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The interplay between immune system and microbiota in gynecological diseases: a narrative review

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Abstract. – OBJECTIVE: The vaginal microbiome is a dynamic environment, depending on the results of a complex interplay between microbiota and the host. In physiological conditions, *Lactobacillus species* are the most represented, regulating glycogen metabolism in order to maintain normal pH. Vaginal flora has been divided into five subtypes. Pattern recognition receptors are present on both squamous epithelial cells lining the vagina and columnar cells lining the upper female genital tract. They respond directly to bacterial product expressed by vaginal microbiome. The vagina contains different immune related cells and receptors which can recognize and react with the microbial environment. Altered microbiota and altered interplay between microbiota and immune system underlie several gynecologic diseases.

MATERIALS AND METHODS: In this review, literature data related to vaginal microbiota, vaginal inflammation, immune system and menopause, preterm labor and miscarriage, were summarized. Relevant publications were retrieved from: PubMed, Medline, Scopus and Web of Science.

RESULTS: The vaginal microbiome and the relationship with immune system has been analyzed in different gynecologic conditions. Menopause is associated to estrogen loss which causes vaginal atrophy, reduced abundance of Lactobacilli and increased amount of other bacterial species. Estrogens influence vaginal immunity through known and unknown mechanisms. In bacterial vaginosis (BV), due to many bacterial species, there has been found an inhibition of the chemotaxis and cytokine secretion. A decreased concentration of Lactobacilli seems to be playing a role in preterm labor as well as the increased levels of pro-inflammatory cytokines. Finally, the disequilibrium in the Th1/Th2 immune adaptive response, with a shift from Th2 to Th1, appears to be playing a role in miscarriage.

CONCLUSIONS: The interplay between microbiota and the host closely involves the immune system. In particular, the vaginal microbiota is classically characterized by Lactobacilli even if vaginal microbiome of asymptomatic woman of reproductive age includes multiple aerobic and facultative or obligate anaerobic species. The role of microbiota and immune system in determining gynecological and obstetric events has been studied throughout recent years reaching new advancements. Therefore, additional studies are needed to better comprehend the complexity of the issue.

Key Words:
Vaginal microbiota, Immune system, Lactobacilli, Menopause, Inflammation; miscarriage, Preterm labor.

Introduction

The vaginal microbiome is a dynamic environment, depending on the results of a complex interplay between microbiota (the core of microbial communities) and the host. Several modifying factors are thought to impact the microbiome throughout life. The first contact with microbiota

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may begin during late gestation, with the largest exposure at the time of delivery. Over time, the abundance and diversity of the infant microbiome increase with life, stabilize around the time that the infant begins to eat solid foods, and persist throughout adulthood. Several modifying factors are thought to have an influential role in shaping the identity and abundance of the infant microbiota throughout life.

Lactobacillus species are the most represented[1] in non-pregnant woman reaching the concentration of 10^7 to 10^8 CFU/g of vaginal fluid, together with *Lactobacillus Crispatus*, *Lactobacillus Iners*, *Lactobacillus Jensenii*, and *Lactobacillus Gasseri*[2].

Lactobacilli regulate the glycogen metabolism, converting glycogen from vaginal epithelial cells into glucose and lactic acid to maintain the typical acidic vaginal pH (pH $\leq$ 4.5, range 3.8-4.4). Hence, creating an unfavorable environment for the growth of pathogens or other "un-healthy" bacteria[3]. Lactobacilli may also prevent the adherence of pathogenic microorganisms to vaginal epithelial cells through 'competitive exclusion' and 'bacterial interference'[3]. In addition, Lactobacilli produce various metabolites, such as bacteriocins and H_2O_2, which may help to stimulate the immune response during vaginal infections. Lactobacilli reduce local production of interleukin (IL)-1β, IL-6, and IL-8 and increase anti-inflammatory cytokines, such as IL-2 and IL-17[3]. In addition to Lactobacilli, the vaginal core microbiota account also for other multiple aerobic or facultative aerobic species as well as obligate anaerobic species.

In healthy women, the transition from puberty to menopause as well as transient hormonal changes, such as pregnancy and menstruation are characterized by major changes in vaginal microbiome. Furthermore, external factors, such as antibiotic usage, sexually transmitted infections, and vaginal irrigation can affect vaginal flora composition as well[4]. In particular, estrogens play a central role. The estrogen environment helps in the maintenance of the right balance among different vaginal bacterial communities. Indeed, it has been showed that the hormone replacement therapy (HRT) in menopause relieves the symptoms of vulvovaginal atrophy and supports the enrichment of vaginal microbiota[5].

Vaginal infections are the most common cause of abnormal functional status. Thus, while evaluating these infections, several characteristics should be considered:

- Bacterial density, which refers to the degree of bacterial distribution. It reflects the total biomass of the vaginal flora[6].
- Flora diversity, which represents the total number of bacterial species in the vaginal flora. This reflects the vaginal flora variety[7].
- Vaginal H_2O_2 is mainly produced by Lactobacilli, such as *L. Crispatus*, *L. Gasseri*, *L. Jensenii* and *L. Acidophilus*. Thus, as these Lactobacilli are often the predominant bacteria in healthy women, H_2O_2 levels may reflect the function of Lactobacilli[8].
- Enzymatic activity, such us leukocyte esterase activity indicates the presence of inflammation in the vagina. Sialidase is a specific marker of BV, whereas β-glucuronidase and coagulase activity may represent bacterial vaginitis[9,10].

