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REVIEW ARTICLE

Female reproductive tract microbiome and early miscarriages

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Miscarriage is one of the main causes of reproductive loss, which can lead to a number of physical and psychological complications and other long-term consequences. However, the role of vaginal and uterine microbiome in such complications is poorly understood. To review the published data on the function of the female reproductive tract microbiome in the pathogenesis of early miscarriages. The articles published over the past 20 years and deposited in PubMed, Google Academy, Scopus, Elibrary, ResearchGate, and EBSCO databases were analyzed. The review presents new data on the impact of the vaginal and uterine microbiome on the local immunity, including defense against sexually transmitted infections, and its association with other factors of miscarriages. The studies on the microbiome of non-pregnant women with recurrent miscarriages in the anamnesis, patients undergoing IVF, and pregnant women with miscarriages, as well as new directions in the microbiome research are discussed. The majority of studies have demonstrated that the dominant species of the vaginal and uterine microbiome in patients with early miscarriages are non-*Lactobacillus* bacteria. As many of these bacteria have not previously been detected by cultural studies and their role in obstetric complications is not well defined, further research on the female reproductive tract microbiome, including the microbiome of the cervix uteri, is needed to develop new approaches for the prognosis and prevention of miscarriages.

Key words: Vaginal microbiome; uterine microbiome; local immunity; early miscarriage; early pregnancy loss; missed abortion; spontaneous abortion; anembryonic pregnancy; *Lactobacillus*; *Atopobium*; *Bifidobacterium*; *Gardnerella*; *Megasphaera*; sexually transmitted diseases.

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INTRODUCTION

Miscarriages occur in 15% of clinically recognized pregnancies in the general population [1,2]. Miscarriage has several physical (bleeding, sepsis, infertility) and psychological (depression, anxiety, suicide) consequences and might be a risk marker of severe obstetrical complications during the following pregnancies and various long-term pathological conditions, such as venous thromboembolism and cardiovascular diseases [2].

Up to 80% of reproductive losses occur in the first trimester [3]. Miscarriage may be caused by chromosomal abnormalities [4,5], antiphospholipid syndrome [6], thrombophilias [7,8], and immune and endocrine disorders [9–11]. One of the leading causes of miscarriages is inflammation [12,13]. However, in up to 50% of cases, the cause of the miscarriage remains unknown [14].

In this regard, studying the role of the female reproductive tract microbiome in the pathogenesis

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of spontaneous early miscarriages is of particular interest.

FEMALE REPRODUCTIVE TRACT MICROBIOME

The microbiocenosis of the female reproductive tract is an ecological niche that includes the epithelial barrier, secretions of the epithelial glands, immunocompetent cells, and specific microflora formed under the influence of exogenous and endogenous factors [15].

The development of non-cultural methods in the last two decades, such as the high-throughput sequencing of the 16 S rRNA, has resulted in significant progress in the studies of female reproductive tract microbiocenosis [15,16]. Thus, several microorganisms not previously associated with the vaginal microbiocenosis (*Sneathia*, *Leptotrichia*, *Atopobium vaginae*, *Dialister*, *Eggerthela*, *Megasphera*) have been identified [17], some of which may be etiological factors in a number of gynecological and obstetrical complications [18,19].

Vaginal microbiome

The studies of the vaginal microbiome by the high-throughput sequencing of 16 S rRNA have identified five types of vaginal communities (community state types, CSTs) based on the predominance of *Lactobacillus* species: CST I (*Lactobacillus crispatus*), CST II (*Lactobacillus gasseri*), CST III (*Lactobacillus iners*), CST V (*Lactobacillus jensenii*), and CST IV consisting mainly of obligate and facultative anaerobes without predominance of lactobacilli [20,21].

