Nakanjako D. Diversity of vaginal microbiota in sub-Saharan Africa and its effects on HIV transmission and prevention. *Am J Obstet Gynecol.* (2019) 220:155–66. doi: 10.1016/j.ajog.2018.10.014
24. Borgdorff H, van der Veer C, van Houdt R, Alberts CJ, de Vries HJ, Bruisten SM, et al. The association between ethnicity and vaginal microbiota composition in Amsterdam, the Netherlands. *PLoS One* (2017) 12:e0181135. doi: 10.1371/journal.pone.0181135
25. van de Wijgert JHHM, Jespers V. The global health impact of vaginal dysbiosis. *Res Microbiol.* (2017) 168:859–64. doi: 10.1016/j.resmic.2017.02.003
26. Kroon SJ, Ravel J, Huston WM. Cervicovaginal microbiota, women's health, and reproductive outcomes. *Fertil Steril.* (2018) 110:327–36. doi: 10.1016/j.fertnstert.2018.06.036
27. Parnell LA, Briggs CM, Mysorekar IU. Maternal microbiomes in preterm birth: recent progress and analytical pipelines. *Semin Perinatol.* (2017) 41:392–400. doi: 10.1053/j.semperi.2017.07.010
28. Anahtar MN, Gootenberg DB, Mitchell CM, Kwon DS. Cervicovaginal microbiota and reproductive health: the virtue of simplicity. *Cell Host and Microbe.* (2018) 23:159–68. doi: 10.1016/j.chom.2018.01.013
29. Romero R, Hassan S, Gajer P, Tarca AL, Fadrosh DW, Nikita L, et al. The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* (2014) 2:4. doi: 10.1186/2049-2618-2-4
30. Freitas AC, Chaban B, Bocking A, Rocco M, Yang S, Hill JE, et al. The vaginal microbiome of pregnant women is less rich and diverse, with lower prevalence of mollicutes, compared to non-pregnant women. *Sci Rep.* (2017) 7:9212. doi: 10.1038/s41598-017-07790-9
31. Avershina E, Slangsvold S, Simpson MR, Storrø O, Johnsen R, Øien T, et al. Diversity of vaginal microbiota increases by the time of labor onset. *Sci Rep.* (2017) 7:17558. doi: 10.1038/s41598-017-17972-0
32. Walther-António MRS, Jeraldo P, Berg Miller ME, Yeoman CJ, Nelson KE, Wilson BA, et al. Pregnancy's stronghold on the vaginal microbiome. *PLoS One* (2014) 9:e98514. doi: 10.1371/journal.pone.0098514
33. Nasioudis D, Forney LJ, Schneider GM, Gliniewicz K, France M, Boester A, et al. Influence of pregnancy history on the vaginal microbiome of pregnant women in their first trimester. *Sci Rep.* (2017) 7:10201. doi: 10.1038/s41598-017-09857-z
34. Aagaard K, Riehle K, Ma J, Segata N, Mistretta T-A, Coarfa C, et al. A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. *PLoS One* (2012) 7:e36466. doi: 10.1371/journal.pone.0036466
35. Verstraelen H, Verhelst R, Claeys G, De Backer E, Temmerman M, Vaneechoutte M. Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol.* (2009) 9:116. doi: 10.1186/1471-2180-9-116
36. Hernández-Rodríguez C, Romero-González R, Albani-Campanario M, Figueroa-Damián R, Meraz-Cruz N, Hernández-Guerrero C. Vaginal microbiota of healthy pregnant mexican women is constituted by four *Lactobacillus* species and several vaginosis-associated bacteria. *Infect Dis Obstetr Gynecol.* (2011) 2011:851485. doi: 10.1155/2011/851485
37. Muzny CA, Schwebke JR. Pathogenesis of bacterial vaginosis: discussion of current hypotheses. *J Infect Dis.* (2016) 214(Suppl. 1):S1–5. doi: 10.1093/infdis/jiw121
38. DiGiulio DB, Callahan BJ, McMurdie PJ, Costello EK, Lyell DJ, Robaczewska A, et al. Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci USA.* (2015) 112:11060–5. doi: 10.1073/pnas.1502875112
39. MacIntyre DA, Chandiramani M, Lee YS, Kindinger L, Smith A, Angelopoulos N, et al. The vaginal microbiome during pregnancy and the postpartum period in a European population. *Sci Rep.* (2015) 5:8988. doi: 10.1038/srep08988
40. Harris JW, Brown JHLR. The bacterial content of the uterus at cesarean section. II. *Am J Obstet Gynecol.* (1927) 13:133–43.
41. Koedooder R, Mackens S, Budding A, Fares D, Blockeel C, Laven J, et al. Identification and evaluation of the microbiome in the female and male reproductive tracts. *Hum Reprod Update.* (2019) 25:298–325. doi: 10.1093/humupd/dmy048
42. Mitchell CM, Haick A, Nkwopara E, Garcia R, Rendi M, Agnew K, et al. Colonization of the upper genital tract by vaginal bacterial species in nonpregnant women. *Am J Obstet Gynecol.* (2015) 212:611.e1–9. doi: 10.1016/j.ajog.2014.11.043
43. Moreno I, Codoñer FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazán J, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am J Obstet Gynecol.* (2016) 215:684–703. doi: 10.1016/j.ajog.2016.09.075
44. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Sci Transl Med.* (2014) 6:ra65–237. doi: 10.1126/scitranslmed.3008599
45. Hornef M, Penders J. Does a prenatal bacterial microbiota exist? *Mucosal Immunol.* (2017) 10:598–601. doi: 10.1038/mi.2016.141
46. Bushman FD. De-discovery of the placenta microbiome. *Am J Obstet Gynecol.* (2019) 220:213–4. doi: 10.1016/j.ajog.2018.11.1093
47. Theis KR, Romero R, Winters AD, Greenberg JM, Gomez-Lopez N, Alhousseini A, et al. Does the human placenta delivered at term have a microbiota? Results of cultivation, quantitative real-time PCR, 16S rRNA gene sequencing, and metagenomics. *Am J Obstet Gynecol.* (2019) 220:267.e1–39. doi: 10.1016/j.ajog.2018.10.018
48. Vanterpool SF, Been JV, Houben ML, Nikkels PGJ, De Krijger RR, Zimmermann LJI, et al. *Porphyromonas gingivalis* within placental villous mesenchyme and umbilical cord stroma is associated with adverse pregnancy outcome. *PLoS One.* (2016) 11:e0146157. doi: 10.1371/journal.pone.0146157
49. Bulmer JN, Williams PJ, Lash GE. Immune cells in the placental bed. *Int J Dev Biol.* (2010) 54:281–94. doi: 10.1387/ijdb.082763jb
50. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann N Y Acad Sci.* (2011) 1221:80–7. doi: 10.1111/j.1749-6632.2010.05938.x
51. Bonney EA. Immune regulation in pregnancy. *Obst Gynecol Clin North Am.* (2016) 43:679–98. doi: 10.1016/j.ogc.2016.07.004
52. Frew L, Stock SJ. Antimicrobial peptides and pregnancy. *Reproduction.* (2011) 141:725–35. doi: 10.1530/REP-10-0537
53. Hancock REW, Rozek A. Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol Lett.* (2002) 206:143–9. doi: 10.1111/j.1574-6968.2002.tb11000.x
54. Underwood MA, Gilbert WM, Sherman MP. Amniotic fluid: not just fetal urine anymore. *J Perinatol.* (2005) 25:341–8. doi: 10.1038/sj.jp.7211290
55. Baker JM, Chase DM, Herbst-Kralovetz MM. Uterine microbiota: residents, tourists, or invaders? *Front Immunol.* (2018) 9:208. doi: 10.3389/fimmu.2018.00208
56. King AE, Critchley HO, Kelly RW. Presence of secretory leukocyte protease inhibitor in human endometrium and first trimester decidua suggests an antibacterial protective role. *Mol Hum Reprod.* (2000) 6:191–6. doi: 10.1093/molehr/6.2.191
57. Trundley A, Moffett A. Human uterine leukocytes and pregnancy. *Tissue Antigens.* (2004) 63:1–12. doi: 10.1111/j.1399-0039.2004.00170.x
58. McIntire RH, Hunt JS. Antigen presenting cells and HLA-G – a review. *Placenta.* (2005) 26:S104–9. doi: 10.1016/j.placenta.2005.01.006
59. Racicot K, Kwon J-Y, Aldo P, Silasi M, Mor G. Understanding the complexity of the immune system during pregnancy. *Am J Reprod Immunol.* (2014) 72:107–16. doi: 10.1111/aji.12289
60. Faas MM, de Vos P. Uterine NK cells and macrophages in pregnancy. *Placenta.* (2017) 56:44–52. doi: 10.1016/j.placenta.2017.03.001

61. Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med.* (2000) 192:259–70. doi: 10.1084/jem.192.2.259
62. Boulenouar S, Doisne J-M, Sferruzzi-Perri A, Gaynor LM, Kieckbusch J, Balmas E, et al. The residual innate lymphoid cells in NFIL3-deficient mice support suboptimal maternal adaptations to pregnancy. *Front Immunol.* (2016) 7:43. doi: 10.3389/fimmu.2016.00043
63. Nagamatsu T, Schust DJ. Review: the immunomodulatory roles of macrophages at the maternal—-fetal interface. *Reprod Sci.* (2010) 17:209–18. doi: 10.1177/1933719109349962
64. Mor G, Abrahams VM. Reproductive biology and endocrinology. *Reprod Biol Endocrinol.* (2003) 1:119–8. doi: 10.1186/1477-7827-1-119
65. Faas MM, Spaans F, De Vos, P. Monocytes and macrophages in pregnancy and pre-eclampsia. *Front Immunol.* (2014) 5:298. doi: 10.3389/fimmu.2014.00298/abstract
66. Swartwout B, Luo XM. Implications of probiotics on the maternal-neonatal interface: gut microbiota, immunomodulation, and autoimmunity. *Front Immunol.* (2018) 9:2840. doi: 10.3389/fimmu.2018.02840
67. Blois SM, Barrientos G, Garcia MG, Orsal AS, Tometten M, Cordo-Russo RI, et al. Interaction between dendritic cells and natural killer cells during pregnancy in mice. *J Mol Med.* (2008) 86:837–52. doi: 10.1007/s00109-008-0342-2
68. Kämmerer U, Eggert AO, Kapp M, McLellan AD, Geijtenbeek TBH, Dietl J, et al. Unique appearance of proliferating antigen-presenting cells expressing DC-SIGN (CD209) in the decidua of early human pregnancy. *Am J Pathol.* (2003) 162:887–96. doi: 10.1016/S0002-9440(10)63884-9
69. Krey G, Frank P, Shaikly V, Barrientos G, Cordo-Russo R, Ringel F, et al. In vivo dendritic cell depletion reduces breeding efficiency, affecting implantation and early placental development in mice. *J Mol Med.* (2008) 86:999–1011. doi: 10.1007/s00109-008-0379-2
70. Williams PJ, Searle RF, Robson SC, Innes BA, Bulmer JN. Decidual leucocyte populations in early to late gestation normal human pregnancy. *J Reprod Immunol.* (2009) 82:24–31. doi: 10.1016/j.jri.2009.08.001
71. Singer M, Borg M, Ouburg S, Morré SA. The relation of the vaginal microbiota to early pregnancy development during in vitro fertilization treatment-A meta-analysis. *J Gynecol Obstet Hum Reprod.* (2019) 48:223–9. doi: 10.1016/j.jogoh.2019.01.007
72. Chaouat G. The Th1/Th2 paradigm: still important in pregnancy? *Semin Immunopathol.* (2007) 29:95–113. doi: 10.1007/s00281-007-0069-0
73. Tilburgs T, Roelen DL, van der Mast BJ, de Groot-Swings GM, Kleijburg C, Scherjon SA, et al. Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. *J Immunol.* (2008) 180:5737–45. doi: 10.4049/jimmunol.180.8.5737
74. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *Am J Reprod Immunol.* (2010) 63:601–10. doi: 10.1111/j.1600-0897.2010.00852.x
75. Zenclussen AC, Gerlof K, Zenclussen ML, Ritschel S, Zambon Bertoja A, Fest S, et al. Regulatory T cells induce a privileged tolerant microenvironment at the fetal-maternal interface. *Eur J Immunol.* (2006) 36:82–94. doi: 10.1002/eji.200535428
76. Polese B, Gridelet V, Araklioti E, Martens H, Perrier d'Hauterive S, Geenen V. The endocrine milieu and CD4 T-lymphocyte polarization during pregnancy. *Front Endocrinol.* (2014) 5:106. doi: 10.3389/fendo.2014.00106
77. Vacca P, Cantoni C, Vitale M, Prato C, Canegallo F, Fenoglio D, et al. Crosstalk between decidual NK and CD14+ myelomonocytic cells results in induction of Tregs and immunosuppression. *Proc Natl Acad Sci USA.* (2010) 107:11918–23. doi: 10.1073/pnas.1001749107
78. Gaudino SJ, Kumar P. Cross-talk between antigen presenting cells and T cells impacts intestinal homeostasis, bacterial infections, and tumorigenesis. *Front Immunol.* (2019) 10:360. doi: 10.3389/fimmu.2019.00360
79. Taddei CR, Cortez RV, Mattar R, Torloni MR, Daher S. Microbiome in normal and pathological pregnancies: a literature overview. *Am J Reprod Immunol.* (2018) 80:e12993. doi: 10.1111/aji.12993
80. Brotman RM. Vaginal microbiome and sexually transmitted infections: an epidemiologic perspective. *J Clin Invest.* (2011) 121:4610–7. doi: 10.1172/JCI57172
81. Petrova MI, Lievens E, Malik S, Imholz N, Lebeer S. *Lactobacillus* species as biomarkers and agents that can promote various aspects of vaginal health. *Front Physiol.* (2015) 6:81. doi: 10.3389/fphys.2015.00081
82. Rose WA, McGowin CL, Spagnuolo RA, Eaves-Pyles TD, Popov VL, Pyles RB. Commensal bacteria modulate innate immune responses of vaginal epithelial cell multilayer cultures. *PLoS One.* (2012) 7:e32728. doi: 10.1371/journal.pone.0032728
83. van de Wijgert JHHM. The vaginal microbiome and sexually transmitted infections are interlinked: consequences for treatment and prevention. *PLoS Med.* (2017) 14: e1002478. doi: 10.1371/journal.pmed.1002478
84. 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. doi: 10.1073/pnas.1002611107
85. McKinnon LR, Achilles SL, Bradshaw CS, Burgener A, Crucitti T, Fredricks DN, et al. The evolving facets of bacterial vaginosis: implications for HIV transmission. *AIDS Res Hum Retroviruses.* (2019) 35:219–28. doi: 10.1089/aid.2018.0304
86. Torcia M. Interplay among vaginal microbiome, immune response and sexually transmitted viral infections. *Int J Mol Sci.* (2019) 20:266–213. doi: 10.3390/ijms20020266
87. Amabebe E, Anumba DOC. The vaginal microenvironment: the physiologic role of *Lactobacilli*. *Front Med.* (2018) 5:181. doi: 10.3389/fmed.2018.00181
88. Wessels JM, Felker AM, Dupont HA, Kaushic C. The relationship between sex hormones, the vaginal microbiome and immunity in HIV-1 susceptibility in women. *Dis Model Mech.* (2018) 11:dmm035147. doi: 10.1242/dmm.035147
89. Vitali D, Wessels JM, Kaushic C. Role of sex hormones and the vaginal microbiome in susceptibility and mucosal immunity to HIV-1 in the female genital tract. *AIDS Res Ther.* (2017) 14:39. doi: 10.1186/s12981-017-0169-4
90. 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. *Am J Reprod Immunol.* (2018) 80:e12863. doi: 10.1111/aji.12863
91. Murphy K, Keller MJ, Anastos K, Sinclair S, Devlin JC, Shi Q, et al. Impact of reproductive aging on the vaginal microbiome and soluble immune mediators in women living with and at-risk for HIV infection. *PLoS One* (2019) 14:e0216049. doi: 10.1371/journal.pone.0216049
92. Sansonetti PJ. To be or not to be a pathogen: that is the mucosally relevant question. *Mucosal Immunol.* (2010) 4:8–14. doi: 10.1038/mi.2010.77
93. 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.* (2015) 9:621–33. doi: 10.1038/mi.2015.86
94. 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
95. Jespers V, Kyongo J, Joseph S, Hardy L, Cools P, Crucitti T, et al. A longitudinal analysis of the vaginal microbiota and vaginal immune mediators in women from sub-Saharan Africa. *Sci Rep.* (2017) 7:11974. doi: 10.1038/s41598-017-12198-6
96. Smith SB, Ravel J. The vaginal microbiota, host defence and reproductive physiology. *J Physiol.* (2016) 595:451–63. doi: 10.1113/JP271694
97. Kirjavainen PV, Pautler S, Baroja ML, Anukam K, Crowley K, Carter K, et al. Abnormal immunological profile and vaginal microbiota in women prone to urinary tract infections. *Clin Vaccine Immunol.* (2009) 16:29–36. doi: 10.1128/CVI.00323-08
98. Valore EV, Wiley DJ, Ganz T. Reversible deficiency of antimicrobial polypeptides in bacterial vaginosis. *Infect Immun.* (2006) 74:5693–702. doi: 10.1128/IAI.00524-06
99. Dunn AB, Dunlop AL, Hogue CJ, Miller A, Corwin EJ. The microbiome and complement activation: a mechanistic model for preterm birth. *Biol Res Nurs.* (2017) 19:295–307. doi: 10.1177/1099800416687648