Patients with the following features are considered to have a normal micro-ecological status:

- pH values ranging from 3.8 to 4.5;
- Bacterial density degree II to III;
- Flora diversity degree II to III;
- Gram-positive rods as predominant flora;
- Nugent and AV score $\leq$3;
- Absence of pathogens and negative specific enzymes.

Gajer et al[4] and Srinivasan et al[11] showed that the diversity, the composition, and the relative abundance of vaginal microbial species change dramatically, during different periods of life. Ravel et al[14] have demonstrated that reproductive-aged women can be grouped into five different categories referred to as Community State Types (CSTs). Four of these CSTs are dominated by Lactobacilli, namely, *L. Crispatus* (CST-I), *L. Iners* (CST-III), *L. Gasseri* (CST-II) or *L. Jensenii* (CST-V). One category, CST-IV, does not contain a significant number of Lactobacillus, but is composed of a polymicrobial mixture of strict and facultative anaerobes including species of the genera *Gardnerella*, *Atopobium*, *Mobiluncus*, *Prevotella* and other species in the order of *Clostridiales*[12-15].

CST IV was recently divided into two subtypes, termed CST IV-A and CST IV-B; by Gajer et al[4] CST IV-A is characterized by various species of anaerobic bacteria belonging to the genera *Anaerococcus*, *Peptoniphilus*, *Prevotella*, and *Streptococcus*. However, CST IV-B has higher proportions of the genera *Atopobium* and *Megasphaera*, among others (Figure 1). Species-specific diffe-

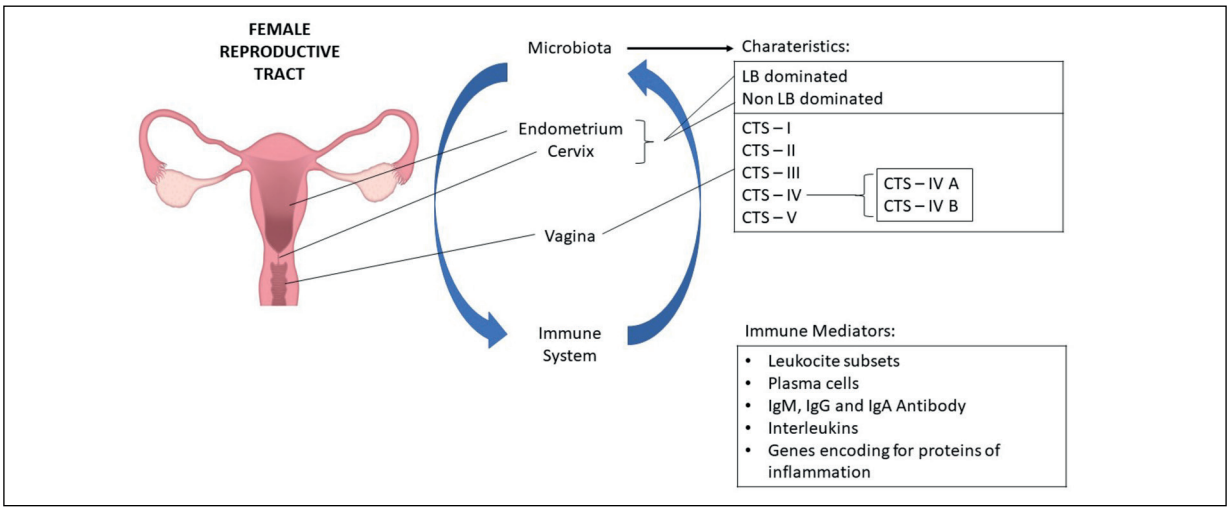

Figure 1. Microbiome composition is not equally expressed over the female reproductive tract. Components of the female reproductive system and their respective microbiome population; the uterus appears to be mostly occupied by *Lactobacillus spp*, the uterine cervix is predominated by non-*Lactobacillus spp*, and the vagina is normally predominated by *Lactobacillus spp* of the Community State Types (CST) I,II, III and V. CST-IV is mainly composed by a polymicrobial mixture of anaerobes. Therefore, a vaginal predominance of CST-IV can manifest clinically as bacterial vaginosis. It was demonstrated that the innate immune response is largely driven by vaginal bacterial community states, with CST-IV potentially having a greater pro-inflammatory response than CST-I or CST-II, and with CST-III triggering an intermediate response. The interplay between the immune system and the microbiome involves diverse immune factors such as leukocyte subsets, plasma cells, IgG , IgM and IgA anti-bodies, interleukins and inflammatory proteins. Note: LB, lactobacillus; CTS, Community State Types.

rences in the vaginal microbiota have been shown to be significant, as demonstrated by Srinivasan et al[11]. In this study, the various bacterial species were associated differently with each of the four signs constituting the Amsel criteria for the diagnosis of BV. This suggests a link between specific vaginal bacteria and clinical signs.