Using the new vaginal microbiome classification tool VALENCIA (*VA*gina*L* community state typ*E* *N*earest *C*entro*I*d cl*A*ssifier), which is based on the nearest centroid classification, the above-mentioned CSTs were recently divided into subtypes. CST I and CST III were split into subtypes A and B (with a higher and lower relative abundance of the focal species, respectively) [22]. CST IV was divided into 3 subtypes: CST IV-A [high relative abundance of *Candidatus Lachnocurva vaginae* (formerly known as BVAB1), a moderate relative abundance of *Gardnerella vaginalis*, a moderate relative abundance of *A. vaginae*], CST IV-B (high relative abundance of *G. vaginalis*, low relative abundance of *Ca. L. vaginae*, moderate relative abundances of *A. vaginae*), and CST IV-C (low relative abundance of *Ca. L. vaginae*, *G. vaginalis*, *A. vaginae*, and *Lactobacillus* spp., and prevalence of facultative and strictly anaerobic bacteria) [22]. CST IV-C was subdivided into five groups: CST IV-C0 (moderate abundance of *Prevotella*), CST IV-C1 (*Streptococcus*-dominated community), CST IV-C2 (*Enterococcus*-dominated community), CST IV-C3 (*Bifidobacterium*-dominated community), and CST IV-C4 (*Staphylococcus*-dominated community) [22].

Lactobacillus crispatus has the highest capacity to produce lactic acid and, therefore, provides the lowest vaginal pH among all CSTs [20]. *L. iners* has the lowest capacity for lactic acid production because it converts glucose through pyruvate exclusively into L-lactate, but not into D-lactate [23,24]. Therefore, the vaginal pH of patients with CST III is higher than in women with other *Lactobacillus*-dominated communities [20]. According to the data of France et al. [22], *L. iners*-dominated (CST III) and *L. jensenii*-dominated (CST V) communities have the second lowest vaginal pH after *L. crispatus*-dominated community (CST I). *L. gasseri*-dominated communities (CST II) have the highest pH among *Lactobacillus* spp.-dominated communities.

In the *L. iners*-dominated communities (CST III), the ratio between L- and D-lactic acid strongly correlates with the levels of the vaginal extracellular matrix metalloproteinase inducer (EMMPRIN) and matrix metalloproteinase-8 (MMP-8) in the vaginal secretions. EMMPRIN and MMP-8 facilitate the breakdown of the extracellular matrix, which leads to the ascending infection due to bacterial migration from the vagina to the uterus [25]. *L. iners* has almost a half-size genome compared to *L. crispatus* [26]. Therefore, it has fewer enzymes, including those involved in carbohydrate metabolism and production of essential amino acids. This makes *L. iners* more vulnerable to exogenous factors compared to *L. crispatus*. In bacterial vaginosis (BV) environment, *L. iners* upregulates the expression of proteins involved in glycerol transport and related metabolic enzymes, cholesterol-dependent cytolysin, and mucin *vs.* healthy individuals [27]. It was suggested that this can be an adaptation aimed to promote survival during the BV episodes. During early pregnancy, vaginal epithelial cells in the presence of *L. iners* exhibit a lower level of autophagy, produce more stress-related HSP70 protein, and release higher amounts of pro-inflammatory mediators compared to the cells in the presence of *L. crispatus* [28].

CST IV-A has the highest pH among all CSTs, followed by CST IV-B. The CST IV-C subtypes dominated by *Bifidobacterium*, *Enterococcus*, and *Staphylococcus* are associated with lower pH, while the *Streptococcus*-dominated communities have higher pH [22].

CST IV-A and CST IV-B are the BV-associated communities [20,22], whereas other CST IV

communities have no direct correlation with the Nugent score. Therefore, they were assigned to the CST IV-C group in the new classification. The most common CST IV-C communities in reproductive-age women are CST IV-C1 (*Streptococcus*-dominated) and CST IV-C3 (*Bifidobacterium*-dominated). Despite that both *Streptococcus* spp. and *Bifidobacterium* spp. can produce lactic acid, vaginal pH in these communities is higher, than in the *Lactobacillus*-dominated communities [22].

It was shown that in early pregnancy, vaginal microbiome becomes less diverse and *Lactobacillus* dominates in the majority of women [29]. The vaginal microbiome in the first trimester of pregnancy can depend on ethnicity. The highest rate of *L. inners*-dominated communities in the first trimester was found in the Chinese population [30]; *L. crispatus* was the most common in the Canadian population [31]. MacIntyre et al. [29] found that CST I, CST III, and CST IV were represented in the same proportion in women of European, Asian, and African origin in the UK population, while CST II was not found in women of African origin. Therefore, the presence of the control group is important in every research. In multicenter studies, control groups from different regions of the world should be initially checked for significant differences between them, and if these differences are present, such groups cannot be united in one control group.

