

Review

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Review

Microbiota and Recurrent Pregnancy Loss (RPL); More Than a Simple Connection

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Abstract: Recurrent Pregnancy Loss (RPL) affects 1-2% of women, and its triggering factors are unclear. Several studies have shown the vaginal, endometrial, and gut microbiota may play a role in RPL. A decrease in the quantity of *Lactobacillus crispatus* in local microbiota has been associated with an increase in local (vaginal and endometrial) inflammatory response and immune cell activation that leads to pregnancy loss. The inflammatory response may be triggered by gram-negative bacteria, lipopolysaccharides (LPS), viral infections, mycosis, or atypia (tumor growth). Bacterial structures and metabolites produced by microbiota could be involved in immune cell modulation and may be responsible for immune cell activation and molecular mimicry. Gut microbiota metabolic products may increase the amount of circulating pro-inflammatory lymphocytes, which, in turn, will migrate into vaginal or endometrial tissues. Local proinflammatory Th1 and Th17 subpopulations along with a decrease in Treg and tolerogenic NK cells are accountable for the increase in pregnancy loss. Local microbiota may modulate the local inflammatory response increasing pregnancy success. Analyzing local and gut microbiota may be necessary to characterize some RPL patients. Although oral supplementation of probiotics has not been shown to modify vaginal or endometrial microbiota, the metabolites produced by it may benefit patients. *Lactobacillus crispatus* transplantation into the vagina may enhance the required immune tolerogenic response to achieve a normal pregnancy. The effect of hormone stimulation and progesterone to maintain early pregnancy on microbiota has not been adequately studied, and more research is needed in this area. Well-designed clinical trials are needed to ascertain the benefit of microbiota modulation in RPL.

Keywords: recurrent pregnancy loss (RPL); vaginal microbiota; uterine microbiota; gut microbiota; probiotic supplementation

1. Introduction

Recurrent pregnancy loss (RPL) is defined as the pregnancy loss before 20 weeks of gestation of two (US) or three (UK) consecutive clinical pregnancies. The loss must be documented by ultrasound or histopathology. This condition affects 1–2% of women of reproductive age [1–3] and is linked to genetic, anatomical, endocrine, autoimmune, and infectious factors [1,3]. Most cases of RPL lack clear etiology, and little is known about its associated factors [1–3].

There are two types of RPL: primary and secondary [3]. Primary RPL occurs in women who have never given birth to a live infant, while secondary RPL occurs in women who have given birth to a live infant. More research is required to define the differences between the two entities. It is generally assumed that the mechanisms of primary and secondary RPL differ, but this has not been proven.

Great efforts have been made to analyze vaginal and endometrial/uterine conditions in RPL [3]. The analysis of local tissue along with resident immune cells and the plausible role of infectious diseases in modifying local homeostasis has rendered exciting results [4–6]. Except for explainable

problems with the spermatozoa, low amount, low migration, impaired function, and genetic mutations of the zygote/fetus, the rest mainly involve maternal physiological responses. In primary RPL, there is a need to understand the key elements that are involved in the increase of recurrent abortion; in secondary RPL, there is a need to know why, after a pregnancy, it has been impossible to achieve another normal pregnancy. Local factors may play a critical role in the process.

Microbiota are the diverse microbial communities that home in the human body. This comprises bacteria, archaea bacteria, fungi, viruses, and protists. These communities vary significantly in composition and function among different body sites and individuals. The changes in the microbiota (dysbiosis) are associated with various diseases [4]. Normal microbiota and the lack of inflammatory mediators due to bacterial, fungal, or viral infections or cancer are crucial to succeed in a normal pregnancy. Local microbiota plays a role in the defence of external pathogens and the modulation of local immune response [4–7]. Two key elements have been defined: the importance of zygote implantation and immune tolerogenic response to avoid fetal rejection [3–7]. In both permissive conditions, local microbiota plays a critical role against foreign pathogens, protecting tissues and promoting a vigilant response from local immune cells.

This brief overview will discuss the importance of genital and gut microbiota in RPL.