The polymicrobial condition known as BV is compositionally similar to CST-IV since it is defined by a loss of *Lactobacillus spp*., presence of anaerobes, strict anaerobes, and occasionally accompanying clinical manifestations, including discharge, odor and irritation. Clinically, the evaluation of vaginal discharge, malodor, clue cells and vaginal pH > 4.5 is necessary for the diagnosis of BV, as defined by the Amsel's criteria[15]. The frequency of these CSTs varies according to different ethnic backgrounds, with CST-IV being more common (40%) in black and Hispanic populations[14]. Vaginal flora is thought to be, in some way, correlated with gut microbiome as well. Some bacterial species have been identified with b-glucuronidase activity that might potentially increase intestinal reabsorption of estrogens into the bloodstream[16]. This consideration has led to the idea of the so called "estrogen-gut microbiome axis".

Data Sources

We searched MEDLINE (PubMed), Web of Science, SCOPUS, and Grey literature (Google Scholar; British Library) from January 1980 to June 2019. We used the terms "vaginal flora", "vaginal microbiota", "vaginal microbiome", "vaginal bacteria", as text words and as appropriate medical subject headings or equivalent subject headings/thesaurus terms. These terms were combined with "immune system", "immune response", "immune adaptive response", "immune native response" and terms "gynecological disease", vaginal disease", "vaginal atrophy". The reference lists of all available primary studies were reviewed to identify additional relevant citations.

Screening of Abstract for Eligibility

Abstracts and titles identified from the search were screened by 3 investigators. Disagreements about the inclusion or exclusion of studies were primarily solved by consensus, and when this was not possible, a fourth reviewer resolved them.

Study Selection and Eligibility Criteria

A set of specific criteria were used for selection of literature: randomized controlled trials (RCT); prospective or retrospective cohort

studies; reviews and meta analyses; international societies' guidelines; and study with characterization of the role of microbiota in gynecological and obstetric conditions. Only studies written in English with an available abstract were accepted.

How to Investigate the Vaginal Microflora

The historical and most traditional method of microbiota analysis in gynecology is represented by Pap smear microscopy. This method has been widely used throughout the years both in clinical setting and research, due to its simplicity, quickness and effectiveness.

Vaginal swab samples are stained with Gram stain and examined microscopically using the Vaginal Micro-ecology Evaluation System (VMES)[6]. This tool is mainly composed of morphological and functional micro-ecological indicators:

- Morphological indicators include bacterial density, flora diversity, dominant bacterial flora, indicators of inflammation, and pathogenic microorganisms. The system also includes, both Nugent score and Aerobic Vaginitis (AV) score used for bacterial vaginitis and aerobic vaginitis, respectively[7,8].
- Functional indicators reflect microbial functional status, consisting of three main components: vaginal factors (pH value), metabolites (for example H_2O_2) and microbial enzymes, such as sialidase, β-glucuronidase, leukocyte esterase, and acetylglucosaminidase. It should be noted that if the functional indicators are inconsistent with the morphological indicators, the latter should be taken as reference indicators[6].

Pap smear microscopy has been flanked, especially over the last decade, by new methods of microbiota composition analysis. Among these, the most spread one is the 16S rRNA analysis, that allows a rapid and effective analysis of diversity, as well *phyla*, *genera* and *species*, giving a complete overall view on the microbiota composition. For this reason, the introduction of this method has led to the so-called Next Generation Sequencing (NGS), with the aim to study and characterize the entire composition of microbiota.

Hong et al[17] tested NGS as a diagnostic tool in vaginitis, finding out a total correspondence between NGS and microbiological culture of 56.7%, whereas a correspondence of 73.1% in detecting *Lactobacilli*, whose role in maintaining the homeostasis and eubiosis of vaginal flora is long time known.

Virtanen et al[18] compared the findings of Pap Smear analysis and NGS in 50 asymptomatic women, finding high correspondence between the two methods, especially in determining the prevalence or absence of Lactobacilli.

Smidt et al[19] compared culture-based methods, quantitative PCR and next-generation sequencing (NGS) in detecting Lactobacilli. Good concordance for *L. Crispatus* was also found between the results of the culture-based method and qPCR. Finally, good overlap between the results of the culture-based method and NGS was revealed: in case of a positive NGS result for *L. Crispatus*, the same species was isolated in 95% of samples. The corresponding percentages were 82% for *L. Jensenii* and 86% for *L. Gasseri*.

The rRNA analysis, by the NGS methods, has undoubtedly opened new perspectives in the study of the vaginal microbiota, even if its role in clinical practice is not defined yet.

Although its cost has been decreasing over the last few years, this method still lacks the right amount of standardization in order to be totally inserted in everyday clinical practice. For this reason, classic cultural and microbiological methods keep playing the most important role, especially in clinic, whilst NGS is gradually opening its way thanks to its accuracy in microbiota characterization[19].

Immune System and Vaginal Microbiota

The vagina contains different immune related cells and receptors which can recognize and react with the microbial environment[20]. Surveillance for microbes within the female genital tract of both commensal and pathogenic microbes is generally achieved by microbial specimen recognition through pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), dectin-1 receptor, and nucleotide-binding oligomerization domain (NOD). These receptors are present on both squamous epithelial cells lining the vagina and columnar cells lining the upper female genital tract[21-25].

Microbial stimulation of PRRs initiates cytokine/chemokine signaling cascades, leading to secretion of IL-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α), in order to recruit or activate specialized cells, including NK cells, macrophages, CD4+ helper T-cells, and CD8+ cytotoxic

T-cell lymphocytes, and B lymphocytes. Genetic variants of PRRs, such as the IL-1R antagonist gene, TLR4, TLR9, IL-1R2 and TNF-α may play a role in individual woman's response to a particular microbial challenge or pregnancy outcome (Figure 2)[26,27].