100. Chu DM, Seferovic M, Pace RM, Aagaard KM. The microbiome in preterm birth. *Best Pract Res Clin Obstet Gynaecol.* (2018) 52:103–13. doi: 10.1016/j.bpobgyn.2018.03.006
101. Chen X, Liu S, Tan Q, Shoenfeld Y, Zeng Y. Microbiome, autoimmunity, allergy, and helminth infection: the importance of the pregnancy period. *Am J Reprod Immunol.* (2017) 78:e12654. doi: 10.1111/aji.12654
102. Mori M, Bogdan A, Balassa T, Csabai T, Szekeres-Bartho J. The decidua-the maternal bed embracing the embryo-maintains the pregnancy. *Semin Immunopathol.* (2016) 38:635–49. doi: 10.1007/s00281-016-0574-0
103. PrabhuDas M, Bonney E, Caron K, Dey S, Erlebacher A, Fazleabas A, et al. Immune mechanisms at the maternal-fetal interface: perspectives and challenges. *Nat Immunol.* (2015) 16:328–34. doi: 10.1038/ni.3131
104. D'Ippolito S, Di Nicuolo F, Pontecorvi A, Gratta M, Scambia G, Di Simone N. Endometrial microbes and microbiome: recent insights on the inflammatory and immune "players" of the human endometrium. *Am J Reprod Immunol.* (2018) 80:e13065. doi: 10.1111/aji.13065
105. Benner M, Ferwerda G, Joosten I, van der Molen RG. How uterine microbiota might be responsible for a receptive, fertile endometrium. *Hum Reprod Update.* (2018) 24:393–415. doi: 10.1093/humupd/dmy012
106. Radtke AL, Quayle AJ, Herbst-Kralovetz MM. Microbial products alter the expression of membrane-associated mucin and antimicrobial peptides in a three-dimensional human endocervical epithelial cell model. *Biol Reprod.* (2012) 87:132. doi: 10.1095/biolreprod.112.103366
107. Harding CV, Ramachandra L, Wick MJ. Interaction of bacteria with antigen presenting cells: influences on antigen presentation and antibacterial immunity. *Curr Opin Immunol.* (2003) 15:112–9. doi: 10.1016/S0952-7915(02)00008-0
108. Barnea ER, Kirk D, Todorova K, McElhinney J, Hayrabedyan S, Fernández N. PIF direct immune regulation: blocks mitogen-activated PBMCs proliferation, promotes TH2/TH1 bias, independent of Ca(2+). *Immunobiology.* (2015) 220:865–75. doi: 10.1016/j.imbio.2015.01.010
109. Barnea ER, Vialard F, Moindjie H, Ornaghi S, Dieudonne MN, Paidas MJ. PreImplantation Factor (PIF*) endogenously prevents preeclampsia: promotes trophoblast invasion and reduces oxidative stress. *J Reprod Immunol.* (2016) 114:58–64. doi: 10.1016/j.jri.2015.06.002
110. Di Simone N, Di Nicuolo F, Marana R, Castellani R, Ria F, Veglia M, et al. Synthetic preimplantation factor (PIF) prevents fetal loss by modulating LPS induced inflammatory response. *PLoS One.* (2017) 12:e0180642. doi: 10.1371/journal.pone.0180642
111. Mueller M, Zhou J, Yang L, Gao Y, Wu F, Schoeberlein A, et al. Preimplantation factor promotes neuroprotection by targeting microRNA let-7. *Proc Natl Acad Sci USA.* (2014) 111:13882–7. doi: 10.1073/pnas.1411674111
112. Goodale LF, Hayrabedyan S, Todorova K, Roussev K, Ramu S, Stamatkin C, et al. Preimplantation factor (PIF) protects cultured embryos against oxidative stress: relevance for recurrent pregnancy loss (RPL) therapy. *Oncotarget.* (2017) 20:32419–32. doi: 10.18632/oncotarget.16028
113. Barnea ER, Lubman DM, Liu YH, Absalon-Medina V, Hayrabedyan S, Todorova K, et al. Insight into preimplantation factor (PIF*) mechanism for embryo protection and development: target oxidative stress and protein misfolding (PDI and HSP) through essential RIKP [corrected] binding site. *PloS One* (2014) 7:e100263. doi: 10.1371/journal.pone.0100263
114. Schoenmakers S, Steegers-Theunissen R, Faas M. The matter of the reproductive microbiome. *Obstet Med.* (2019) 12:107–15. doi: 10.1177/1753495X18775899
115. Franasiak JM, Scott RT. Introduction: microbiome in human reproduction. *Fertil Steril.* (2015) 104:1341–3. doi: 10.1016/j.fertnstert.2015.10.021
116. Brotman RM, Klebanoff MA, Nansel TR, Yu KF, Andrews WW, Zhang J, et al. Bacterial vaginosis assessed by gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection. *J Infect Dis.* (2010) 202:1907–15. doi: 10.1086/657320
117. Wiesenfeld HC, Hillier SL, Krohn MA, Landers DV, Sweet RL. Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. *Clin Infect Dis.* (2003) 36:663–8. doi: 10.1086/367658
118. 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. doi: 10.1136/sextrans-2017-053133
119. Tuominen H, Rautava S, Nen SSX, Collado MC, Rautava J. HPV infection and bacterial microbiota in the placenta, uterine cervix and oral mucosa. *Sci Rep.* (2018) 8:9787. doi: 10.1038/s41598-018-27980-3
120. Martin HL, Richardson BA, Nyange PM, Lavreys L, Hillier SL, Chohan B, et al. *Vaginal lactobacilli*, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *J Infect Dis.* (1999) 180:1863–8. doi: 10.1086/315127
121. Di Paola M, Sani C, Clemente AM, Iossa A, Perissi E, Castronovo G, et al. Characterization of cervico-vaginal microbiota in women developing persistent high-risk human papillomavirus infection. *Sci Rep.* (2017) 7:10200. doi: 10.1038/s41598-017-09842-6
122. Shannon B, Yi TJ, Perusini S, Gajer P, Ma B, Humphrys MS, et al. Association of HPV infection and clearance with cervicovaginal immunology and the vaginal microbiota. *Mucosal Immunol.* (2017) 10:1310–9. doi: 10.1038/mi.2016.129
123. Dareng EO, Ma B, Famooto AO, Adebamowo SN, Offiong RA, Olaniyan O, et al. Prevalent high-risk HPV infection and vaginal microbiota in Nigerian women. *Epidemiol Infect.* (2016) 144:123–37. doi: 10.1017/S0950268815000965
124. Lee JE, Lee S, Lee H, Song Y-M, Lee K, Han MJ, et al. Association of the vaginal microbiota with human papillomavirus infection in a Korean twin cohort. *PLoS One.* (2013) 8:e63514. doi: 10.1371/journal.pone.0063514
125. Chen Y, Hong Z, Wang W, Gu L, Gao H, Qiu L, et al. Association between the vaginal microbiome and high-risk human papillomavirus infection in pregnant Chinese women. *BMC Infect Dis.* (2019) 19:677. doi: 10.1186/s12879-019-4279-6
126. Ritu W, Enqi W, Zheng S, Wang J, Ling Y, Wang Y. Evaluation of the associations between cervical microbiota and HPV infection, clearance, and persistence in cytologically normal women. *Cancer Prev Res (Phila).* (2019) 12:43–56. doi: 10.1158/1940-6207.CAPR-18-0233
127. van de Wijgert JHHM, Morrison CS, Cornelisse PGA, Munjoma M, Moncada J, Awio P, et al. Bacterial vaginosis and vaginal yeast, but not vaginal cleansing, increase HIV-1 acquisition in African women. *J Acquir Immune Defic Syndr.* (2008) 48:203–10. doi: 10.1097/QAI.0b013e3181743936
128. Borgdorff H, Tsivtsivadze E, Verhelst R, Marzorati M, Jurriaans S, Ndayisaba GF, et al. *Lactobacillus*-dominated cervicovaginal microbiota associated with reduced HIV/STI prevalence and genital HIV viral load in African women. *ISME J.* (2014) 8:1781–93. doi: 10.1038/ismej.2014.26
129. 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. doi: 10.1111/1574-6976.12029
130. Stapleton AE. The vaginal microbiota and urinary tract infection. *Microbiol Spectr.* (2016) 4:1–6. doi: 10.1128/microbiolspec.UTI-0025-2016
131. Kyono K, Hashimoto T, Nagai Y, Sakuraba Y. Analysis of endometrial microbiota by 16S ribosomal RNA gene sequencing among infertile patients: a single-center pilot study. *Reprod Med Biol.* (2018) 17:297–306. doi: 10.1002/rmb2.12105
132. Bernabeu A, Lledo B, Díaz MC, Lozano FM, Ruiz V, Fuentes A, et al. Effect of the vaginal microbiome on the pregnancy rate in women receiving assisted reproductive treatment. *J Assist Reprod Genet.* (2019) 36:2111–9. doi: 10.1007/s10815-019-01564-0
133. Koedooder R, Singer M, Schoenmakers S, Savelkoul PHM, Morré SA, de Jonge JD, et al. The vaginal microbiome as a predictor for outcome of in vitro fertilization with or without intracytoplasmic sperm injection: a prospective study. *Hum Reprod.* (2019) 34:1042–54. doi: 10.1093/humrep/dez065
134. Practice Committee of American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss: a committee opinion. *Fertil Steril.* (2013) 99:63. doi: 10.1016/j.fertnstert.2012.09.023