2. Immune Cells in the Female Reproductive Tract

Immune cells vary among different parts of the female reproductive tract [7,8]. In the vagina, contrary to expectation, T cells (CD4⁺ and CD8⁺ subpopulations, memory cells) predominate at around 50 % of the total leucocyte, NK cells are the second highest population, close to 20 %, the number of B cells is only 1 %, and the rest of the cells, approximately 30 %, are granulocytes and macrophages [7,8]. As a comparison, semen contains preferentially granulocytes and macrophages and a small amount of lymphocytes (~ 5 %) [9].

T cells (CD4⁺ and CD8⁺) are around 50 % of the leucocytes in the ecto and endocervix, with macrophages and NK cells representing around 12 % [7,8]. The rest are granulocytes and B lymphocytes. Finally, the cell type in the endometrium differs depending on the hormonal cycle. From the early follicular to the early secretory phase, the number of leucocytes (CD45⁺) in the endometrium remains low, but during the secretory phase, they increase about 5-fold [7]. Therefore, the total number of leukocytes peaks premenstrually. In the late secretory phase, NK cells (especially CD3⁺/CD56^{bright}/CD16⁺) are predominant, compromising approximately 80% of CD45⁺ cells, while CD3⁺ T cells (predominantly CD8⁺) decrease to less than 10% [7]. The percentage of B cells is low in the proliferative and middle secretory phases and increases in the late secretory phase [7]. Macrophages, neutrophils, and eosinophils increase at the late secretory phase. Mast cells represent 1 -2% of the total endometrial cells during the menstrual cycle. T cells are high in the Fallopian tube, followed by granulocytes, NK cells, macrophages, and B cells [7]. In summary, in the endometrium, cells have high mobility depending on the hormonal cycle, which may change in case of infection. As expected, the immune cell response will differ according to the type (viral, bacterial, fungal), infection site, and the hormonal cycle stage [7,8].

It is also important to clarify that mucosal T cells (MAIT) and innate lymphoid cells will migrate to the reproductive tissues in the presence of infection [7,8,10–14]. Three types of innate lymphoid cells are precursors of Th1, Th2, and Th17/Th22, depending on the tissue milieu [11]. The role of immune cells, particularly uterine and decidual NK cells, has been reviewed before [13].

The role of interferon (IFN) signaling upon viral infection may condition endometrial local immune response [12]. Cell activation by Toll-like receptors or danger cell signals and inflammasome [10] activation negatively impacts RPL [11–14]. The production of local cytokines may partially explain the local response [16], and semen impacts local cytokine production [17].

The resolution of chronic inflammation induced by pathogens [19,20] remains uncertain. In addition, the role of immunoglobulins, complement, and antibacterial peptides in local infection resolution requires more research.