When compared to CST-I, women with CST-IV demonstrate elevated levels of IL-1α, IL-1β, TNF-α, IFN-γ, IL-4, IL-8, IL-10, IL-12p70, and fms-like tyrosine kinase 3 ligand. Furthermore, significantly higher levels of IFN-$\gamma$are found in CST-III, relative to CST-I. Particularly, Anahtar et al[28] have demonstrated that *Prevotella Amnii*, *Mobiluncus Mulieris*, *Sneathia Amnii*and *Sneathia Sanguinegens* (all commonly found in CST-IV) were found to induce higher levels of IL-1α, IL-1β, and IL-8 secretion, relative to *L. Crispatus* dominated communities (in CST-I). In addition, L. *Iners* dominated communities (CST-III) induced moderate IL-8 levels relative to CST-I. Nevertheless, a significant increase in IL-1α, IL-1β and TNF-α was noted during transition from a CST-I, to CST-III and to a CST-IV.

However, mock communities dominated by *L. Crispatus* (CST-I) and *L. Jensenii* (CST-V) on reconstructed three-dimensional vaginal epithelial models do not cause cytokine IL-1β or IL-8 secretion relative to medium control, and also inhibit some pro-inflammatory responses after TLR 2/6 and 3 agonist induction[29].

Therefore, these studies demonstrate that the innate immune response is largely driven by vaginal bacterial community states, with CST-IV potentially having a greater pro-inflammatory response than CST-I or CST-II, and with CST-III triggering an intermediate response.

Other factors contributing to vaginal defense include mannose binding lectin (MBL), immunoglobulin A and G (IgA, IgG) and vaginal antimicrobial peptides (AMPs):

– MBL binds mannose, N-acetyl-glucosamine and fucose carbohydrate moieties present on microbial cell surfaces. Eventually, this interaction leads to cell lysis or targeting for the immune system[30].

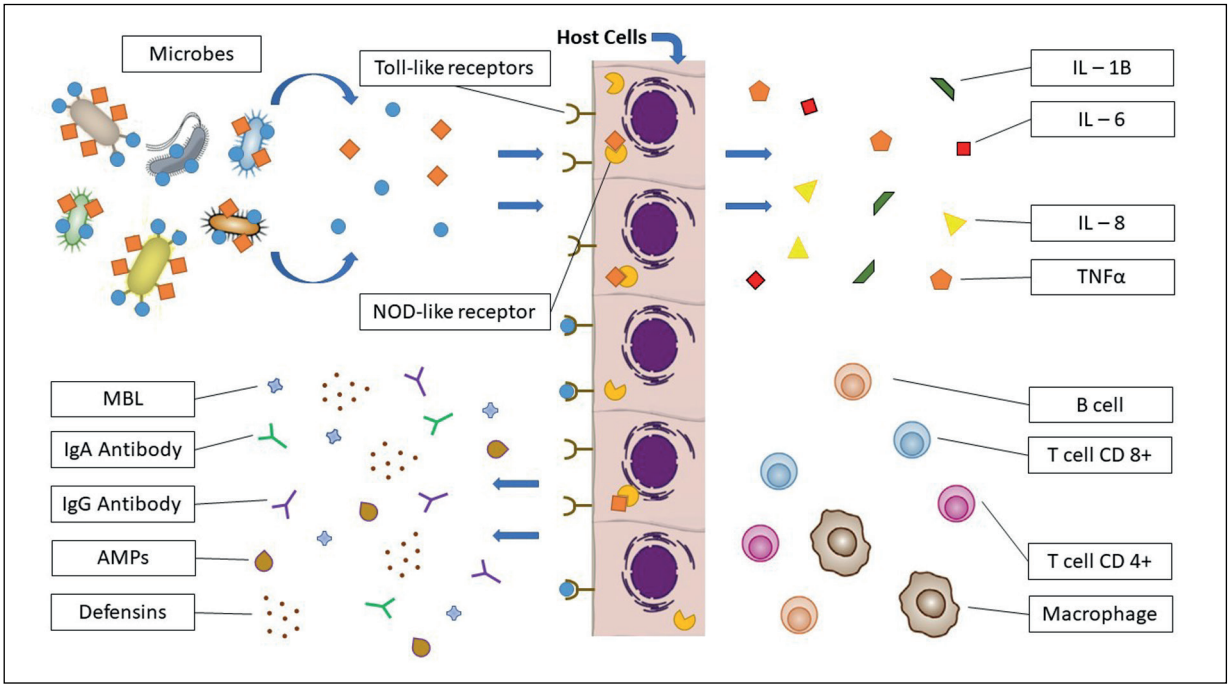

Figure 2. The female genital tract is constantly exposed to microbes. Variable defense factors of the innate immune response including mannose binding lectin (MBL), immunoglobulin A (IgA), immunoglobulin G (IgG), vaginal antimicrobial peptides (AMPs) and defensins contribute to clearance of infectious microbes by different mechanisms of action. Surveillance for both commensal and pathogenic microbes is generally achieved by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and NOD like receptors (NLR). Microbial stimulation of PRRs initiates cytokine/chemokine signaling cascades, leading to secretion of interleukin and tumor necrosis factor-α (TNF-α), in order to recruit or activate specialized cells including macrophages, CD4+ helper T-cells, CD8+ cytotoxic T-cell and B lymphocytes.