135. Practice Committee of the American Society for Reproductive. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. *Fertil Steril.* (2012) 5:1103–11. doi: 10.1016/j.fertnstert.2012.06.048
136. Kitaya K, Matsubayashi H, Yamaguchi K, Nishiyama R, Takaya Y, Ishikawa T, et al. Chronic endometritis: potential cause of infertility and obstetric and neonatal complications. *Am J Reprod Immunol.* (2015) 75:13–22. doi: 10.1111/aji.12438
137. Cicinelli E, Matteo M, Tinelli R, Pinto V, Marinaccio M, Indraccolo U, et al. Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage as confirmed by improved pregnancy outcome after antibiotic treatment. *Reprod Sci.* (2014) 21:640–7. doi: 10.1177/1933719113508817
138. Kitaya K. Prevalence of chronic endometritis in recurrent miscarriages. *Fertil Steril.* (2011) 95:1156–8. doi: 10.1016/j.fertnstert.2010.09.061
139. Johnston-MacAnanny EB, Hartnett J, Engmann LL, Nulsen JC, Sanders MM, Benadiva CA. Chronic endometritis is a frequent finding in women with recurrent implantation failure after in vitro fertilization. *Fertil Steril.* (2010) 93:437–41. doi: 10.1016/j.fertnstert.2008.12.131
140. Kasius JC, Broekmans FJM, Sie-Go DMDS, Bourgain C, Eijkemans MJC, Fauser BC, et al. The reliability of the histological diagnosis of endometritis in asymptomatic IVF cases: a multicenter observer study. *Hum Reprod.* (2011) 27:153–8. doi: 10.1093/humrep/der341
141. Vitagliano A, Saccardi C, Noventa M, Di Spiezio Sardo A, Saccone G, Cicinelli E, et al. Effects of chronic endometritis therapy on in vitro fertilization outcome in women with repeated implantation failure: a systematic review and meta-analysis. *Fertil Steril.* (2018) 110:103–12.e1. doi: 10.1016/j.fertnstert.2018.03.017
142. Kitaya K, Tada Y, Hayashi T, Taguchi S, Funabiki M, Nakamura Y. Comprehensive endometrial immunoglobulin subclass analysis in infertile women suffering from repeated implantation failure with or without chronic endometritis. *Am J Reprod Immunol.* (2014) 72:386–91. doi: 10.1111/aji.12277
143. Solt I. The human microbiome and the great obstetrical syndromes: a new frontier in maternal-fetal medicine. *Best Pract Res Clin Obstet Gynaecol.* (2015) 29:165–75. doi: 10.1016/j.bpobgyn.2014.04.024
144. Vinturache AE, Gyamfi-Bannerman C, Hwang J, Mysorekar IU, Jacobsson B, Prebic TPBIC. Maternal microbiome – a pathway to preterm birth. *Semin Fetal Neonatal Med.* (2016) 21:94–9. doi: 10.1016/j.siny.2016.02.004
145. Brown RG, Marchesi JR, Lee YS, Smith A, Lehne B, Kindinger LM, et al. Vaginal dysbiosis increases risk of preterm fetal membrane rupture, neonatal sepsis and is exacerbated by erythromycin. *BMC Med.* (2018) 16:9. doi: 10.1186/s12916-017-0999-x
146. Peelen MJ, Luef BM, Lamont RF, de Milliano I, Jensen JS, Limpens J, et al. The influence of the vaginal microbiota on preterm birth: a systematic review and recommendations for a minimum dataset for future research. *Placenta.* (2019) 79:30–9. doi: 10.1016/j.placenta.2019.03.011
147. Fettweis JM, Serrano MG, Brooks JP, Edwards DJ, Girerd PH, Parikh HI, et al. The vaginal microbiome and preterm birth. *Nat Med.* (2019) 25:1–28. doi: 10.1038/s41591-019-0450-2
148. Dunlop AL, Mulle JG, Ferranti EP, Edwards S, Dunn AB, Corwin EJ. Maternal microbiome and pregnancy outcomes that impact infant health. *Adv Neonat Care.* (2015) 15:377–85. doi: 10.1097/ANC.0000000000000218
149. Romero R, Hassan S, Gajer P, Tarca AL, Fadrosh DW, Bieda J, et al. The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. *Microbiome.* (2014) 2:31–15. doi: 10.1186/2049-2618-2-18
150. Callahan BJ, DiGiulio DB, Goltsman DSA, Sun CL, Costello EK, Jeganathan P, et al. Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc Natl Acad Sci USA.* (2017) 114:9966–71. doi: 10.1073/pnas.1705899114
151. Son K-A, Kim M, Kim YM, Kim SH, Choi S-J, Oh S-Y, et al. Prevalence of vaginal microorganisms among pregnant women according to trimester and association with preterm birth. *Obstet Gynecol Sci.* (2018) 61:38–10. doi: 10.5468/ogs.2018.61.1.38
152. Stout MJ, Zhou Y, Wylie KM, Tarr PI, Macones GA, Tuuli MG. Early pregnancy vaginal microbiome trends and preterm birth. *Am J Obstet Gynecol.* (2017) 217:356.e1–e8. doi: 10.1016/j.ajog.2017.05.030
153. Solt I, Cohavy O. The great obstetrical syndromes and the human microbiome – a new frontier. *Rambam Maimonides Med J.* (2012) 3:e0009. doi: 10.5041/RMMJ.10076
154. Lawn JE, Cousens S, Zupan J. Lancet neonatal survival steering team. 4 million neonatal deaths: when? where? why? *Lancet.* (2005) 365:891–900. doi: 10.1016/S0140-6736(05)71048-5
155. Kim CJ, Romero R, Chaemsaithong P, Chaiyasit N, Yoon BH, Kim YM. Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance. *Am J Obstet Gynecol.* (2015) 213:S29–52. doi: 10.1016/j.ajog.2015.08.040
156. Fortner KB, Grotegut CA, Ransom CE, Bentley RC, Feng L, Lan L, et al. Bacteria localization and chorion thinning among preterm premature rupture of membranes. *PLoS One* (2014) 9:e83338. doi: 10.1371/journal.pone.0083338
157. Steel JH, Malatos S, Kennea N, Edwards AD, Miles L, Duggan P, et al. Bacteria and inflammatory cells in fetal membranes do not always cause preterm labor. *Pediatr Res.* (2005) 57:404–11. doi: 10.1203/01.PDR.0000153869.96337.90
158. Watts DH, Krohn MA, Hillier SL, Eschenbach DA. The association of occult amniotic fluid infection with gestational age and neonatal outcome among women in preterm labor. *Obstet Gynecol.* (1992) 79:351–7. doi: 10.1097/00006250-199203000-00005
159. Ilekis JV, Tsilou E, Fisher S, Abrahams VM, Soares MJ, Cross JC, et al. Placental origins of adverse pregnancy outcomes: potential molecular targets: an executive workshop summary of the eunice kennedy shriver national institute of child health and human development. *Am J Obstet Gynecol.* (2016) 15(Suppl. 1):S1–46. doi: 10.1016/j.ajog.2016.03.001
160. Barker DJ. Adult consequences of fetal growth restriction. *Clin Obstet Gynecol.* (2006) 49:270–83. doi: 10.1097/00003081-200606000-00009
161. Budding AE, Grasman ME, Lin F, Bogaards JA, Soeltan-Kaersenhout DJ, Vandenbroucke-Grauls CMJE, et al. IS-pro: high-throughput molecular fingerprinting of the intestinal microbiota. *FASEB J.* (2010) 24:4556–64. doi: 10.1096/fj.10-156190
162. Riscuta G, Xi D, Pierre-Victor D, Starke-Reed P, Khalsa J, Duffy L. Diet, microbiome, and epigenetics in the era of precision medicine. *Methods Mol Biol.* (2018) 1856:141–56. doi: 10.1007/978-1-4939-8751-1_8
163. Ruiz L, Moles L, Gueimonde M, Rodríguez JM. Perinatal microbiomes" Influence on Preterm Birth and Preterms" health: influencing factors and modulation strategies. *J Pediatr Gastroenterol Nutr.* (2016) 63:e193–203. doi: 10.1097/MPG.0000000000001196
164. Sharma H, Tal R, Clark N, Segars J. Microbiota and pelvic inflammatory disease. *Semin Reprod Med.* (2014) 32:43–9. doi: 10.1055/s-0033-1361822
165. Wallace DC. Bioenergetics and the epigenome: interface between the environment and genes in common diseases. *Dev Disabil Res Rev.* (2010) 16:114–9. doi: 10.1002/ddrr.113
166. Jasiulionis MG. Abnormal epigenetic regulation of immune system during aging. *Front Immunol.* (2018) 9:197. doi: 10.3389/fimmu.2018.00197
167. Niemarkt HJ, De Meij TG, van Ganzewinkel C-J, de Boer NKH, Andriessen P, Hütten MC, et al. Necrotizing enterocolitis, gut microbiota, and brain development: role of the brain-gut axis. *Neonatology.* (2019) 115:423–31. doi: 10.1159/000497420
168. Baldassarre ME, Di Mauro A, Capozza M, Rizzo V, Schettini F, Panza R, et al. Dysbiosis and prematurity: is there a role for probiotics? *Nutrients.* (2019) 11:1273–1212. doi: 10.3390/nu11061273
169. Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, et al. Is meconium from healthy newborns actually sterile? *Res Microbiol.* (2008) 159:187–93. doi: 10.1016/j.resmic.2007.12.007
170. Rautava S, Kainonen E, Salminen S, Isolauri E. Maternal probiotic supplementation during pregnancy and breast-feeding reduces the risk of eczema in the infant. *J Allergy Clin Immunol.* (2012) 130:1355–60. doi: 10.1016/j.jaci.2012.09.003
171. Gomez de Agüero M, Ganal-Vonarburg SC, Fuhrer T, Rupp S, Uchimura Y, Li H, et al. The maternal microbiota drives early postnatal innate immune development. *Science.* (2016) 351:1296–302. doi: 10.1126/science.aad2571
172. Goldenberg RL, McClure EM, Saleem S, Reddy UM. Infection-related stillbirths. *Lancet.* (2010) 375:1482–90. doi: 10.1016/S0140-6736(09)61712-8

173. Willyard C. Could baby's first bacteria take root before birth? *Nature.* (2018) 553:264–6. doi: 10.1038/d41586-018-00664-8
174. Stinson LF, Payne MS, Keelan JAA. Critical review of the bacterial baptism hypothesis and the impact of cesarean delivery on the infant microbiome. *Front Med.* (2018) 5:135. doi: 10.3389/fmed.2018.00135
175. Dominguez-Bello MG, De Jesus-Laboy KM, Shen N, Cox LM, Amir A, Gonzalez A, et al. Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. *Nat Med.* (2016) 22:250–3. doi: 10.1038/nm.4039
176. Reardon S. Do C-section babies need mum's microbes? Trials tackle controversial idea. *Nature.* (2019) 572:423–4. doi: 10.1038/d41586-019-02348-3
177. Committee on Obstetric Practice. Committee opinion no. 725 vaginal seeding. *Obstet Gynecol.* (2017) 130:e274–8.

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Immunological Role of the Maternal Uterine Microbiota in Postpartum Hemorrhage

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Recent metagenomics and microbiology studies have identified microorganisms that are typical of the fetoplacental unit. Considering this emerging evidence, the placenta, uterus, and the amniotic cavity are not sterile and not immune privileged. However, there is evidence for a beneficial interaction between active maternal immune system and the presence of commensal pathogens, which lead to an immune-tolerant state, thereby preventing fetal rejection. Multiple conditions associated with the loss of the normal flora are described (dysbiosis), which could result in perinatal and puerperal adverse events, including, directly or indirectly, postpartum hemorrhage. Altered flora when associated with a severe proinflammatory state and combined with patient's genetic and environmental factors confers a high-risk adverse outcome. Better understanding of the adverse role of dysbiosis in pregnancy outcome will improve maternal outcome.

Keywords: flora, microorganisms, placenta, uterus, pregnancy, preterm labor, postpartum hemorrhage

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IMMUNOLOGY OF PREGNANCY AND THE ROLE OF UTERINE AND PLACENTAL MICROBIOTA

In pregnancy, there is an increased predisposition toward infectious complications secondary to the transient and physiological immunocompromise. The amniotic cavity and choriodecidual junction have been traditionally described as immune-privileged entities, free of inflammatory response or microbiological growth. Nevertheless, the presence of infiltrating cells belonging to the maternal immune system in the choriodecidual region has been recently reported. Approximately 70% of decidual leukocytes are natural killer (NK) cells, 20 to 25% are macrophages, 3 to 10% are T cells, and 1.7% are dendritic cells. The described immune cell composition suggests that the rejection response that would be triggered by the implanted semi/allogeneic blastocyst, just like an allotransplant, is prevented. Paternal contribution has been considered as an exogenous component, translated to persistent immune tolerance to maternal cell infiltrates, thereby avoiding rejection processes (1). However, the theory of a decrease in immune response still remains controversial, especially because immune tolerance is necessary for a favorable pregnancy outcome. It is known that inactivation of NK cells could interfere with regulation of trophoblastic invasion contributing to abnormal placentation, which is a predisposing factor to postpartum hemorrhage (PPH) (2). Additionally, transduction of inhibitory factors involved in the inflammatory cascade such as FAS and FAS-L–associated ligands could result in miscarriage or stillbirth due to difficulties to maintain pregnancy (1). There are two proinflammatory processes related to pregnancy, predominantly represented by a T_H1 response (it was previously considered to be absent during

gestation and associated with adverse perinatal outcome). However, both the implantation process and labor involve, to some degree, inflammatory processes (1).

On the other hand, partial suppression of the T_H2 response is required for fetal growth and development. Maternal dendritic cells that are found in the decidua present fetal antigens to maternal T lymphocytes seeking immunological tolerance. In contrast, in organ transplants, dendritic cells belonging to the transplanted organ present foreign antigens to the host's T lymphocytes (1). During this stage of gestation, anti-inflammatory cytokines are also synthesized to avoid adverse outcome. Regardless of the presence of decidual inflammatory cell infiltrates, trophoblastic development stimulates cytokine/chemokine release, such as [CXCL12, interleukin 18 (IL-18), and transforming growth factor β], promoting infiltration of immune system cells. This has been thought to improve pregnancy homeostasis, and any disturbance with this process could alter the pregnancy's outcome (3).

Additionally, recent advances in metagenomics have shown that the placenta harbors its own rich and diverse microbiota, which has been studied and described in healthy pregnancies (4). Altered placental microbiota (dysbiosis) induces a proinflammatory state leading to adverse maternal outcomes such as preterm labor, chorioamnionitis, premature rupture of membranes, intrauterine growth restriction, and even PPH (5).

The precise role of the uterine and placental microbiota in inducing immune tolerance to maternal and paternal antigens during gestation is still not clear. It is viewed that the microbiota works synergistically with the maternal inflammatory infiltrate and the trophoblast, preventing rejection (1). This likely related to the trophoblastic, macrophage, and decidual cell synthesis of the Toll-like receptor 4. This receptor induces interferon type 1 when exposed to microbial lipopolysaccharides that is present in the normal microbiota (1). Interferons are polypeptides that have 3 basic roles: (1) develop antimicrobial environment, (2) modulate the innate immune system, and (3) activate the adaptive immune system. In this way, the microbiota present in the maternal–fetal interface could be responsible for the basal expression of peptides, allowing modulation of the maternal immune system during fetal development and preventing the colonization by pathogenic microorganisms (1).

Yet, it is fundamental to recognize the existence of microorganisms that are present in fetal meconium and umbilical cord blood (6, 7). This confirms that the fetus is not sterile and that possibly the maternal microbiota is essential for development of the fetal microbiota, as well as acts as initiating stimulus for the fetal and neonatal innate immune system (4).