The microbiome of the cervix and endometrium

The endometrial microbiome plays a key role in several obstetrical complications [32]. However, cervical and uterine microbiomes are less studied compared to the vaginal microbiome [33].

To our knowledge, there are no studies on normal uterine and cervical microbiome during the first trimester of pregnancy, for example, in patients, admitted for legal abortions.

Meanwhile, there are studies in which uterine samples were collected with a catheter for the embryo transfer with an outer sheath, with the previous rinsing of the cervix by an antiseptic solution to prevent the catheter from contamination [34–38]. In one study, cervical mucus was removed before extraction of the catheter from the uterine cavity [35].

Moreno et al. [35] showed that the dominant species in the endometrial fluid of healthy fertile women were *Lactobacillus* spp. The other most common species of the uterine microbiome of healthy reproductive-age women were *Gardnerella*, *Bifidobacterium*, *Streptococcus*, and *Prevotella*. The authors classified uterine microbiomes as *Lactobacillus*-dominated (>90% bacteria belong to *Lactobacillus* spp.) and non-*Lactobacillus*-dominated (<90% bacteria belong to *Lactobacillus* spp. and >10% bacteria are pathogenic or dysbiotic).

In the study of Kyono et al. [37], 6 out of 7 healthy volunteers were found to have the *Lactobacillus*-dominated uterine microbiome (>90% *Lactobacillus* spp.).

Fang et al. (2016) showed that the most abundant phyla in the endometrium at the first stage of the menstrual cycle are *Firmicutes*, *Proteobacteria*, and *Actinobacteria*. The dominant genera were *Lactobacillus*, *Enterobacter*, and *Pseudomonas* [38].

Franasiak et al. [34] demonstrated that the prevalent species in the uterine microbiome of reproductive-age women were *Flavobacterium* and *Lactobacillus*. In the study of Tao et al. [36], *Lactobacillus*, *Corynebacterium*, *Bifidobacterium*, *Staphylococcus*, and *Streptococcus* were found in the uterine cavity. However, only infertile women admitted for embryo transfer were examined in these two studies.

A number of articles describe the cervical and uterine microbiomes after hysterectomy [39–43] and hysteroscopy [42,44]. Indications for hysterectomy included benign proliferative conditions (uterine fibroids, endometrial hyperplasia, *etc*.), and indications for hysteroscopy were menorrhagia and dysmenorrhea. These conditions are caused by hormonal changes. As the microbiome highly depends on the menstrual cycle and other changes in the hormonal status, the samples obtained in patients with gynecological disorders cannot be considered as normal [45]. Due to the same reason, we did not consider as normal the cervical and uterine microbiomes of endometriosis patients that have not undergone hysterectomy [46]. Besides, in the case of hysteroscopy, contamination of uterine samples by cervical microbiota cannot be excluded [33].

MICROBIOME AND MAIN CAUSES OF MISCARRIAGE

Microbiome and local immunity

It was shown that in vaginal epithelial cell (VEC) culture, *A. vaginae* promoted expression of mucin 1 (responsible for antibacterial defense) and mucin 3 (responsible for Th1 lymphocyte apoptosis), but not mucin 4, mucin 16, and mucin 5AC. *L. crispatus* and *L. iners* did not have a significant impact on mucin production compared to sterile VEC culture [47].

Lactobacillus crispatus and *L. jensenii* did not effect on cytokine production compared to non-

colonized VECs [48]. Clinical observations have confirmed that the presence of *L. crispatus* and *L. jensenii* does not lead to inflammation and even decreases the levels of pro-inflammatory cytokines [49].

Cervical epithelial cells produce more proinflammatory cytokines interleukin (IL)-6 and IL-8 in the presence of *G. vaginalis*, *Prevotella bivia*, and *Prevotella amnii* and more cytokine PC1 in the presence of *Megasphaera*, *Clostridium*, *Prevotella*, *A. vaginae*, and *Sneathia* compared to *L. crispatus* [50].