– IgA and IgG may help to prevent adherence to vaginal epithelial cells and subsequent uptake of bacteria, as well as contribute to the neutralization and clearance of infectious microbes from the vagina[31].
– Vaginal AMPs exist in various classes and may recruit immune cells *via* chemotaxis and possess anti-endotoxin activity. Defensins are a class of cationic and amphipathic AMPs with diverse mechanisms of action against common vaginal bacteria as well as pathogenic bacteria and viruses including HIV, herpes simplex virus, and human papillomavirus. In organotypic models of the vaginal epithelium, human β-defensin (HBD)-2 expressions, but not that of HBD-1, was associated with colonization by *L. Iners*, *Atopobium Vaginae* and *Prevotella Bivia*. Other studies[32,33] with similar experimental *in vitro* conditions has shown that L. *Jensenii* but not *G. Vaginalis* induce HBD-2 transcription.

In addition to defensins, other AMPs are found in the human vagina and include the secretory leukocyte protease inhibitor (SLPI), human epididymis protein 4 (HE4), LL-37, surfactant protein (SP)-A and SP-D. SLPI expression is associated with bacterial vaginitis organisms but not with *L. Crispatus*, *L. Iners*, *A. Vaginae* or *P. Bivia*. HE4 is associated with *G. Vaginalis,* and LL-37 inactivates the sexually transmitted pathogen *Neisseria Gonorrhoeae* while having little or no effect on *L.* Iners, *L. Crispatus*, and *L. Jensenii*[34,35].

Like defensins, SP-A and SP-D contribute to viral inhibition, including HIV. These AMPs bind to the viral protein gp120 and human CD4 receptor, enhancing attachment to dendritic cells and therefore facilitating HIV uptake by immune cells[36].

Altered Interplay Between Microbiota and Immune System

Menopause

Menopause is characterized by the loss of estrogen function on vaginal cells metabolism leading to less cell layers, thinner mucus layer, decreased glycogen production. Furthermore, the estrogen deprivation influences the microbiome susceptibility as well as the mucosal immunity. Changes in the immune system accompanying ageing and menopause are known as immune-senescence, the process is characterized by a decrease in cell-mediated immune function and humoral response[37]. In fact, in post-menopause, the NK cell activity decrease significantly and hormone replacement therapy (HRT) was recently shown to restore NK cytotoxicity[38].

Several studies have recently assessed the vaginal microbiome in postmenopausal women. Largely, these studies[39-44] sustain the perception that diversity and abundance of Lactobacilli are declining following menopause. In a portion of these studies, the postmenopausal women were considered healthy (asymptomatic) and not treated with HRT[39,40,42].

Gustaesson et al[40] found that fertile women had a great diversity in the subspecies of Lactobacilli present. They concluded that fertile women were more commonly colonized with *L. Crispatus*, compared to menopausal women ($p = 0.0036$). Similarly, Zhang et al[42] observed a lower diversity of *Lactobacillus spp.* In postmenopausal relative to premenopausal women ($p < 0.05$). Mirmonsef et al[45] demonstrated similar results, showing that premenopausal women had significantly higher free glycogen levels and higher Lactobacillus levels in comparison with postmenopausal women.

In some cases, but not always, the depletion of *Lactobacillus spp.* and the increase in diverse microbial species (CST IV-A and CST IV-B), results in symptoms of VVA or the genitourinary syndrome of menopause (GSM), which describes a number of menopausal symptoms in relation to changes of the vulva, vagina, and lower urinary tract[46].

Many researchers[47,48] have begun to investigate the vaginal microbiome exploring VVA and GSM through the protective features of the vaginal microbiome.

Vaginal microbiota plays an important role in preventing colonization by pathogenic organisms but the predominant connection between the vaginal microbiome and menopause occurs through the influential action of estrogen. The role of estrogen has been highlighted in menopause with the estrogen replacement therapy. Gliniewicz et al[5] characterized the vaginal bacterial communities of women in three groups: postmenopausal women undergoing HRT who had a vaginal pH ≤ 5 and a vaginal atrophy score ≤ 2; postmenopausal women with a vaginal pH ≥ 5 and vaginal atrophy score ≥ 6, and premenopausal women with a vaginal pH ≤ 5 and vaginal atrophy score ≤ 2. In premenopausal women three sorts of communities were commonly found that were dominated by either *L.*

Crispatus, *Gardenerella Vaginalis*, or *L. Iners*. The vaginal communities of most postmenopausal women receiving HT were dominated by these species of lactobacilli, whereas this was usually not the case in untreated postmenopausal women. The authors suggest that HRT may lead to preferential enrichment of *L. Crispatus*. It has been speculated that estrogen stimulates that proliferation of squamous epithelial cells, which is accompanied by the increased production of glycogen by these cells. Glucose, maltose, and maltodextrins produced through the hydrolysis of glycogen are thought to serve as carbon sources that support the proliferation of vaginal Lactobacilli[5].

It was demonstrated that all routes of estrogen administration (oral, transdermal, and vaginal) are effective for relief of menopausal symptoms.