It becomes evident that the presence of microorganisms in these areas might not lead to adverse perinatal outcomes. In contrast, it could lead to the development of protective mechanisms to avoid such adverse outcomes. Different environmental or innate factors could alter the usual composition and function of microbiota during pregnancy. These processes allow pregnancy preservation and the normal uterine function during the immediate puerperium.

COMPOSITION AND VARIATIONS OF UTERINE, PLACENTAL, AND VAGINAL MICROBIOTA

Generally, human microbiota's composition depends on its location, the host's genetic composition, dietary intake, and immune status (4). The female's reproductive system's microbiota changes according to her hormonal status during the menstrual cycle, as well as during the perigestational period (preconception and postconception) (4). Non-pregnant woman's vaginal microbiome has a great variety of microorganisms, with significant changes related to hormonal influx, age, and estrogen concentrations, which condition the acidity of the vaginal environment. This composition prevents the colonization of pathogens that do not belong to the vaginal milieu (5). More than 20 species of *Lactobacillus* species have been described, a dominant species in quantity as compared to the other commensal microorganisms. They oversee the production of lactic acid and compete for the vaginal wall surface (nutrients and cellular receptors), avoiding colonization by other pathogens. In a lesser proportion, there are also anaerobic organisms such as *Prevotella*, *Megasphaera*, *Gardnerella vaginalis*, *Sneathia*, and *Atopobium vaginae* (5).

The interaction between host microorganisms and those taking place among microorganisms start before birth and tend to vary among individuals especially due to their hormonal status. This leads to a high concentration of *Lactobacillus* species, *Propionibacterium* species, and Enterobacteriaceae presence during pregnancy in the healthy placental tissue (infection-free) (8–10). It is still unclear how microorganisms manage to enter the fetoplacental compartment. It is believed that the bacteria can ascend through the vagina and can be transported by antigen-presenting cells and carried into the amniotic cavity or colonize by hematogenous dissemination. The ascending path gains relevance considering the great portion of the vaginal microbiota that are incorporated in the placental microbiome are *Lactobacillus* species (5).

In a pregnant woman, it has been demonstrated that the composition of vaginal microbiota tends to fluctuate less than in a non-pregnant woman. During this period, *Lactobacillus* bacteria continue to prevail, but with the increase in four additional strains (*Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, and *Lactobacillus vaginalis*), leading to a decrease in anaerobic species. The increased estrogen levels in pregnant women lead to increased deposits of glycogen in the vaginal epithelium, which provides a better substrate for the growth of *Lactobacillus*, as well as the possibility for higher protective lactic acid production (5).

Several studies confirmed that during pregnancy *Lactobacillus* strain-specific vaginal microbiome could change according to the gestational age (11, 12). In a longitudinal study of the vaginal microbiota of 22 patients with non-complicated births, slight and occasional changes among diverse communities of *Lactobacillus* were found. However, the changes related to anaerobic bacteria predominance were rather rare (13). These changes were associated with lesser hormonal variability during

gestation, decrease in sexual activity, or changes in production of vaginal secretions (5).

Microorganisms from the oral cavity and the gastrointestinal tract such as Enterobacteriaceae have been found in the placental environment. This has been associated with increased immune tolerance during pregnancy, which enables bacterial translocation from the gastrointestinal system to the bloodstream. This would create an access through the blood stream from different organ systems toward the uterine cavity (4).

PLACENTAL MICROBIOTA BEHAVIOR IN PREGNANCY WITH ADVERSE OUTCOMES

The microbial invasion (with non-commensal microorganisms) of the fetoplacental junction has been associated with maternal and neonatal morbidity. Studies showed the existence of different microorganisms in the placenta and the amniotic cavity with miscarriage, chorioamnionitis, premature rupture of the membranes, preterm birth, and stillbirth (14, 15). However, new evidence has also determined that the same type of pathogens might be present in births without associated complications. Therefore, genetic and/or environmental mechanisms may enable the progress of adverse perinatal outcomes due to the existence of the germs in a specific gestational stage (4). Alterations in the placental and amniotic microbiome can be associated with different pathologies during gestation, as well as can be associated with bacterial vaginosis (4).

Studies based on the analysis of nucleic acids of the placental tissue in pathologic pregnancies showed a predominance of anaerobic germs over beneficial *Lactobacillus*. The placental tissue analyzed in cases of preterm birth have found a higher number of *Prevotella*, *Bacteroides*, *Peptostreptococcus*, *Gardnerella*, *Mobiluncus*, and *Mycoplasma* species (16–19). *Streptococcus agalactiae*, *Fusobacterium nucleatum*, and *Ureaplasma parvum* species have been found in women with chorioamnionitis at a higher proportion (4). These microorganisms tend to have lower virulence outside the intrauterine environment. For example, *F. nucleatum* is an anaerobic microorganism that is generally located in the oral cavity mucosa. Nevertheless, the hematogenous spread of the same bacteria toward the placenta modifies the endothelial permeability of placental vasculature, which allows the entrance of other pathogenic organisms locally including *Escherichia coli* (5).

The analysis of oral bacteria such as *Fusobacterium* or *Capnocytophaga* in the placenta samples of women with preterm birth has been linked to periodontal disease that developed toward the end of gestation (20, 21). However, it is important to recognize that not only microorganisms from the oral cavity have been associated with these types of modified flora. As pregnancy progresses, there is a change in the gastrointestinal microbiota related to hormonal variations. Studies have shown dramatic changes in the composition of fecal flora of pregnant women from the first trimester until the third trimester, with increases in Proteobacteria and Actinobacteria content and decreases in *Lactobacillus* (5). This modification in colonizing germs may benefit from physiological changes in the maternal immune milieu, mostly becoming evident toward the end of the pregnancy. Such altered flora is translocated toward the bloodstream, reaching the amniotic and placental cavity, and creating a proinflammatory environment.

The ascent of vaginal colonizing pathogens to the uterine cavity has been etiologically linked with preterm birth. Hormonal and changing maternal environments lead to decrease in *Lactobacillus* species, allowing the colonization and access of pathogenic germs to the uterine cavity (5). A prospective cohort of 88 patients examining this association concluded that there is a correlation between microbial vaginal diversity and the progress to preterm birth (5). The pathogens that do not belong to the vaginal microbiota promote inflammatory mechanisms affecting the fetus and choriodecidual tissues. For example, secondary intra-amniotic infection due to unusual microorganisms triggers an inflammatory cascade associated with a large increase in the release of prostaglandins, metalloproteinases, and proinflammatory cytokines, which promote uterine activity leading to a major decrease in cervical collagen content (5).

THE RELATION BETWEEN PLACENTAL AND UTERINE MICROBIOTA AND POSTPARTUM HEMORRHAGE

The changes in the uterine and placental microbiota could determine an increase in PPH risk. After delivery, a state of acute postpartum myometritis with local inflammatory phenomena is seen, and the secondary dysbiosis causes muscular fatigue leading to PPH. This causality has been demonstrated in patients having significant hemorrhagic episodes of unclear etiology (22).

The most frequent cause of PPH is uterine atony (75% of cases). Multiple risk factors antepartum and intrapartum have been described for this clinical presentation (23), in those where it could be correlated with modification of the microbiota. For some of these risk factors, it is plausible and of clear etiology that explains the myometrial fiber dysfunction inability to contract. However, in many cases (up to 30% of the patients do not have risk factors), the phenomena could be secondary to local dysbiosis (22). Through immunofluorescence studies of uterine cavity samples, an increased association of inflammatory cells such as neutrophils, macrophages, and mastocytes was evidenced in patients with PPH of unclear etiology as compared to patients without associated hemorrhagic episodes. These immune cells are a principal source for chemical mediators exerting potent effect on the smooth vascular and uterine muscle. The accumulation of local uterine exudate that is associated with inflammatory response in interstitial spaces progresses to stromal edema and damage to uterine contractile function (22).

The term "acute postpartum myometritis" has been proposed to define local inflammation when there is no association with infection. Therefore, many theories related to the exposure

TABLE 1 | PPH risk factors and uterine microbiome alterations.

Medium risk	Possibly related to dysbiosis	High risk	Possibly related to dysbiosis
ANTEPARTUM RISK FACTORS			
Previous cesarean section, uterine surgery, or multiple laparotomy	Yes	Placenta previa (24, 25)	Yes
Multiple gestation	No data	Placenta accreta	Yes
>4 Previous births	No	Platelets <70,000	Yes
Uterine fibroids	Yes	Known coagulopathy	Yes
Fetus estimated <4,000 g	No	Abruptio placentae	Yes
Obesity high (body mass index >40 kg/m^2)	No	>2 Intermediate risk factors	Factor dependent
Hematocrit <30% plus another risk factor	No data	–	–
INTRAPARTUM RISK FACTORS			
Chorioamnionitis	Yes	Active vaginal bleeding	Yes
Oxytocin drip during >24 h	Yes	Preeclampsia with severity criteria	Yes
Prolonged second stage of labor	Yes	≥2 Intermediate risk factors antepartum/intrapartum	Factor dependent
Use of magnesium sulfate	No	–	–

of the maternal blood to amniotic fluid or fetal tissue have been suggested. This might promote the activation of the complement system, leading to increased neutrophil and macrophage activation and infiltration, as well as mastocyte degranulation in uterine and placental tissues, with the same deleterious effect on uterine contraction (22). Nevertheless, changes in the uterine microbiota and infiltration by pathogenic organisms (infection) may lead to the same response as described above. With this emerging insight, the local dysbiosis followed by activations in inflammatory cells and changes described in myometrial tissue could directly be associated with the development of PPH.

Similarly, **Table 1** lists the diverse risk factors medium and high resulting in PPH as they possibly are related to the altered microbiota. Most of such direct causality has not been established yet. At this point, they remain presumptive based on the paucity of available evidence while it opens the possibility for pursuing new lines of clinical investigation.

For some risk factors, the relationship with the altered microbiota is better defined. Maternal obesity or overweight is directly related to inflammatory mechanisms active in placental tissue, conditioning dysbiosis, and anomalies regarding fetal well-being (4). The increase in adipose tissue and the associated peripheral insulin resistance lead to an increase in cytokine levels that act directly to increase pathology severity (5). For instance, in diabetic patients, a decrease in *Acinetobacter* load in the gastrointestinal tract (a commensal organism in this location) was coupled with a decrease in eosinophilic levels and reduced placental anti-inflammatory gene expression, including IL-10 (4). These potential pathological mechanisms are currently being further investigated (26).

Acute and chronic abruptio placentae cause significant maternal and perinatal morbidity. However, at present, it is frequently difficult to establish a direct correlation because of the absence of appropriate placental monitoring (27). However, the clinical manifestations with altered patterns of uterine contractility and the increase in incidence of preterm birth cases, premature rupture of membrane, and preeclampsia support the hypothesis of a contribution by the presence of dysbiosis as an underlying physiopathology.

The most accepted hypothesis for placenta accreta etiology states that it is a spectrum of endometrial interface imperfection that leads to failed decidualization in the area of uterine scar, allowing an abnormal and profound anchoring of the villous trophoblast. What is the role of the microbiota in these abnormal implantation phenomena? That is a question that has not been addressed yet. However, there is evidence for the presence of proinflammatory biomarkers that identify such increased risk, which suggest involvement of dysbiosis in the placenta and amniotic cavity in patients with placenta accreta (28).

CONCLUSION

Microbiological load, typical of the human being, turns out to be beneficial and necessary in all areas in the body. Nevertheless, until recently, the amniotic cavity and the fetoplacental unit were considered as an immune-privileged area and free of associated inflammatory processes. The current evidence indicates that inflammatory mechanisms and the immunotolerance together are both necessary and coexist. It is possible that modification of the beneficial microbiota that is required for maintaining pregnancy leads to immune intolerance. Such enables development of a proinflammatory state leading to preterm birth, premature rupture of membranes, stillbirth, and PPH. It is very important

to broaden the microbiology and metagenomics investigations that allow recognizing the high impact of these types of associated phenomena, especially PPH, recognized worldwide to be associated with high lethality. Possibly by avoiding development of maternal dysbiosis, this huge clinical burden could be reduced.

AUTHOR CONTRIBUTIONS

ME, JB, and MH: contribution to the conception of the work, intellectual production, writing of the manuscript and critical revision, final approval of the manuscript, agreement to be accountable for all aspects of the work, research of information.