3. Local (Vaginal and Uterine) Microbiota and RPL

Evidence suggests that vaginal and uterine microbiota may play a role in RPL [21–27]. The microbial population that lives in the vagina is composed of facultative and obligate anaerobes that form a symbiotic relationship with the host [15,22–34]. These bacteria maintain healthy vaginal tissue by promoting and supporting an ideal pH of ~4, producing hydrogen peroxide [31,32] and a proteinaceous outer protective layer [6,33,34]. Different species of *Lactobacillus* appear to be most prevalent among women [35,36], with *L. crispatus* being categorized as the most protective species and found in the highest proportions in healthy individuals who had successful pregnancies, and *L. nervosa* being the least protective. It is unclear whether this confers a negative effect [37,38]. Recurrent implantation failure (RIF) and RPL are associated with increased microbiome diversity and a loss of *Lactobacillus* dominance in the lower female reproductive system [25,29]. First-trimester miscarriage has been associated with reduced prevalence of *Lactobacillus* spp [39] and *Lactobacillus* spp. depleted vaginal microbiota was related to pro-inflammatory cytokine (IL-1 β , IL-6, IL-8) levels most strongly in euploid miscarriage compared to viable term pregnancy [40]. On the other hand, *Lactobacillus crispatus* was less abundant in the endometrial samples of women with RPL compared with controls, and *Gardnerella vaginalis* was more abundant in the RPL group than in controls in both endometrial and vaginal samples [41]. Furthermore, Vomstein et al. [28] observed a lower abundance of *Lactobacillaceae* in the uterus of RPL and RIF patients at three points of their menstrual cycle. They found an increase in *Proteobacteria* in the RSA and RIF groups towards the end of the menstrual cycle. In this study, the RIF group exhibited a remarkably diverse composition, unlike the control and the RPL group [42]. In the same way, a relative dominance rate of *Ureaplasma* species in the endometrial microbiome was an independent risk factor for subsequent miscarriage with normal karyotype in a cohort of patients with a history of RPL [24]. Also, *Proteobacteria* and *Firmicutes* were significantly elevated in the endometrium of RPL patients in comparison with women requesting termination of normal pregnancy [43]. Further, the abundance of *Bacteroides* and *Helicobacter* in the vagina in an early embryonic arrest group was higher than that in the standard pregnancy-induced abortion group. Furthermore, the abundance of *lactobacilli* in the normal pregnancy-induced abortion group was higher than that in the embryonic arrest group. In this last group, the abundance of *L. iners* was significantly lower than that in the normal pregnancy group [44]. Therefore, women with an unbalanced population of bacteria, mainly composed of *Gardnerella*, *Prevotella*, *Atopobium*, *Sneathia*, *Megasphaera*, *Delftia*, *Cutibacterium*, *Peptoniphilus*, *Anaerobacillus*, and unknown bacteria, are at higher risk for premature birth and RPL [30,41,45–47]. In summary, Smith and Ravel [48] proposed a hierarchical clustering of the vaginal microbiota of reproductive-aged women into five distinct community state types (CST), four of which are dominated by *Lactobacillus* spp. (*Lactobacillus crispatus* (CST-I), *L. iners* (CST-III), *L. gasseri* (CST-II) or *L. jensenii* (CST-V)) and the fifth (CST-IV) is composed of a polymicrobial mixture of strict and facultative anaerobes, including species of the genera *Atopobium*, *Megasphaera*, *Mobiluncus*, *Prevotella*, and other taxa in the order *Clostridiales*. CST I correlates with low obstetric-gynecological risk, and CST IV correlates mostly with vaginal discomfort and/or obstetric-gynecological diseases [49].

The microbiota is sensitive to environmental, hormonal, and dietary changes [48–52]. Microbiota plasticity, the ability to change the composition better to suit the host's and bacteria's needs, is implemented during the menstrual cycle [48–50]. In the genital tract of women of reproductive age, *Prevotella* spp. is linked to the proliferative phase of the menstrual cycle, whereas *Sneathia* spp. is related to the secretory phase [53]. On the other hand, *Lactobacillus* species are found in the highest numbers during the luteal phase and decrease during menstruation [50,54]. Interestingly, a prevalence of *Prevotella* spp. over *Lactobacillus* spp. and poor microbiota plasticity (little to no changes in bacterial population in response to different signals) are associated with an increased risk of infections and RPL [42].

Human leukocyte antigen genes (HLA) play a role in selecting the population of intestinal bacteria [55,56]; certain HLA haplotype carriers might be more susceptible to having a dysbiotic microbial population; in fact, women with the HLA-DQ2/DQ8 haplotype appear to have an altered microbiota [57].

A dysbiotic microbiota is less protective against sexually and non-sexually transmitted diseases such as HIV [58], syphilis, chlamydia, gonorrhea, *Trichomonas* [59,60], human papillomavirus (HPV) [61], herpes simplex virus (HSV) [62,63], pelvic inflammatory disease [64], aerobic vaginitis (AV) [65], bacterial vaginosis (BV) [20] and candidiasis [66]; all of which can negatively affect gestation [67–71] by causing inflammation and tissue destruction [72,73].