Ginkel et al[48] comparing women receiving estrogen replacement therapy to those taking non hormone therapy, showed that women on HRT were less likely to be colonized with anaerobic bacteria. Therefore, a longitudinal study in women treated with oral estrogens found that after three months of treatment, 20% of women on placebo and 80% of women on oral estrogens treatment reported improvement in vaginal dryness and irritation concurrent with increased vaginal lactobacilli and lower vaginal pH.

Probiotics (including *Lactobacillus spp.*) potentially work through a variety of mechanisms to reinstate homeostasis by enhancing epithelial barrier function, commensal colonization, blocking adhesion of pathogenic bacteria, reduction pH, influencing antimicrobial peptide production/secretion and overall mucosal immunity and vaginal health[49-51].

Changes in vaginal microbiota can influence the vaginal microenvironment, and so decrease the efficacy of different potential therapies. Consequently, even the local estrogen administration which is effective for relief of menopausal symptoms may have better result with the probiotics therapy taken at the same time.

Probiotics potentially work to reinstate homeostasis by enhancing epithelial barrier function, commensal colonization, blocking adhesion of pathogenic bacteria, reducing pH, influencing antimicrobial peptide production/secretion and overall mucosal immunity and vaginal health[52-54]. Both oral and vaginal routes of Lactobacillus (based probiotic formulation) are effective for reinstating vaginal homeostasis[55,56]

Few studies analyzed the combination treatment of probiotics and estrogen or antibiotics therapy. A recent study included 60 postmenopausal women, with atrophic vaginitis and chronic recurrent bacterial cystitis in the acute stage. Patients receiving antibiotic therapy in combination with vaginal local estriol were compared with patients receiving antibiotics in combination with a lyophilized culture of *lactobacilli L. Casei Rhamnosus Doderleini* for 3 months. The association therapy of antibiotics and Lactobacilli contributed to the normalization of pH, and reduce the severity of vaginal dryness and burning. The rate of patients with improvement of symptoms was significantly higher in the group receiving antibiotics and Lactobacilli than in the group receiving antibiotics and estriol (96.7% *vs.* 83.3% respectively)[57].

In another study it has been evaluated the efficacy of lyophilized lactobacilli in combination with estriol when compared to metronidazole in the treatment of bacterial vaginal infections. The authors concluded that lyophilized lactobacilli in combination with low dose estriol are equivalent to metronidazole in the short-term treatment of bacterial vaginal infections, but have less effect after 1 month, and so further studies are required to evaluate the long-term efficacy of lactobacilli when applied repeatedly[58].

Vaginal Inflammation

The vaginal microbiota can be characterized by different CSTs, with CST-IV lacking a significant number of *Lactobacillus spp.* Generally, CST-IV can clinically manifest as aerobic vaginitis or BV. Aerobic vaginitis is mainly differentiated from BV by the presence of an inflammatory response predominately associated with aerobes, such as group B *Streptococcus*, *Staphylococcus Aureus*, *Escherichia coli*, and *Enterococcus*[8].

The aerobic vaginitis inflammatory response is characterized by symptoms, including itching or burning, molecular changes, such as increased IL-6 and IL-1β, and presence of cells, such as leukocytes or primary blood cells in a microscopic wet mount. In contrast, BV is not characterized by inflammatory responses and therefore, recruitment of neutrophils, redness, itching or burnings assent[52].

Several cytokines as well as other immune-related factors [IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ, chemokine C-C motif ligand 5 (CCL5)] and SLPI have been variably and inconsistently associated with BV. These conflicting findings may be due to different study design different definitions of BV (symptomatic *vs.* asymptomatic BV or Nugent BV *vs.* BV diagnosed according to the Amsel criteria). Moreover,

it is possible that immune-related factors, such as IgA degradation, TLR expression inhibition, or immune-related genetic variants can suppress the inflammatory response in BV[53,54].

Bacteria-derived short chain fatty acids (SCFAs), namely acetate, butyrate, propionate and succinate, some of which exist at relatively higher proportions during BV, can induce a pro-inflammatory response under the hypothesis that SCFAs may act to ultimately inhibit chemotaxis and inflammation in BV. Relatively high concentrations (2-20 mM) of acetate and butyrate, but not propionate, induce cytokine IL-6, IL-8 and IL-1β secretions and also induce IL-8 and TNF-α with TLR2 and TLR7 ligand stimulation in a dose- and time-dependent manner *in vitro*[59].

Lactic acid which is produced mainly by vaginal microbes inactivates a broad range of BV-associated microbes at pH <4.5[60]. When *Lactobacillus spp.* dominate the vaginal microbiota, they acidify the vagina to a highly acidic mean pH of 3.5 ± 0.2 that is likely to help protect against a broad range of infections.

Recent studies aimed to uncover the mechanism by which lactic acid can affect host immune functions have found diverse effects, including direct inhibition of pro-inflammatory responses IL-6, IL-8 and IL-1RA, induction of the Th17 lymphocyte pathway *via* IL-23 in a dose-dependent manner upon lipopolysaccharide co-stimulation, stimulation of mediators from vaginal epithelial cells, and upon transforming growth factor-β, activation of antiviral response[53,54,59].