REFERENCES

1. Mor G, Aldo P, Alvero A. The unique immunological and microbial aspects of pregnancy. *Early Life Immunol Rev*. (2017) 17:469–81. doi: 10.1038/nri.2017.64
2. van Beekhuizen HJ, Joosten I, Lotgering FK, Bulten J, van Kempen LC. Natural killer cells and HLA-G expression in the basal decidua of human placenta adhesiva. *Placenta*. (2010) 31:1078–84. doi: 10.1016/j.placenta.2010.09.016
3. Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, et al. The preterm parturition syndrome. *BJOG*. (2006) 113(Suppl. 3):17–42. doi: 10.1111/j.1471-0528.2006.01120.x
4. Pelzer E, Gomez L, Barret H, Dekker M. Review: maternal health and the placental microbiome. *Placenta*. (2017) 54:30–7. doi: 10.1016/j.placenta.2016.12.003
5. Fox C, Eichelberg K. Maternal microbiome and pregnancy outcomes. *Fertil Steril*. (2015) 104:1358–63. doi: 10.1016/j.fertnstert.2015.09.037
6. Martin R, Makino H, Yavuz AC, Ben-Amor K, Roelofs M, Ishikawa E, et al. Early- life events, including mode of delivery and type of feeding, siblings and gender, shape the developing gut microbiota. *PLoS ONE*. (2016) 11:e0158498. doi: 10.1371/journal.pone.0158498
7. Jiménez E, Fernández L, Marín ML, Martín R, Odriozola JM, Nueno-Palop C, et al. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Curr Microbiol*. (2005) 51:270–4. doi: 10.1007/s00284-005-0020-3
8. Prince AL, Ma J, Kannan PS, Alvarez M, Gisslen T, Harris RA, et al. The placental membrane microbiome is altered among subjects with spontaneous preterm birth with and without corioamnionitis. *Am J Obstet Gynecol*. (2016) 214:627.e1–627.e16. doi: 10.1016/j.ajog.2016.01.193
9. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci Rep*. (2016) 6:23129. doi: 10.1038/srep23129
10. DiGiulio DB, Callahan BJ, McMurdie PJ, Costello EK, Lyell DJ, Robaczewska A, et al. Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci USA*. (2015) 112:11060–5. doi: 10.1073/pnas.1502875112
11. Aagaard K, Riehle K, Ma J, Segata N, Mistretta TA, Coarfa C, et al. A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. *PLoS One*. (2012) 7:e36466. doi: 10.1371/journal.pone.0036466
12. Prince A, Antony K, Ma J, Aagaard K. The microbiome and development: a mother's perspective. *Semin Reprod Med*. (2014) 32:14–22. doi: 10.1055/s-0033-1361818
13. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Nikita L, et al. The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome*. (2014) 2:4. doi: 10.1186/2049-2618-2-10
14. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Sci Transl Med*. (2014) 6:237ra65. doi: 10.1126/scitranslmed.3008599
15. Solt I. The human microbiome and the great obstetrical syndromes: a new frontier in maternal-fetal medicine. *Best Pract Res Clin Obstet Gynaecol*. (2015) 29:165–75. doi: 10.1016/j.bpobgyn.2014.04.024
16. Onderdonk AB, Delaney ML, DuBois AM, Allred EN, Leviton A, Extremely Low Gestational Age Newborns (ELGAN) Study Investigators. Detection of bacteria in placental tis- sues obtained from extremely low gestational age neonates. *Am J Obstet Gynecol*. (2008) 198:110.e1–7. doi: 10.1016/j.ajog.2007.05.044
17. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture- based investigation. *PLoS ONE*. (2008) 3:e3056. doi: 10.1371/journal.pone.0003056
18. Onderdonk AB, Hecht JL, McElrath TF, Delaney ML, Allred EN, Leviton A, et al. Colonization of second-trimester placenta parenchyma. *Am J Obstet Gynecol*. (2008) 199:52.e1–52.e10. doi: 10.1016/j.ajog.2007.11.068
19. DiGiulio DB, Romero R, Kusanovic JP, Gómez R, Kim CJ, Seok KS, et al. Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm pre-labor rupture of membranes. *Am J Reprod Immunol*. (2010) 64:38–57. doi: 10.1111/j.1600-0897.2010.00830.x
20. Collado MC, Isolauri E, Laitinen K, Salminen S. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. *Am J Clin Nutr*. (2008) 88:894–9. doi: 10.1093/ajcn/88.4.894
21. Fardini Y, Wang X, Temoin S, Nithianantham S, Lee D, Shoham M, et al. Fu- sobacterium nucleatum adhesion FadA binds vascular endothelial cadherin and alters endothelial integrity. *Mol Microbiol*. (2011) 82:1468–80. doi: 10.1111/j.1365-2958.2011.07905.x
22. Farhana M, Tamura N, Mukai M, Ikuma K, Koumura Y, Furuta N, et al. Histological characteristics of the myometrium in the postpartum hemorrhage of unknown etiology: a possible involvement of local immune reactions. *J Reprod Immunol*. (2015) 110:74–80. doi: 10.1016/j.jri.2015.04.004
23. Andrikopoulou M, DÁlton ME. Postpartum hemorrhage: early identification challenges. *Semin Perinatol*. (2019) 43:11–7. doi: 10.1053/j.semperi.2018.11.003
24. Aydogan P, Kahyaoglu S, Saygan S, Kaymak O, Mollamahmutoglu L, Danisman N. Does cervical ureaplasma/mycoplasma colonization increase the lower uterine segment bleeding risk during cesarean section among patients with placenta previa? A cross-sectional study. *Eur Rev Med Pharmacol Sci*. (2014) 18:2243–7. Available online at: https://www.europeanreview.org/article/7688
25. Park C, Moon K, Park J, Jun J, Yoon B. The frequency and clinical significance of intra-uterine infection and inflammation in patients with placenta previa and preterm labor and intact membranes. *Placenta*. (2009) 30:613–8. doi: 10.1016/j.placenta.2009.04.005
26. Taricco E, Radaelli T, Rossi G, Nobile de Santis M, Bulfamante G, Avagliano L, et al. Effects of gestational diabetes on fetal oxygen and glucose levels *in vivo*. *BJOG*. (2009) 116:1729–35. doi: 10.1111/j.1471-0528.2009.02341.x
27. Downes KL, Grantz KL, Shenassa ED. Maternal, labor, delivery, and perinatal outcomes associated with placental abruption: a systematic review. *Am J Perinatol*. (2017) 34:935–57. doi: 10.1055/s-0037-1599149
28. American College of Obstetricians and Gynecologists and the Society for Maternal– Fetal Medicine. Obstetric Care Consensus No. 7: placenta accreta spectrum. *Obstet Gynecol*. (2018) 132:e259–75. doi: 10.1097/AOG.0000000000002983

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REVIEW
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Recent Insights on the Maternal Microbiota: Impact on Pregnancy Outcomes

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Hormonal changes during and after pregnancy are linked with modifications in the maternal microbiota. We describe the importance of the maternal microbiota in pregnancy and examine whether changes in maternal microbiotic composition at different body sites (gut, vagina, endometrium) are associated with pregnancy complications. We analyze the likely interactions between microbiota and the immune system. During pregnancy, the gastrointestinal (gut) microbiota undergoes profound changes that lead to an increase in lactic acid–producing bacteria and a reduction in butyrate-producing bacteria. The meaning of such changes needs clarification. Additionally, several studies have indicated a possible involvement of the maternal gut microbiota in autoimmune and lifelong diseases. The human vagina has its own microbiota, and changes in vaginal microbiota are related to several pregnancy-related complications. Recent studies show reduced lactobacilli, increased bacterial diversity, and low vaginal levels of beta-defensin 2 in women with preterm births. In contrast, early and healthy pregnancies are characterized by low diversity and low numbers of bacterial communities dominated by *Lactobacillus*. These observations suggest that early vaginal cultures that show an absence of *Lactobacillus* and polymicrobial vaginal colonization are risk factors for preterm birth. The endometrium is not a sterile site. Resident endometrial microbiota has only been defined recently. However, questions remain regarding the main components of the endometrial microbiota and their impact on the reproductive tract concerning both fertility and pregnancy outcomes. A classification based on endometrial bacterial patterns could help develop a microbiota-based diagnosis as well as

personalized therapies for the prevention of obstetric complications and personalized treatments through nutritional, microbiotic, or pharmaceutical interventions.

Keywords: microbiota, pregnancy, immunity, gut, vagina, endometrium, inflammasome

INTRODUCTION

The human body is host to a community of microorganisms, including viruses, bacteria, and fungi. The bacterial component of this community, the microbiota, is known to influence health given its symbiotic relationship with the human host.

Vertical transmission of bacteria from mother to newborn contributes to developing the microbiota of the infant gastrointestinal (gut); emerging evidence suggests that this influence may begin in utero (1).

Important changes in the maternal gut microbiota have been observed during pregnancy. These changes are associated with an increase in maternal body weight and dietary changes. Physiological maternal metabolic modifications maintain maternal hyperglycemia and provide glucose to the growing fetus (2). The growth of bacterial species that can synthesize glycogen is stimulated in the presence of increased concentrations of glucose (3). In fact, the transcriptomic pattern of the maternal gut microbiota shows microbiotic changes related to hyperglycemia, especially in the third trimester (4).

The maternal gut microbiota may influence the growth of bacteria in the newborn's gut, affecting its function and the development of the immune system (5). How the microbiota impacts the immune system in the short and long terms is a critical concern. The microbiota is involved in the regulation of T cell expansion, the development and function of macrophages, and neutrophil chemotaxis (6–8). A major contribution to this debate illustrates how the transient colonization of pregnant female mice with engineered *Escherichia coli* may modify the levels of intestinal innate immune composition in mother and offspring. This suggests that gut maternal microorganisms may play a role in the regulation of the immune system of newborns (9). Vitamin synthesis, gut barrier function, and development of the immune system are essential functions of human health that develop alongside the expansion of the gut microbiota (9). Thus, any changes in the maturation of the gut microbiota in the infant may influence future bacterial colonization and the development of the immune system with possible health consequences.

Despite various scholarly contributions on the maternal microbiota in late pregnancy, only empirical evidence can capture changes in the maternal microbiota at this stage. To date, several factors have been known to influence the human microbiota, such as ancestry, antibiotic use, lifestyle, dietary habits, exercise frequency, and body mass index (BMI) (10). This means that there is no unique health or disease indicator related to the microbiota, yet each individual has a different microbiota from that of others. Consequently, evaluating the microbiota in early pregnancy or even before pregnancy may be a useful tool to enable a personalized approach. Demonstration that an altered microbiota may be linked to maternal and fetal complications is an important target in personalized medicine.

THE MATERNAL MICROBIOTA AND PREGNANCY OUTCOMES

The Gastrointestinal Microbiota

During pregnancy, maternal fat deposition and food intake increase progressively. In the second and third trimesters, maternal metabolic changes include increased gluconeogenesis, lipolysis, and insulin resistance. Such an acquired diabetogenic condition is functional and induces maternal physiological hyperglycemia, which, in turn, increases glucose availability for the growing fetus. Therefore, significant changes in the maternal gut microbiota occur during pregnancy (2, 4, 11). Although scholars have explored the maternal microbiota in the third trimester of pregnancy, data on the changes in maternal microbiota during early pregnancy are scarce (2, 4, 11, 12).

The gut microbiota in the first trimester of pregnancy resembles the microbiota of healthy nonpregnant women (2, 4, 11). Women have unique gut microbiota that can be classified into different classes or enterotypes. In turn, these are characterized by different groups of bacteria (13, 14). Currently, three classes of enterotypes are recognized, each with its dominant group of bacteria: enterotype I, which is characterized by the presence of *Bacteroides*; enterotype II, characterized by *Prevotella*; and enterotype III, dominated by *Ruminococcus*. The three enterotypes have different and specific functions, producing energy from carbohydrates or proteins (13, 14). A different enterotype characterizes each individual and can be modified by various factors, including diet and BMI (15, 16). Recently, Barret et al. (16) analyze the maternal intestinal microbiota in early pregnancy by comparing an omnivorous diet to a vegetarian one. Women consuming a vegetarian diet showed an increase of bacterial clusters involved in lipid synthesis, which suggests alteration of fermentation and presence of bacterial species producing large amounts of short-chain fatty acids (enterotype II). Studies on gut microbiota in a sample of African women detect the prevalence of enterotype II (*Prevotella*). A diet rich in vegetables with low consumption of animal proteins and lipids allows the growth of bacterial clusters, which degrade the mucin-type glycoproteins that cover the gut mucosal layer. Conversely, a European diet rich in animal protein and lipids is associated with enterotype I (*Bacteroides*). This enterotype produces energy from proteins and carbohydrates (13, 14, 16).

An obese state has also been associated with microbiotic composition during gestation (2, 11, 17). Levels of *Bacteroides* and *Staphylococcus* are higher in the feces produced by overweight pregnant women compared to those with a healthy

weight (17). Additionally, in overweight and obese pregnant women, insulin and adipokines (adipose tissue–derived cytokines) correlate with alterations in bacterial abundance, confirming an association between the microbiota and the level of metabolic hormones and cytokines in pregnancy (18). The pregestational BMI contributes to an increased risk of obstetric complications through cellular and molecular processes that are poorly understood (19). Normal placental development and pregnancy success are largely dependent on angiogenic and vascular remodeling events that take place within the maternal–fetal interface (20–22). Several populations of leukocytes in the decidual microenvironment control the early stages of trophoblast invasiveness; uterine natural killer cells are the most abundant immune cell subtype within the decidua (23–26). These cells are key players in uterine vascular growth through the production of proangiogenic factors and tissue-remodeling cytokines (25–29). Obesity, accompanied by increased adipose tissue richness in macrophages, T and B lymphocytes, mast cells, and neutrophils, is associated with altered levels of proinflammatory cytokines. In addition, obese women show decreased levels of decidual uterine natural killer cells with reduced production of proangiogenic factors (30, 31).

Several studies support the hypothesis that changes in the gut microbiota during early pregnancy are associated with an increased risk of gestational diabetes and hypertension (32–35). Enriched abundance of *Blautia* and *Ruminococcus* has been observed in patients with diabetes (35). Gomez-Arango et al. (36) find that the abundance of *Odoribacter*, a butyric acid–producing bacterium, is negatively correlated with systolic blood pressure in pregnant women at 16 weeks of pregnancy. Lv et al. (35) find an important association between alterations in gut microbiota (dysbiosis) and early-onset preeclampsia (PE). They show that the composition of gut microbiota in patients with early-onset PE differed significantly from that in healthy pregnant women. They identified that the bacteria associated with PE were also associated with other host morbidities, including obesity, higher incidence of glucose metabolic disorders, proinflammatory states, and intestinal barrier dysfunction. In addition, these microorganisms correlated with host immune parameters, such as interleukin-6 and lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria. Overall, these findings suggest that an altered gut microbiota during early pregnancy (by acting on the maternal immune system and affecting the production of proinflammatory cytokines) may be involved in the development of pregnancy-related complications, such as early-onset PE.

In a recent study, we hypothesized that, if abnormal bacterial translocation across the epithelium occurs early in pregnancy (with LPS being a marker of increased bacterial translocation across the intestinal epithelium), then uterine innate immunity and obstetric outcome may be affected. We find that increased intestinal permeability in early pregnancy is associated with increased maternal levels of LPS, excessive inflammasome-mediated production of cytokines at the endometrial level, and last, increased risk of pregnancy loss (37, 38). Therefore, we suggest that, during early pregnancy, gut bacterial products from the intestinal lumen are translocated into the maternal circulation. This is likely associated with increased intestinal permeability and may increase the risk of obstetric complications (**Figure 1**).

During the third trimester of pregnancy, butyrate-producing bacteria with anti-inflammatory activities decline, whereas bifidobacteria, proteobacteria, and lactic acid–producing bacteria increase (2, 4, 11, 39). Additionally, the maternal gut microbiota grows less actively, reaching a stationary phase accompanied by reduced gut motility and increased intestinal permeability (2, 11, 39). Gastrointestinal modifications in the third trimester concern the host immune system of the gastrointestinal mucosa. Together with changes in metabolic, hormonal, and gastrointestinal permeability, these modifications may increase the diffusion of glucose from the gut epithelium toward the lumen and bacterial translocation. Collectively, these changes impact the composition of the gut microbiota and, consequently, maternal weight gain (4, 39, 40). Some of the proposed mechanisms by which the gut microbiota plays a role in host weight gain during pregnancy include enhanced absorption of glucose and fatty acids, increased fasting-induced adipocyte factor secretion, induction of catabolic pathways, and immune system stimulation (2, 11, 17, 39, 40).