The composition of uterine endometrium microbiota (UEM) might predict pregnancy outcomes [24]. A dysbiotic UEM, consisting of *Lactobacillus iners* and *Ureaplasma* species, is associated with inflammatory conditions like chronic endometritis (CE) [26], and women with this condition are likely to be diagnosed with RPL, RIF, and infertility [74]. CE occurs when plasma cells and B lymphocytes migrate to the uterine endometrial stroma, creating permanent inflammation [75,76]. The administration of antibiotics has been shown to improve implantation outcomes [77] but has not improved miscarriage rates [78]. These results suggest that other bacteria may be essential to induce the tolerogenic response of the immune cells required for a normal pregnancy.

The gut microbiome might directly influence the vaginal and uterine microbiomes. Recently, Zhu et al. [31] provided evidence suggesting a connection between a dysbiotic gut microbiome and RPL. The gut microbiome, composed of symbiotic bacteria, is involved in metabolism, inflammation, and immunity [29]. To protect the host from infections, bacteria release factors and form a physical barrier by attaching to the intestinal wall [79]. They convert food particles into essential nutrients [80] and neurotransmitters and help modulate insulin response. The association between a dysbiotic gut microbiome and RPL is complex. Gut microbiota participates in numerous pathologies associated with higher incidences of RLP, such as chronic inflammation, vulnerability to infections, obesity, diabetes, and polycystic ovary syndrome (PCOS) [29]. The type of bacterial population in the gut microbiota is involved in local inflammatory responses due to abnormal production of cytokines [81].

A decrease in gut bacterial population diversity may result in the infiltration of gram-harmful bacteria and, consequently, the production of lipopolysaccharides (LPS). This endotoxin can enter the systemic circulation and promote the expression of pro-inflammatory genes [81,82]. LPS is also known to increase the production of reactive oxygen species (ROS), resulting in oxidative stress as well as insulin resistance (IR) [83,84]. Moreover, an abundance of non-protective bacterial species such as *Prevotella* [85] promotes inflammation that leads to immune cell migration and activation, which might induce the formation of autoantibodies [81] and excessive NK cell migration [86,87]. Autoimmune diseases such as Systemic Lupus Erythematosus (SLE), autoimmune thyroid disease (AITD), and celiac disease (CD) are all associated with higher incidences of RPL and a dysbiotic gut [57,86–88]. It is yet unclear whether treatment with the “beneficial” *Lactobacillus* species (*L. crispatus*) can improve autoimmunity and thus reduce the likelihood of fetal rejection [89]. A report has shown a direct association between regulated production and stimulation of NK cells and downregulation of pro-inflammatory cells, even in the placenta [90]. Interestingly, a higher prevalence of *Gardnerella vaginalis* and gram-negative bacteria has been associated with an increased number of circulating NK cells and recurrent miscarriage [91].

Changes in local microbiota not only affect RPL patients. In preeclampsia, an increase in *Escherichia* species was reported along with *Rothia*, *Actinomyces*, and *Enterococcus*, and a lower abundance of *Coprococcus* compared to pregnant women with normotension [92]. Changes may also occur in eclampsia and diabetic pregnancies. There are only a few reports relating RPL with metabolic syndrome after 40 years of age [93]. This association can be due to different events but may involve the gut microbiota. More research is required in this area.

4. Perspectives of Microbiota Modulation on RPL

The main problem in understanding the changes in local microbiota is the analysis of cultured samples. The molecular diagnosis assessment should aid in defining quantitative differences in the species encountered. In general, the changes in specific species of *Lactobacillus* and the increase in *L. crispatus* in women with successful pregnancy means that local microbiota changes may be necessary but not essential [94]. It is also difficult to ascertain the role of sperm microbiota in the process, the

possible consequences of hormones like progesterone, and the relationship between different microbiotas, vaginal and endometrial, based on current knowledge.