Lactic acid exists in the vagina in both D-(–)- and L-(+)-isomers, with the host contributing only about 4-30% of the total lactate level. Women with BV were found to be deficient in both isomers, while those with vulvovaginal candidiasis have elevated L-(+)-lactic acid as well as CD147 and MMP-8 genes[61]. *L. Iners* does not produce D-(–)-lactic acid and fails to produce as high the L- (+)-lactic acid as seen with *L. Crispatus*, *L. Gasseri.* However, *L. Jensenii* produces only D-(–)-lactic acid[62], suggesting potential *Lactobacillus* species-specific effects on the host. Consequently, the composition of the vaginal microbiota, and specifically the ability of vaginal microbes to produce D-(–)-lactic acid, may help to inhibit inflammatory responses while also favoring *Lactobacillus spp.* survival by using host cells resources for carbon source.

Pregnancy

During pregnancy, in parallel to the dramatic hormonal, weight, immunological and metabolic changes, significant changes in the microbiome occur. These changes affect all the districts where the human microbiota is expressed: gut, vagina, endometrium, and other sites oral cavity. Several authors[63] have linked pregnancy complications with microbial changes. The different body sites harbor different microbial populations according to different pH, oxygen, nutrients and temperature. Pregnancy is associated to an increased gut bacterial load[64] and modifications of the composition of gut microbiota, including reduced α-diversity (individual richness) and increased ß-diversity (between-subject diversity), increased *Actinobacteria* and *Proteobacteria phyla* and reduced *Faecali bacterium* and other short-chain fatty-acid producers[65]. These gut modifications resemble those observed in metabolic syndrome and have been suggested to play an important contribute to changes in host immunology and in metabolism, *via* increased absorption of glucose fatty acids, increased fasting-induced adipocyte factor secretion, and stimulation of the immune system. The vaginal microbiome shows a significant reduction in overall diversity, increased stability and increased abundance of *Lactobacillus species*, with the final result of decreased vaginal pH creating a barrier against pathogenic bacteria and viral infections[66]. It is important to note that pregnancy is a healthy physiological process in which beneficial microbial alterations are expected. In contrast, pregnancy complications like preterm birth and miscarriage, have been associated with some bacterial infections, through mechanisms not completely understood. Antibiotics administered during pregnancy have been shown to affect the microbiome composition and diversity[67]. However, further research is needed in order to explain the impact of microbiome changes on pregnancy as well as the importance of recommending antibiotic treatments or probiotic for pregnancy complications.

Preterm Labor

The vaginal microbiota in combination with other factors is associated with adverse reproductive and obstetric outcomes.

The association between an abnormal maternal vaginal microbiome and an increased risk of preterm birth (PTB) is still controversial. Several studies on the vaginal microbiome and PTB show a small sample size; often there is absence of data collection on vaginal swabs across pregnancy, necessary information on spontaneous PTB is lacking, and studies investigating the vaginal

microbiome and PTB show a small sample size. A homogeneous Lactobacillus-dominated microbiota has been considered a marker of a healthy female reproductive tract. In contrast, a vaginal microbiome with high species diversity, as in BV, has been associated with increased risk of infections, PTB and pelvic inflammatory disease[68,69].

DiGiulio et al[70] showed that the risk of PTB was observed to be higher in patients with abundant *Gardnerella* or *Mycoplasma* and poor in Lactobacillus. Otherwise, in a cohort study[71] no correlation was observed between absence of Lactobacillus and the risk of PTB. Kindinger et al[72] have reported that a dominance of *L. Crispatus* in the vaginal microbiota seems to be protective against PTB, while *L. Iners* seems to be a risk factor for PTB in high risk patients. In a paper recently published in Nature[73], *L. Crispatus* was greatly reduced in PTB samples, whereas *Prevotella*, *BVAB1*, *BVAB-TM7* and *Sneathia Amnii*, were more abundant in vaginal PTB samples. Considering that PTB might be related to an ascension of pathogenic microbes from the vagina, these observations suggest that the vaginal microbiome composition, early in pregnancy, might assist in prediction of adverse outcomes and serve as risk marker for PTB.

Nevertheless, it is noteworthy that the association between the vaginal microbiome and PTB is population-dependent. Specifically, the association between lower Lactobacillus, higher *Gardnerella* and increased risk of PTB was detected only in African Americans and in white populations[74]. Women of African descent frequently have vaginal *L. Crispatus* predominance and they often show an increased vaginal microbial diversity[75].

Analysis of vaginal cytokines showed that vaginal bacterial taxa, generally associated with dysbiosis, are highly correlated with increased levels of pro-inflammatory cytokines, which play a role in the induction of labor. Recently, Fettweiss et al[76] observed that vaginal inflammatory cytokine CXCL10 levels were inversely correlated with *L. Crispatus* and positively correlated with *L. Iners* in PTB patients, suggesting a cytokine/lactobacillus ratio as a possible prediction marker of preterm birth.

It is evident that an early prediction of PTB risk is critical for the development of new strategies for prevention and intervention. All the available data support that population-specific studies might be helpful to assess the impact of the vaginal microbiome on the risk of PBT and to identify high risk vaginal microbiota specific for a subset of women. Moreover, it is evident that an early prediction of risk for PTB is critical for the development of new strategies for prevention and intervention[75,76].