The meaning of modifications in the gut microbiota has been investigated (2, 11, 41, 42). Notably, fecal transplantation of first- and third-trimester fecal microbiotas to germ-free mice revealed that mice transplanted with third-trimester microbiota gained significantly more weight, developed insulin resistance, and had a greater inflammatory response compared to mice transplanted with first trimester-microbiota (11). These findings demonstrate the direct role of microbiotic components in inducing changes in host immunology and metabolism. Interestingly, such modifications resemble those seen in metabolic syndrome, despite occurring in a physiological rather than pathological condition, such as being 7–9 months pregnant.

The gut microbiota during pregnancy is a critical determinant of offspring health (5, 18, 41, 42). Potentially, it determines the development of atopy and autoimmune phenotypes in the offspring (5). The commensal microbiota has a role in regulating host immunity to pathogens and autoimmune responses. Indeed, the microbiota is a source of metabolites and peptide ligands for T cell recognition, known as pathogen-associated molecular patterns (PAMPs), which are recognized by immune receptors. Microbiota-derived metabolites and PAMPs can affect target organs and activate the autoimmune cascade. This does not start after birth but may occur in the womb, engendering a predisposition of the progeny to disease (43). Despite this, the relationship between the immune system, gut microbiota, and metabolism of pregnant women is unclear.

The Vaginal Microbiota

The vaginal microbiota changes throughout a woman's reproductive life from puberty to menopause with variations during the menstrual cycle (44). In the healthy female reproductive tract, lactobacilli are dominant. One of the key

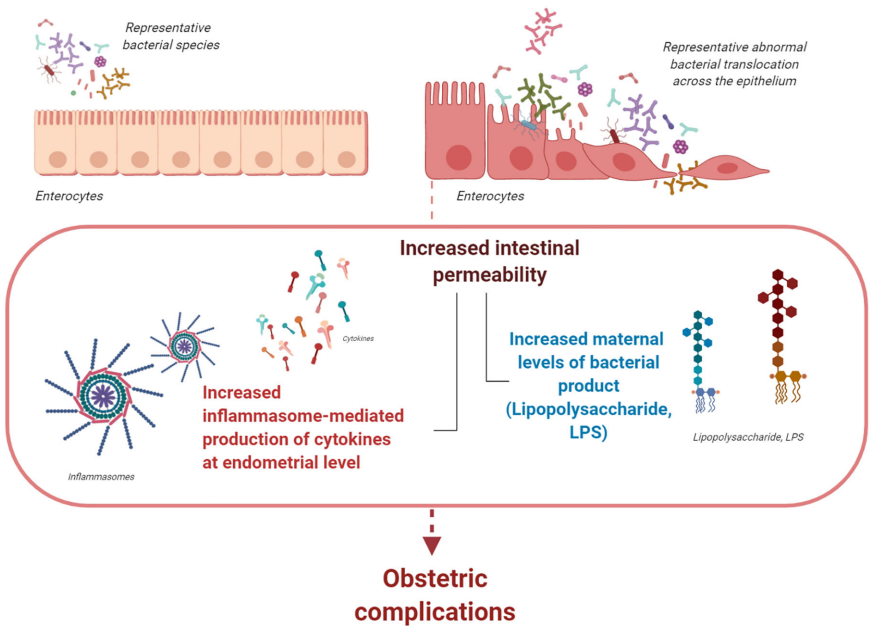

FIGURE 1 | The gastrointestinal (gut) microbiota. During pregnancy, the gut microbiota undergoes profound changes with different enterotypes characterizing each woman. If abnormal bacterial translocation across the epithelium that is associated with increased levels of LPS occurs during early pregnancy, uterine innate immunity and obstetric outcome may be affected. Abnormally increased intestinal permeability during early pregnancy is associated with increased levels of circulating bacterial products and cytokines. Both events might increase inflammasome activation at the endometrial level; consequently, they increase the risk of obstetric complications during early pregnancy (figure created with BioRender.com).

functions of lactobacilli is to activate glycogen metabolism. Glycogen produced by vaginal epithelial cells is transformed into lactic acid, inducing a low vaginal pH (3.8–4.4). This creates an unfavorable environment for the growth of pathogenic bacteria (45). Vaginal dysbiosis, which is linked to inflammatory states, is associated with adverse obstetric outcomes (**Figure 2**) (46). In the presence of dysbiosis, the vaginal microbiota increases the levels of vaginal inflammatory cytokines, which, in turn, increases the risk of spontaneous preterm birth (sPTB) (46–48). However, the debate on the relationship between vaginal dysbiosis and an increased risk of obstetric complications is ongoing. Several studies on the vaginal microbiota and sPTB rely on small sample sizes, primarily because data on vaginal swabs throughout pregnancy are often absent; where they exist, they show limited information on sPTB.

Alterations in lactobacilli dominance are likely to influence a patient's reproductive potential. In the presence of a microbiota with high bacterial diversity, as in bacterial vaginosis, an increased risk of infections, sPTB, and pelvic inflammatory disease have been observed (47–56). Consistent with these data, an increased risk of sPTB was detected in patients with low levels of *Lactobacillus* and increased bacterial diversity with *Gardnerella vaginalis* and *Mycoplasma* (47–56). Presence of *Lactobacillus* and low bacterial diversity were detected in women with term deliveries (56, 57). *Lactobacillus iners* is a risk factor for sPTB in high-risk patients; however, some studies suggest that the presence of *Lactobacillus crispatus* in the vaginal microbiota is protective against sPTB (56, 57). Because sPTB might be related to pathogenic microbes able to ascend from the vagina, these observations suggest that early characterization of the vaginal microbiota might be a predictive marker for obstetric complications, such as sPTB.

The relationship between the vaginal microbiota and obstetric complications is population-dependent. Women of European ancestry are more likely to harbor a *Lactobacillus*-dominated microbiome, whereas African American women are more likely to exhibit a diverse microbiotic profile. These women are also twice as likely to be diagnosed with bacterial vaginosis and twice as likely to experience preterm birth (53). By comparing African American women with women of European ancestry, Fettweis et al. (53) find that vaginal microbiotic diversity is significantly greater in African American women. In these women, the most common profile was *L. iners* followed by *G. vaginalis*, *Candidatus* Lachnocurva vaginae (also known as bacterial vaginosis-associated bacterium 1), and *L. crispatus*. In contrast, the most common profile in women of European ancestry was *L. crispatus*, followed by *L. iners* and *G. vaginalis*. These results suggest that there are significant differences in vaginal microbiota related to ancestry (53); such differences might explain the observed prevalence of bacterial vaginosis and preterm birth. Vaginal dysbiosis is associated with increased levels of proinflammatory cytokines (58). Recently, Fettweis et al. (53) observed that levels of the vaginal inflammatory cytokine CXCL10 were related to the *L. crispatus*/*L. iners* ratio in patients at increased

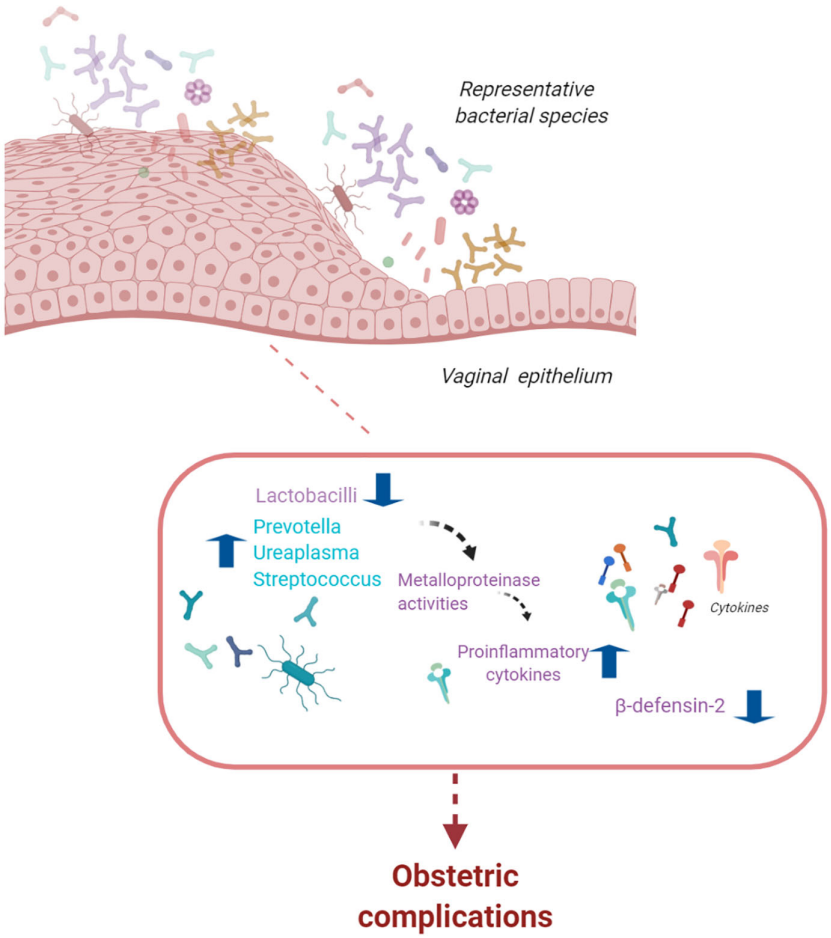

FIGURE 2 | The vaginal microbiota. The vaginal microbiota is composed of a variety of bacterial species. Alterations in lactobacilli dominance and a microbiota with high bacterial diversity are associated with an increased risk of infections, spontaneous preterm birth, and pelvic inflammatory disease (figure created with BioRender.com).

risk of sPTB, indicating a cytokine/*Lactobacillus* ratio as a possible predictive marker for sPTB. However, the difference between preterm and term deliveries cannot be explained only by a lack of *Lactobacillus* species given that many women deliver at term despite lacking *Lactobacillus* species. Conversely, the presence of *Lactobacillus* species does not guarantee a term birth, suggesting that there may be a risk associated with other causes of sPTB. Recently, Elovitz et al. (55) show that immune factors, such as beta-defensin 2, can modulate the risk independently of the presence or absence of *Lactobacillus* species. Indeed, high vaginal levels of beta-defensin 2 have been shown to lower the risk of sPTB. However, the reasons why some women have high or low beta-defensin 2 levels are unknown.

Despite research efforts, sPTB is one of the most common causes of neonatal death and infant mortality with consequences persisting from early childhood into adulthood; this presents families and society with important emotional and financial costs. Existing empirical evidence suggests that future population-specific studies may be able to shed light on the role of the vaginal microbiota, thereby supporting the development of therapeutic strategies. These include immune modulators and microbiome-based therapeutic approaches.

The Endometrial Microbiota

The endometrium is a site of immune surveillance where different components from the immune system work together to prevent infections and allow implantation of the blastocyst during pregnancy (59–61). When pregnancy begins, the endometrium undergoes decidualization; modifications in immune cell composition also occur (59–64). The local immune response, influenced by ovarian steroids, is essential for successful blastocyst implantation. Alteration of the endometrial immunological response during pregnancy has been linked to pregnancy complications, such as early pregnancy loss, preterm delivery, PE, and fetal growth restriction (**Figure 3**) (65–69).

The endometrium is not a sterile site. Recently, due to the sequencing of specific regions of bacterial ribosomal RNA, a

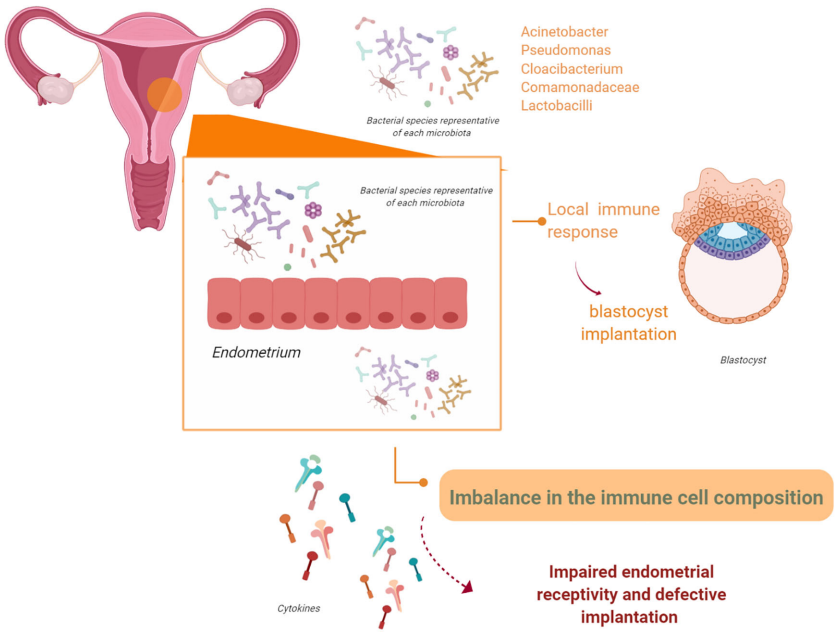

FIGURE 3 | The endometrial microbiota. The endometrium is not a sterile tissue. Resident populations of microorganisms at the endometrial level have been observed. It is possible that these microorganisms might interact with the endometrial epithelium and/or alter endometrial expression of leukocytes and cytokines. Therefore, these events, either in isolation or acting together, may impair endometrial receptivity and affect adequate implantation (figure created with BioRender.com).

resident endometrial microbiota and microbiome have been defined (70–73). Early studies on the endometrial microbiota reported the dominance of *Lactobacillus* species. Moreno et al. (74, 75) report an association between low levels of *Lactobacillus* species (<90% *Lactobacillus* with >10% other bacteria) in the endometrial microbiota and poor pregnancy outcomes regarding implantation success and ongoing and term pregnancy rates. A further study describes an endometrial microbiota mainly dominated by *Bacteroides* (76). Nevertheless, the heterogeneous characteristics of the women included in these studies (obstetric history, number of previous term deliveries, demographics, and medical history) limited this research. More recently, the endometrial microbiota obtained from the tip of the transfer catheter in 70 women undergoing *in vitro* fertilization was analyzed (77). In line with other studies, vaginal bacterial *Lactobacillus* species were dominant (>70% abundance). Furthermore, *Corynebacterium*, *Bifidobacterium*, *Staphylococcus*, and *Streptococcus* were observed (73, 78). However, a key limitation of the study was the heterogeneity of the population analyzed.