Several authors have investigated the impact of *Lactobacillus* spp. on pro-inflammatory cytokine production in the presence of viral and bacterial ligands. Toll-like receptors (TLRs) are signaling receptors of innate immunity cells, which are the first line of pathogen recognition in the female reproductive tract [51–53]. They can induce immune response and regulate its intensity [54]. The expression of TLRs and their signaling adaptor genes can play an important role in the pathogenesis of early miscarriages [55,56].

Using VEC culture, it was shown that *L. crispatus*, but not *L. jensenii*, significantly reduces the secretion of IL-6 and IL-8 induced by the polyinosinic:polycytidylic acid (PIC), a ligand of TLR3 involved in the recognition of double-stranded viral RNA, compared to the non-colonized cultures. The presence of *L. crispatus* or *L. jensenii* in the VEC culture significantly reduced IL-6 and tumor necrosis factor α (TNFα) secretion after stimulation by the fibroblast stimulating ligand-1 (FSL-1, ligand of TLR2/6 involved in the recognition of lipoteichoic acid and peptidoglycan of Gram-positive bacteria and fungal cell wall saccharides) [48]. Hence, *L. crispatus* and *L. jensenii* have several mechanisms allowing to decrease production of proinflammatory cytokines.

Lactobacillus iners and *A. vaginae* upregulated expression of the TLR signaling adaptor genes for IRF1, IRAK2, NFKBIA, and proinflammatory cytokine TNFα in the VEC culture, while the presence of *L. crispatus* or *P. bivia* caused no increase in the expression of the above-mentioned genes [47]. Therefore, *L. iners*, unlike *L. crispatus*, induces TLR-dependent inflammation. The similarity between *A. vaginae* and *L. iners* allows suggesting that the latter behaves more like a BV-associated microorganism than a commensal bacterium.

Colonization of VECs with *Staphylococcus epidermidis* results in a significant increase in the content of IL-1b, IL-1Ra, IL-8, granulocyte colony-stimulating factor (G-CSF), and TNFα, compared to the non-colonized cultures [48,57].

Other important components of the immune defense of the female reproductive tract are antimicrobial peptides (AMPs). They are secreted mostly by the epithelial cells and neutrophils and can be produced constitutively or in response to microbial stimuli [58]. AMPs are involved in the elimination of Gram-positive and Gram-negative bacteria, viruses, and fungi [59] and the modulation of innate and adaptive immune responses, including TLR signaling [60].

The presence of *L. crispatus*, *L. iners*, *A. vaginae*, and *P. bivia* did not affect the expression of such AMPs as human β-defensin 1 (hBD1) and secretory leukocyte peptidase inhibitor (SLPI) in VECs, while the expression of hBD2 was significantly upregulated by *L. iners*, *P. bivia*, and *A. vaginae*. Expression of CCL20 (C-C motif chemokine ligand 20) was significantly increased following colonization with the BV-associated bacteria (*A. vaginae* and *P. bivia*), but not with *Lactobacillus* spp. [47].

It is known that normal pregnancy is associated with the prevalence of T-helpers 2 (Th2), while Th1 dominance stimulates cytotoxicity and is observed in patients with recurrent pregnancy loss. There is an indication that the BV-associated microbiome can shift the Th1/Th2 balance toward Th1, which activates the immune response against the embryo [32].

Another type of T-lymphocytes, regulatory T cells (Tregs), provides tolerance to the allogenic fetus. Th17 lymphocytes have a pro-inflammatory profile and are also associated with miscarriages. Treg and Th17 cells can differentiate into each other under the action of certain stimuli. Patients with the CST-III and CST-IV communities have a higher number of Th17 cells and increased levels of IL-17 produced by these cells [50]. This can lead to a decrease in the content of Treg cells, which are crucial for pregnancy progression.

Microbiome and risk of sexually transmitted diseases

Sexually transmitted diseases are risk factors for early pregnancy loss. The majority of research on the microbiome and early miscarriages analyze the presence of bacteria, which are etiological factors of these diseases. In addition, changes in the genital tract microbiome can be risk factors for sexually transmitted infections, including during the pregnancy (Fig. 1).