Miscarriage

The human endometrium displays a crucial immunological surveillance for the uterus. Indeed, in the human endometrium a complex immune system is able to prevent the risk of infections as well as, when pregnancy occurs, to allow the acceptance of the blastocyst[77,78]. Combinations of chemokines are secreted by endometrial stromal and epithelial cells, which act as "sentinels" able to influence leukocyte endometrial expression. Major immune cells in the human endometrium include uterine Natural Killer (uNK) cells, macrophages, dendritic cells (DCs) and T cells. Each of these cell populations demonstrated[79-84] a specific role. uNK are involved in the success of implantation and maintenance of pregnancy 65-70 through their ability both to interact through inhibitor receptors with HLA-G, HLA-E and HLA-C expressed on trophoblast cells and to produce angiogenic factors[85,86]. Macrophages and DCs are involved in scavenging and degradative functions associated with menstruation[87,88]. Moreover, macrophages are found in the placental bed throughout gestation and likely provide an immediate antigen non-specific host defense to infection, essential for maintaining the integrity of pregnancy[89,90].

A further crucial component of the innate immunity system is the inflammasome, intracellular, multiprotein complex involved in the endometrial surveillance against possible noxious agents[90]. Once recruited, inflammasome increases pro-inflammatory cytokine, such as IL-1β, IL-18 and IL-33, generating their respective mature secretory forms. These events are necessary for the induction of further systemic responses and to spreading of inflammation[91]. Of interest, a significant increased expression of the inflammasome components as well as of IL-1ß and IL-18 in endometrial biopsies obtained from women with recurrent pregnancy loss as compared to controls has been observed.

Uterine T cells represent the most important component of the adaptive immune system counterpart. They include lymphocytes identified by specific markers, transcription factors, cytokine production, and cytotoxic capacity. Uterine T cells consist of CD8+ cells (66%) and CD4+ cells (33%). The CD4+ T cell population includes Th1, Th2, regulatory T cells

(Tregs), and Th17 cells, each of which secretes specific cytokines with wide-ranging effects. By simplifying, Th1 cells are implicated in the cell-mediated reactions (cellular immunity), important in resistance to infections caused by intracellular pathogens and viruses and are involved in promoting inflammation[92,93]. Therefore, Th1 cells are regarded as potential contributors to pregnancy pathologies and major threats to fetal survival. Th2 cells are mostly involved in antibody production (humoral immunity) and resistance to extra-cellular pathogen infections[93]. Th1 and Th2 cells have mutual inhibitory effect on each other. The existing data linking spontaneous abortion with increased decidual Th1/Th2 ratios[94,95] suggest that pregnancy is a Th2-prevalents phenomenon.

It is now well accepted that the human endometrium hosts different populations of microorganisms, reaching only a 30% of those present in the cervical-vaginal flora[96,97]. In recent years, the development of sequencing-based technologies have enabled the evaluation of the endometrial microbiota and microbiome, defined as the totality of the microbes and their genomes existing at endometrial level[98,99]. Moreno et al[100] detected up to 191 operational taxonomic units (OTUs) of bacteria at the endometrial level, with a composition not influenced by steroid hormones fluctuations[101]. They distinguished a Lactobacillus-dominated (> 90% *Lactobacillus spp*.) and non-Lactobacillus-dominated (< 90% Lactobacillus with > 10% of other bacteria) microbiota and, they found that a non-Lactobacillus-dominated microbiota were associated with a significantly lower rate of implantation (60.7% *vs*. 23.1%), pregnancy (70.6 % *vs*. 33.3%), ongoing pregnancy (58.8% *vs*. 13.3%), and live birth (58.8% *vs*. 6.7%) compared to women with a Lactobacillus-dominated microbiota. Verstraelen et al[102], performed on a heterogeneous group of women with different reproductive history, including subfertility, a unique microbiota dominated by Bacteroides has been documented. More recently, using next-generation sequencing technologies, Kitaya et al[103] attempted to characterize the microbiota in the endometrial fluid and vaginal secretions in women with recurrent implantations failure. They found that the endometrial microbiota had higher α-diversity and broader bacterial species than the vaginal microbiota.

These data highlight the efforts made in the recent scientific research to better characterize the endometrial microbiota, recognize the interplay between the vaginal/uterine microbiome and the immune system and to develop predictive markers for possible outcomes.

Additional studies are needed to better understand women genital tract milieu and identify new biomarkers and their health consequences. These will improve gynecological evaluation will design new approaches to diagnosis and, ultimately, will lead to better, personalized new therapies.

Conclusions

The study and characterization of the role of microbiota in gynecological and obstetric conditions is moving its first steps, following the road of other disciplines (such as gastroenterology), in which it plays a predominant role in explaining the onset and offset of several conditions.

If traditionally vaginal flora has been analyzed through classic microbiologic methods, such as culture or colorations, a new step has to be done: in facts, 16S rRNA sequencing can lead to a more efficient, effective and accurate determination of all the phyla and species determining the diversity of microbiome. This method has not got yet the right grade of standardization, and this does not allow its spreading in everyday clinical and research life.

The debate is still controversial on several points, but there are some certainties: healthy vaginal flora is characterized by high concentration of *Lactobacilli*, responsible for lactic acid production and maintenance of vaginal pH < 4.5, whilst several gynecological pathologies are associated to a reduction in *Lactobacilli*, with an increase of other bacterial species.

Another important aspect that must be taken into consideration is immune response. Immune response strictly interacts and strictly regulates microbiota itself.

The study and characterization of vaginal flora opens up the road to new perspective of therapy, such as probiotics, prebiotics, antibiotics, hormonal therapies that together can shape and modulate microbiome in order to restore clinical and microbiological eubiosis, fundamental for a correct working of all the reproductive system.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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