Winters et al. (79) recently questioned these observations. They suggest that the transcervical catheter collection of endometrial microbiota used in previous research was more prone to contamination. To overcome this, they obtained endometrial samples from hysterectomies and found an endometrial microbiota mainly composed of *Acinetobacter*, *Pseudomonas*, *Cloacibacterium*, and Comamonadaceae. Notably, they report that *Lactobacillus* was rare in the endometrial samples they analyzed. Finally, endometrial bacterial composition was different from that of the vagina. Instead, it was correlated to that of the cervix regarding composition and bacterial load. To date, the role of the endometrial microbiota in female reproduction is not fully understood. Liu et al. (80) try to link endometrial microbiota to chronic endometritis (CE) (81–84). The gold standard for the diagnosis of CE relies on histological identification of plasma cells in the endometrial stroma (84). The impact of CE on reproductive capacity is not well known. The prevalence of CE in the general population ranges from 0.8% to 19%. This percentage reaches 30%–45% in patients who are infertile or experience recurrent pregnancy loss (85–90). The mechanisms involved in CE-related poor pregnancy outcomes include imbalance in immune cell composition in the endometrium, lower response to steroid hormones, impairment in glycodelin secretion, and altered expression of pinopodes (91–94).

Liu et al. (80) compare the endometrial microbiota of infertile women with and without CE. They obtained endometrial biopsies and postovulatory-phase endometrial fluid from 130 infertile women. They found that CE was strictly associated with an increased proportion of non-*Lactobacillus* bacterial taxa in the endometrial cavity although the mechanisms underlying such a correlation are unknown.

Additionally, several recent studies suggest the presence of a resident microbiota in the endometrium (95–97). Yet studies that evaluate the role of the endometrial microbiota on reproductive health are in their infancy. We speculate that the endometrial microbiota may interact with the endometrial epithelium and endometrial immune cells, ultimately resulting in impaired endometrial receptivity and defective implantation.

To date, much remains to be understood regarding the ability of bacteria to colonize the endometrium and/or establish a commensal/pathogenic relationship with intrauterine tissues (98, 99).

CONCLUSIONS

The human microbiota plays a central role in health and female morbidity. Therefore, classifying women based on bacterial patterns would allow a personalized, microbiota-based diagnosis, which could then be used to develop personalized therapies for disease prevention and personalized treatments. These treatments could be used to modulate the composition of the microbiota. Women planning to have a family could be asked to consume specific nutrients, foods, and probiotics as well as making appropriate lifestyle changes. Pharmaceutical intervention is another useful adjunct.

AUTHOR CONTRIBUTIONS

NS and SD'I conceived and designed the study. NS, AO, and SD'I drafted and revised the article where appropriate. MS prepared the figures. NS, AO, CT, PV, AG, GS, and SD'I carried out the final revision of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Mackie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr* (1999) 69:1035S–45S. doi: 10.1093/ajcn/69.5.1035s
2. Nuriel-Ohayon M, Neuman H, Koren O. Microbial Changes during Pregnancy, Birth, and Infancy. *Front Microbiol* (2016) 7:1031. doi: 10.3389/fmicb.2016.01031
3. Preiss J, Romeo T. Physiology, biochemistry and genetics of bacterial glycogen synthesis. *Adv Microb Physiol* (1989) 30:183–238. doi: 10.1016/S0065-2911(08)60113-7
4. Mesa MD, Loureiro B, Iglesia I, Fernandez Gonzalez S, Llurba Olivé E, García Algar O, et al. The Evolving Microbiome from Pregnancy to Early Infancy: A Comprehensive Review. *Nutrients* (2020) 12:E133. doi: 10.3390/nu12010133
5. Nyangahu DD, Jaspan HB. Influence of maternal microbiota during pregnancy on infant immunity. *Clin Exp Immunol* (2019) 198:47–56. doi: 10.1111/cei.13331
6. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci USA* (2014) 111:2247–52. doi: 10.1073/pnas.1322269111
7. Vinolo MA, Rodrigues HG, Hatanaka E, Hebeda CB, Farsky SH, Curi R. Short-chain fatty acids stimulate the migration of neutrophils to inflammatory sites. *Clin Sci (Lond)* (2009) 117:331–8. doi: 10.1042/CS20080642
8. Thorburn AN, McKenzie CI, Shen S, Stanley D, Macia L, Mason LJ, et al. Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. *Nat Commun* (2015) 6:7320. doi: 10.1038/ncomms8320
9. Gomez de Aguero M, Ganal-Vonarburg SC, Fuhrer T, Rupp S, Uchimura Y, Li H, et al. The maternal microbiota drives early postnatal innate immune development. *Science* (2016) 351:1296–302. doi: 10.1126/science.aad2571
10. Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms* (2019) 7(1):E14. doi: 10.3390/microorganisms7010014
11. Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Bäckhed HK, et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell* (2012) 150(3):470–80. doi: 10.1016/j.cell.2012.07.008
12. Ferrocino I, Ponzo V, Gambino R, Zarovska A, Leone F, Monzeglio C, et al. Changes in the gut microbiota composition during pregnancy in patients with gestational diabetes mellitus (GDM). *Sci Rep* (2018) 8(1):12216. doi: 10.1038/s41598-018-30735-9
13. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. *Nature* (2011) 473(7346):174–80. doi: 10.1038/nature09944
14. Costea PI, Hildebrand F, Arumugam M, Bäckhed F, Blaser MJ, Bushman FD, et al. Enterotypes in the landscape of gut microbial community composition. *Nat Microbiol* (2018) 3:8–16. doi: 10.1038/s41564-017-0072-8
15. Gohir W, Whelan FJ, Surette MG, Moore C, Schertzer JD, Sloboda DM. Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptional diet. *Gut Microbes* (2015) 6:310–20. doi: 10.1080/19490976.2015.1086056
16. Barrett HL, Gomez-Arango LF, Wilkinson SA, McIntyre HD, Callaway LK, Morrison M, et al. A Vegetarian Diet Is a Major Determinant of Gut Microbiota Composition in Early Pregnancy. *Nutrients* (2018) 10(7):E890. doi: 10.3390/nu10070890
17. Collado MC, Isolauri E, Laitinen K, Salminen S. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. *Am J Clin Nutr* (2008) 88:894–9. doi: 10.1093/ajcn/88.4.894
18. Gomez-Arango LF, Barrett HL, McIntyre HD, Callaway LK, Morrison M, Dekker Nitert M, et al. Connections Between the Gut Microbiome and Metabolic Hormones in Early Pregnancy in Overweight and Obese Women. *Diabetes* (2016) 65:2214–23. doi: 10.2337/db16-0278
19. Zilberlicht A, Feferkorn I, Younes G, Damti A, Auslender R, Riskin-Mashiah S. The mutual effect of pregestational body mass index, maternal hyperglycemia and gestational weight gain on adverse pregnancy outcomes. *Gynecol Endocrinol* (2016) 32:416–20. doi: 10.3109/09513590.2015.1127911
20. Osol G, Moore LG. Maternal Uterine Vascular Remodeling During Pregnancy. *Microcirculation* (2014) 21:38–47. doi: 10.1111/micc.12080
21. Bardin N, Murthi P, Alfaidy N. Normal and Pathological Placental Angiogenesis. *BioMed Res Int* (2015) 2015:354359. doi: 10.1155/2015/354359
22. Cartwright JE, Fraser R, Leslie K, Wallace AE, James JL. Remodelling at the maternal-fetal interface: Relevance to human pregnancy disorders. *Reproduction* (2010) 140:803–13. doi: 10.1530/REP-10-0294
23. Faas MM, de Vos P. Uterine NK cells and macrophages in pregnancy. *Placenta* (2017) 56:44–52. doi: 10.1016/j.placenta.2017.03.001
24. Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol* (2002) 2:656–63. doi: 10.1038/nri886
25. Felker AM, Croy BA. Uterine natural killer cell partnerships in early mouse decidua basalis. *J Leukoc Biol* (2016) 100:645–55. doi: 10.1189/jlb.1HI0515-226R
26. Croy BA, Chen Z, Hofmann AP, Lord EM, Sedlacek AL, Gerber SA. Imaging of vascular development in early mouse decidua and its association with leukocytes and trophoblasts. *Biol Reprod* (2012) 87:125. doi: 10.1095/biolreprod.112.102830
27. Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normalmurine pregnancy. *J Exp Med* (2000) 192:259–70. doi: 10.1084/jem.192.2.259
28. Barber EM, Pollard JW. The uterine NK cell population requires IL-15 but these cells are not required for pregnancy nor the resolution of a Listeria

monocytogenes infection. *J Immunol* (2003) 171:37–46. doi: 10.4049/jimmunol.171.1.37
29. Guimond MJ, Luross JA, Wang B, Terhorst C, Danial S, Croy BA. Absence of Natural Killer Cells during Murine Pregnancy Is Associated with Reproductive Compromise in TgE26 Mice. *Biol Reprod* (1997) 56:169–79. doi: 10.1095/biolreprod56.1.169
30. Patel PS, Buras ED, Balasubramanyam A. The role of the immune system in obesity and insulin resistance. *J Obesity* (2013) 2013:616193. doi: 10.1155/2013/616193
31. Perdu S , Castellana B, Kim Y, Chan K, DeLuca L, Beristain AG. Maternal obesity drives functional alterations in uterine NK cells. *JCI Insight* (2016) 1: e85560. doi: 10.1172/jci.insight.85560
32. Wang J, Zheng J, Shi W, Du N, Xu X, Zhang Y, et al. Dysbiosis of maternal and neonatal microbiota associated with gestational diabetes mellitus. *Gut* (2018) 67:1614–25. doi: 10.1136/gutjnl-2018-315988
33. Zheng W, Xu Q, Huang W, Yan Q, Chen Y, Zhang L, et al. Gestational Diabetes Mellitus Is Associated with Reduced Dynamics of Gut Microbiota during the First Half of Pregnancy. *mSystems* (2020) 5:e00109-20. doi: 10.1128/mSystems.00109-20
34. Ma S, You Y, Huang L, Long S, Zhang J, Guo C, et al. Alterations in Gut Microbiota of Gestational Diabetes Patients During the First Trimester of Pregnancy. *Front Cell Infect Microbiol* (2020) 10:58. doi: 10.3389/fcimb.2020.00058
35. Lv LJ, Li SH, Li SC, Zhong ZC, Duan HL, Tian C, et al. Early-Onset Preeclampsia Is Associated with Gut Microbial Alterations in Antepartum and Postpartum Women. *Front Cell Infect Microbiol* (2019) 9:224. doi: 10.3389/fcimb.2019.00224
36. Gomez-Arango LF, Barrett HL, McIntyre HD, Callaway LK, Morrison M, Dekker Nitert M, et al. Increased Systolic and Diastolic Blood Pressure Is Associated with Altered Gut Microbiota Composition and Butyrate Production in Early Pregnancy. *Hypertens* (2016) 68:974–81. doi: 10.1161/HYPERTENSIONAHA.116.07910
37. Tersigni C, D'Ippolito S, Di Nicuolo F, Marana R, Valenza V, Masciullo V, et al. Correction to: Recurrent pregnancy loss is associated to leaky gut: a novel pathogenic model of endometrium inflammation? *J Transl Med* (2019) 17:83. doi: 10.1186/s12967-019-1823-5
38. D'Ippolito S, Tersigni C, Marana R, Di Nicuolo F, Gaglione R, Rossi ED, et al. Inflammosome in the human endometrium: further step in the evaluation of the "maternal side". *Fertil Steril* (2016) 105:111–8.e1-4. doi: 10.1016/j.fertnstert.2015.09.027
39. Neuman H, Koren O. The Pregnancy Microbiome. *Nestle Nutr Inst Workshop Ser* (2017) 88:1–9. doi: 10.1159/000455207
40. Gosalbes MJ, Compte J, Moriano-Gutierrez S, Vallès Y, Jiménez-Hernández N, Pons X, et al. Metabolic adaptation in the human gut microbiota during pregnancy and the first year of life. *EBioMedicine* (2019) 39:497–509. doi: 10.1016/j.ebiom.2018.10.071
41. Donnet-Hughes A, Perez PF, Doré J, Leclerc M, Levenez F, Benyacoub J, et al. Potential role of the intestinal microbiota of the mother in neonatal immune education. *Proc Nutr Soc* (2010) 69(3):407–15. doi: 10.1017/S0029665110001898
42. Dunlop AL, Mulle JG, Ferranti EP, Edwards S, Dunn AB, Corwin EJ. Maternal Microbiome and Pregnancy Outcomes That Impact Infant Health: A Review. *Adv Neonatal Care* (2015) 15(6):377–85. doi: 10.1097/ANC.0000000000000218
43. Yurkovetskiy LA, Pickard JM, Chervonsky AV. Microbiota and autoimmunity: exploring new avenues. *Cell Host Microbe* (2015) 17:548–52. doi: 10.1016/j.chom.2015.04.010
44. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA* (2011) 108 Suppl 1:4680–7. doi: 10.1073/pnas.1002611107
45. Petrova MI, Lievens E, Malik S, Imholz N, Lebeer S. Lactobacillus species as biomarkers and agents that can promote various aspects of vaginal health. *Front Physiol* (2015) 6:81. doi: 10.3389/fphys.2015.00081
46. Anahtar MN, Gootenberg DB, Mitchell CM, Kwon DS. Cervicovaginal Microbiota and Reproductive Health: The Virtue of Simplicity. *Cell Host Microbe* (2018) 23:159–68. doi: 10.1016/j.chom.2018.01.013
47. Hyman RW, Fukushima M, Jiang H, Fung E, Rand L, Johnson B, et al. Diversity of the vaginal microbiome correlates with preterm birth. *Reprod Sci* (2014) 21(1):32–40. doi: 10.1177/1933719113488838
48. Fettweis JM, Serrano MG, Brooks JP, Edwards DJ, Girerd PH, Parikh HI, et al. The vaginal microbiome and preterm birth. *Nat Med* (2019) 25:1012–21. doi: 10.1038/s41591-019-0450-2
49. Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, Marrazzo JM. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J Clin Microbiol* (2007) 45:3270–6. doi: 10.1128/JCM.01272-07
50. Chavoustie SE, Eder SE, Koltun WD, Lemon TR, Mitchell C, Nyirjesy P, et al. Experts explore the state of bacterial vaginosis and the unmet needs facing women and providers. *Int J Gynaecol Obstet* (2017) 137:107–9. doi: 10.1002/ijgo.12114
51. Di Giulio DB, Callahan BJ, McMurdie PJ, Costello EK, Lyell DJ, Robaczewska A, et al. Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci USA* (2015) 112:11060–5. doi: 10.1073/pnas.1502875112
52. Callahan BJ, Di Giulio DB, Goltsman DSA, Sun CL, Costello EK, Jeganathan P, et al. Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc Natl Acad Sci U S A* (2017) 114(37):9966–71. doi: 10.1073/pnas.1705899114
53. Fettweis JM, Brooks JP, Serrano MG, Sheth NU, Girerd PH, Edwards DJ, et al. Differences in vaginal microbiome in African American women versus women of European ancestry. *Microbiology* (2014) 160:2272–82. doi: 10.1099/mic.0.081034-0
54. Brown RG, Al-Memar M, Marchesi JR, Lee YS, Smith A, Chan D, et al. Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. *Transl Res* (2019) 207:30–43. doi: 10.1016/j.trsl.2018.12.005
55. Elovitz MA, Gajer P, Riis V, Brown AG, Humphrys MS, Holm JB, et al. Cervicovaginal microbiota and local immune response modulate the risk of spontaneous preterm delivery. *Nat Commun* (2019) 10(1):1305. doi: 10.1038/s41467-019-09285-9
56. Abdelmaksoud AA, Koparde VN, Sheth NU, Serrano MG, Glascock AL, Fettweis JM, et al. Comparison of Lactobacillus crispatus isolates from Lactobacillus-dominated vaginal microbiomes with isolates from microbiomes containing bacterial vaginosis-associated bacteria. *Microbiology* (2016) 162:466–75. doi: 10.1099/mic.0.000238
57. Stout MJ, Zhou Y, Wylie KM, Tarr PI, Macones GA, Tuuli MG. Early pregnancy vaginal microbiome trends and preterm birth. *Am J Obstet Gynecol* (2017) 217:356.e1–356.e18. doi: 10.1016/j.ajog.2017.05.030
58. Campisciano G, Zanotta N, Licastro D, De Seta F, Comar M. In vivo microbiome and associated immune markers: New insights into the pathogenesis of vaginal dysbiosis. *Sci Rep* (2018) 8:2307. doi: 10.1038/s41598-018-20649-x
59. Kitaya K, Yasuo T, Tada Y, Hayashi T, Iwaki Y, Karita M, et al. Unusual inflammation in gynecologic pathology associated with defective endometrial receptivity. *Histol Histopathol* (2014) 29(9):1113–27. doi: 10.14670/HH-29.1113
60. Park DW, Yang KM. Hormonal regulation of uterine chemokines and immune cells. *Clin Exp Reprod Med* (2011) 38(4):179–85. doi: 10.5653/cerm.2011.38.4.179
61. Robertson SA, Chin PY, Glynn DJ, Thompson JG. Peri-conceptual cytokines–setting the trajectory for embryo implantation, pregnancy and beyond. *Am J Reprod Immunol* (2011) 66 Suppl 1:2–10. doi: 10.1111/j.1600-0897.2011.01039.x
62. Spencer TE, Hayashi K, Hu J, Carpenter KD. Comparative developmental biology of the mammalian uterus. *Curr Top Dev Biol* (2005) 68:85–122. doi: 10.1016/S0070-2153(05)68004-0
63. Figueira PG, Abrão MS, Krikun G, Taylor HS. Stem cells in endometrium and their role in the pathogenesis of endometriosis. *Ann N Y Acad Sci* (2011) 1221:10–7. doi: 10.1111/j.1749-6632.2011.05969.x
64. Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunol Rev* (2005) 206:306–35. doi: 10.1111/j.0105-2896.2005.00287.x
65. Vinketova K, Mourdjeva M, Oreshkova T. Human Decidual Stromal Cells as a Component of the Implantation Niche and a Modulator of Maternal Immunity. *J Pregnancy* (2016) 2016:8689436. doi: 10.1155/2016/8689436
66. Liu S, Diao L, Huang C, Li Y, Zeng Y, Kwak-Kim JYH. The role of decidual immune cells on human pregnancy. *J Reprod Immunol* (2017) 124:44–53. doi: 10.1016/j.jri.2017.10.045