Recent evidence indicates that diverse populations with a high proportion of *Lactobacillus crispatus*, are positively associated with lower incidences of infections, implantation failures, and RPL [15,23,95,96]. Efforts to modulate and improve the bacterial population in the vagina through the administration of antibiotics, boric acid, lactic acid, and estrogen have been unsuccessful long-term [97–100]. Treatments with vaginal and gut probiotics yielded mixed results [101–104]. Sex hormones may alter vaginal colonization, as shown in the mouse model [105], and using a low dose of estrogen in women with atrophic vaginitis [106]. Metronidazole treatment may also affect the process [107]. Vaginal microbiota transplants are a relatively new yet promising form of therapy and consist of transferring the entire vaginal microbiota of healthy women to patients [110–112]. Oral probiotic supplementation seems to aid vaginal microbiota [113]. A proof-of-concept case study where a woman successfully shifted her microbiota population after microbiota transplant from mostly *Gardnerella spp.* (90%) to 81.2% *Lactobacillus crispatus* and 9% *Lactobacillus jensenii* ameliorated her vaginal symptoms (vaginal irritation and discharge), and she was able to carry a healthy pregnancy to term after three pregnancy losses [114]. However, this topic and the therapies available are still relatively new, and there is room for improvement in the future.

Liu et al. [41] reported a critical drop in IFN- γ and IL-6 levels inside the uterine cavity of RPL women, suggesting a negative association between microbiota and immune response. Interestingly, this group [41] identified that imidazole propionic acid and 1,4-methyl imidazole acetic acid are associated with subsequent recurrent miscarriage. On the other hand, a reduction in butyrate-producing bacteria in the gut microbiota of RPL has been demonstrated, suggesting a link between immune vigilant responses and anti-inflammatory properties of the microbiota [41]. A report studied 20 fecal samples of patients with RPL, and antibody-related factors explained the conditions compared to 20 patients without antibodies [114]. Community richness and phylogenetic diversity in the antibody-positive group were higher than in the opposing group. Bacteroides were prevalent in the positive group, while in the opposing group, *Bacteroides*, *Erysipelotrichaceae*, *Faecalibacterium*, *Enterococcus*, *Prevotella*, *Megasphaera*, and *Anaerostipes* were abundant. Proteomic studies support the results and propose that cytoskeleton proteins may be crucial in the local disarrangements in endometrial tissue, which can lead to a lack of implantation [115]. In summary, well-defined clinical trials are required to identify the importance of gut microbiota and its possible therapeutic role in RPL.

Also, the association between gut bacterial dysbiosis and a Th1/Th17-mediated proinflammatory state in RPL has been demonstrated [116], uterine NK cells and macrophages [13]. Tolerogenic NK cells, dendritic cells, and macrophages [13], as well as a decreased amount of Treg [117], seem responsible for the process.

Supplementary probiotics aid vaginal and endometrial microbiota [118,119]. Moreover, supplementary probiotic treatment benefits couples with RPL because of their capacity to improve aberrant spermatozoa antigenicity [104,120]. Tersigni and coworkers [121] reported that patients with celiac disease may benefit from oral probiotics by decreasing intestinal inflammation and increasing anti-inflammatory metabolites. There are still controversies in the field [122]. The number of well-designed clinical trials must be increased to ascertain the benefits of oral microbiota supplementation.

Omega-3 supplementation has been shown to increase fertility in mice [123] and is now being successfully used to enhance human fertility [124] and possibly reduce RLP [125]. However, in their review, Kello and Cho [126] mentioned that supplementation may only benefit patients with antiphospholipid syndrome. Consequently, it may assist a group of patients with RPL in which immune disorders may be involved. How oral supplementation of omega-3 may affect local microbiota is unknown; however, it can be postulated that a decrease in prostaglandin E2 production, as a product of an inflammatory response, may promote/support the production of other anti-inflammatory intermediates. The role of nutritional supplementation on RPL should be carefully studied [4,6,120,124].

Recently, it has been shown that gut microbiota metabolites affect circulating lymphocytes. There is an increased amount of Th1, Th17, and B cells, with a decrease in Treg and Breg cells [127]. Even though the report is only based on 12 samples, it raises an important question concerning oral supplementation. It should probably be assumed that RPL patients have a subclinical inflammatory condition that may be modulated pharmacologically.

It has been assumed that local prednisone treatment reduces local inflammatory response and consequently decreases abortion risk and increases pregnancy efficiency in IVF [128,129] and also the use of nutritional supplements [130]. Also, topic cytokines such as G-CSF [132] and interferon λ [133] can increase the possibility of normal pregnancy primarily based on the induction of tolerance at the tissue level. However, we know that critical scientific evidence of therapeutic local progesterone and cytokines local microbiota has not been documented.

5. Conclusions

Primary and secondary RPL are complex medical conditions involving 1-2 % of women of reproductive age. Despite numerous efforts to study the possible triggering factors, significant achievements have yet to be reported. Local (vaginal and endometrial) and gut microbiota and metabolites differ in RPL compared to normal pregnancies. There is evidence of cell migration and local tissue disarrangements in RPL. Protective microbiota prevents local inflammatory response and infections and protects endometrial tissue. Transplantation of microbiota and oral probiotics may help maintain vaginal microbiota.

Patients with RPL, especially those with immune disorders, may benefit from oral probiotic supplementation or microbiota transplantation. It is not clear whether antigen mimicry can play a role in RPL.

Figure 1 represents a summary of the events that occur in eubiosis, normal conditions, and dysbiosis in the vagina.

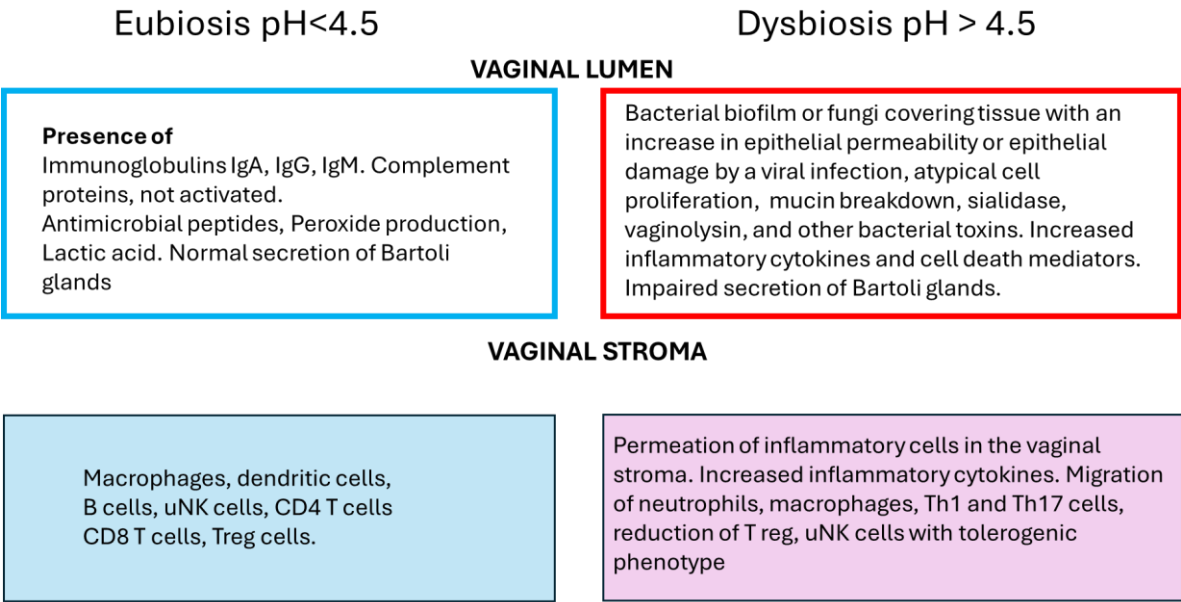


Figure 1. General overview of the differences between eubiosis and dysbiosis in the vagina. In the vaginal lumen, the expected protective effect of immunoglobulins, complement proteins, antimicrobial peptides, peroxide production, and lactic acid. In dysbiosis, the protective effect is lost, and the inflammatory response is due to bacterial proteins, increasing cell death inflammatory mediators. This increase in inflammatory mediators leads to a decrease in vaginal tolerogenic milieu which is the response to the reduction of annidation and increase of pregnancy loss.

Well-planned clinical trials are required to provide evidence of the role of microbiota and microbiota transplantation in RPL. RPL patients may be at risk of developing medical conditions

shortly after reproductive age; therefore, studies on RPL classification and triggers are urgently required.

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