67. Wilczyński JR. Immunological analogy between allograft rejection, recurrent abortion and pre-eclampsia - the same basic mechanism? *Hum Immunol* (2006) 67(7):492–511. doi: 10.1016/j.humimm.2006.04.007
68. Redman CW, Sargent IL. Immunology of pre-eclampsia. *Am J Reprod Immunol* (2010) 63(6):534–43. doi: 10.1111/j.1600-0897.2010.00831.x
69. Lee J, Romero R, Xu Y, Miranda J, Yoo W, Chaemsaithong P, et al. Detection of anti-HLA antibodies in maternal blood in the second trimester to identify patients at risk of antibody-mediated maternal anti-fetal rejection and spontaneous preterm delivery. *Am J Reprod Immunol* (2013) 70(2):162–75. doi: 10.1111/aji.12141
70. Chen C, Song XL, Wei W, Zhong H, Dai J, Lan Z, et al. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat Commun* (2017) 8(1):875. doi: 10.1038/s41467-017-00901-0
71. Green KA, Zarek SM, Catherino WH. Gynecologic health and disease in relation to the microbiome of the female reproductive tract. *Fertil Steril* (2015) 104(6):1351–7. doi: 10.1016/j.fertnstert.2015.10.010
72. Payne MS, Bayatibojakhi S. Exploring preterm birth as a polymicrobial disease: an overview of the uterine microbiome. *Front Immunol* (2014) 5:595. doi: 10.3389/fimmu.2014.00595
73. Franasiak JM, Werner MD, Juneau CR, Tao X, Landis J, Zhan Y, et al. Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. *J Assist Reprod Genet* (2016) 33 (1):129–36. doi: 10.1007/s10815-015-0614-z
74. Moreno I, Franasiak JM. Endometrial microbiota-new player in town. *Fertil Steril* (2017) 108(1):32–9. doi: 10.1016/j.fertnstert.2017.05.034
75. Moreno I, Codoñer FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazán J, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am J Obstet Gynecol* (2016) 215(6):684–703. doi: 10.1016/j.ajog.2016.09.075
76. Verstraelen H, Vilchez-Vargas R, Desimpel F, Jauregui R, Vankeirsbilck NL, Weyers S, et al. Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16S rRNA gene. *PeerJ* (2016) 4:e1602. doi: 10.7717/peerj.1602
77. Tao X, Franasiak JM, Zhan Y, Scott RT, Rajchel J, Bedard J, et al. Characterizing the endometrial microbiome by analyzing the ultra-low bacteria from embryo transfer catheter tips in IVF cycles: Next generation sequencing (NGS) analysis of the 16S ribosomal gene. *Hum Microbiome J* (2017) 3:15–21. doi: 10.1016/j.humic.2017.01.004
78. Fang RL, Chen LX, Shu WS, Yao SZ, Wang SW, Chen YQ. Barcoded sequencing reveals diverse intrauterine microbiomes in patients suffering with endometrial polyps. *Am J Transl Res* (2016) 8(3):1581–92.
79. Winters AD, Romero R, Gervasi MT, Gomez-Lopez N, Tran MR, Garcia-Flores V, et al. Does the endometrial cavity have a molecular microbial signature? *Sci Rep* (2019) 9(1):9905. doi: 10.1038/s41598-019-46173-0
80. Liu Y, Ko EY, Wong KK, Chen X, Cheung WC, Law TS, et al. Endometrial microbiota in infertile women with and without chronic endometritis as diagnosed using a quantitative and reference range-based method. *Fertil Steril* (2019) 112(4):707–17. doi: 10.1016/j.fertnstert.2019.05.015
81. Bouet PE, El Hachem H, Monceau E, Gariépy G, Kadoch IJ, Sylvestre C. Chronic endometritis in women with recurrent pregnancy loss and recurrent implantation failure: prevalence and role of office hysteroscopy and immunohistochemistry in diagnosis. *Fertil Steril* (2016) 105(1):106–10. doi: 10.1016/j.fertnstert.2015.09.025
82. Cicinelli E, De Ziegler D, Nicoletti R, Tinelli R, Saliani N, Resta L, et al. Poor reliability of vaginal and endocervical cultures for evaluating microbiology of endometrial cavity in women with chronic endometritis. *Gynecol Obstet Invest* (2009) 68(2):108–15. doi: 10.1159/000223819
83. Matteo M, Cicinelli E, Greco P, Massenzio F, Baldini D, Falagario T, et al. Abnormal pattern of lymphocyte subpopulations in the endometrium of infertile women with chronic endometritis. *Am J Reprod Immunol* (2009) 61 (5):322–9. doi: 10.1111/j.1600-0897.2009.00698.x
84. Cicinelli E, Matteo M, Tinelli R, Pinto V, Marinaccio M, Indraccolo U, et al. Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage as confirmed by improved pregnancy outcome after antibiotic treatment. *Reprod Sci* (2014) 21(5):640–7. doi: 10.1177/1933719113508817
85. Yoshii N, Hamatani T, Inagaki N, Hosaka T, Inoue O, Yamada M, et al. Successful implantation after reducing matrix metalloproteinase activity in the uterine cavity. *Reprod Biol Endocrinol* (2013) 11:37. doi: 10.1186/1477-7827-11-37
86. Kushnir VA, Solouki S, Sarig-Meth T, Vega MG, Albertini DF, Darmon SK, et al. Systemic Inflammation and Autoimmunity in Women with Chronic Endometritis. *Am J Reprod Immunol* (2016) 75(6):672–7. doi: 10.1111/aji.12508
87. Johnston-MacAnanny EB, Hartnett J, Engmann LL, Nulsen JC, Sanders MM, Benadiva CA. Chronic endometritis is a frequent finding in women with recurrent implantation failure after in vitro fertilization. *Fertil Steril* (2010) 93 (2):437–41. doi: 10.1016/j.fertnstert.2008.12.131
88. Kitaya K. Prevalence of chronic endometritis in recurrent miscarriages. *Fertil Steril* (2011) 95(3):1156–8. doi: 10.1016/j.fertnstert.2010.09.061
89. Yang R, Du X, Wang Y, Song X, Yang Y, Qiao J. The hysteroscopy and histological diagnosis and treatment value of chronic endometritis in recurrent implantation failure patients. *Arch Gynecol Obstet* (2014) 289 (6):1363–9. doi: 10.1007/s00404-013-3131-2
90. Cicinelli E, Matteo M, Tinelli R, Lepera A, Alfonso R, Indraccolo U, et al. Prevalence of chronic endometritis in repeated unexplained implantation failure and the IVF success rate after antibiotic therapy. *Hum Reprod* (2015) 30(2):323–30. doi: 10.1093/humrep/deu292
91. Mikhaleva LM, Boltovskaya MN, Mikhalev SA, Babichenko II, Vandysheva RA. Endometrial dysfunction caused by chronic endometritis: clinical and morphological aspects. *Arkh Patol* (2017) 79(6):22–9. doi: 10.17116/patol201779622-29
92. Hou S, Sun X, Dong X, Lin H, Tang L, Xue M, et al. Chlamydial plasmid-encoded virulence factor Pgp3 interacts with human cathelicidin peptide LL-37 to modulate immune response. *Microbes Infect* (2019) 21(1):50–5. doi: 10.1016/j.micinf.2018.06.003
93. Kitaya K, Tada Y, Hayashi T, Taguchi S, Funabiki M, Nakamura Y. Comprehensive endometrial immunoglobulin subclass analysis in infertile women suffering from repeated implantation failure with or without chronic endometritis. *Am J Reprod Immunol* (2014) 72(4):386–91. doi: 10.1111/aji.12277
94. Di Pietro C, Cicinelli E, Guglielmino MR, Ragusa M, Farina M, Palumbo MA, et al. Altered transcriptional regulation of cytokines, growth factors, and apoptotic proteins in the endometrium of infertile women with chronic endometritis. *Am J Reprod Immunol* (2013) 69(5):509–17. doi: 10.1111/aji.12076
95. Bardos J, Fiorentino D, Longman RE, Paidas M. Immunological Role of the Maternal Uterine Microbiome in Pregnancy: Pregnancies Pathologies and Alterated Microbiota. *Front Immunol* (2020) 10:2823. doi: 10.3389/fimmu.2019.02823
96. Moreno I, Garcia-Grau I, Bau D, Perez-Villaroya D, Gonzalez-Monfort M, Vilella F, et al. The first glimpse of the endometrial microbiota in early pregnancy. *Am J Obstet Gynecol* (2020) 222(4):296–305. doi: 10.1016/j.ajog.2020.01.031
97. Al-Nasiry S, Ambrosino E, Schlaepfer M, Morré SA, Wieten L, Voncken JW, et al. The Interplay Between Reproductive Tract Microbiota and Immunological System in Human Reproduction. *Front Immunol* (2020) 11:378. doi: 10.3389/fimmu.2020.00378
98. Marconi C, de Andrade Ramos BR, Peraçoli JC, Donders GG, da Silva MG. Amniotic fluid interleukin-1 beta and interleukin-6, but not interleukin-8 correlate with microbial invasion of the amniotic cavity in preterm labor. *Am J Reprod Immunol* (2011) 65(6):549–56. doi: 10.1111/j.1600-0897.2010.00940.x
99. Vujaklija DV, Gulic T, Sucic S, Nagata K, Ogawa K, Laskarin G, et al. First trimester pregnancy decidual natural killer cells contain and spontaneously release high quantities of granulysin. *Am J Reprod Immunol* (2011) 66(5):363–72. doi: 10.1111/j.1600-0897.2011.01015.x

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