The *L. iners*-dominated community (CST III) is associated with an increased susceptibility to *Chlamydia trachomatis* [61–63] and *Mycoplasma genitalium* [63]. Women with CST IV have a higher risk of *C. trachomatis* infection compared to other groups, especially CST I [51].

In vitro studies showed the ability of *L. crispatus* (CST-I) and *L. gasseri* (CST-II) to inhibit the growth of *Neisseria gonorrheae* [64].

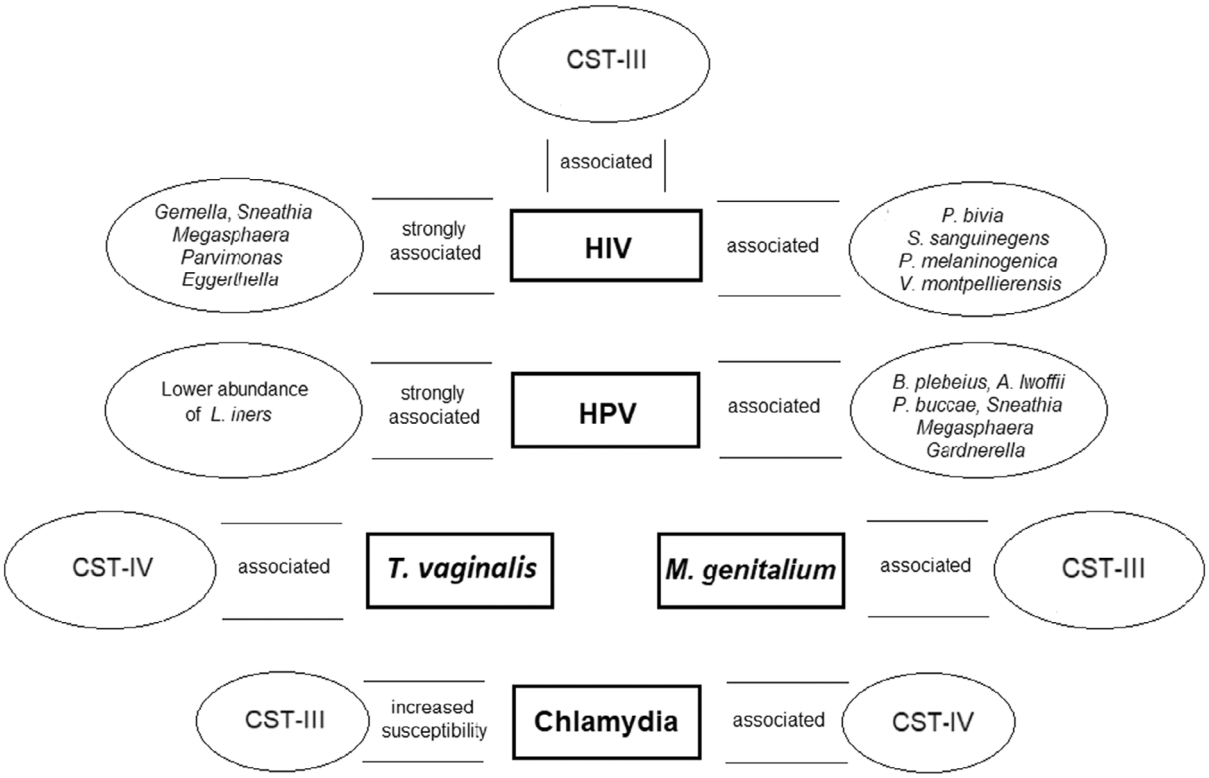

Fig. 1. Vaginal microbiome and the risk of sexually transmitted diseases.

Patients with the CST-IV community have 8-fold higher odds of carrying *Trichomonas vaginalis* compared to women with the *L. crispatus*-dominated communities (CST-I) [65].

In the study of monozygotic human papillomavirus (HPV)-discordant twins, a lower abundance of *L. iners* was strongly associated with the HPV infection, while the decreased *L. crispatus* content was not a risk factor for HPV persistence [66]. The microbiome of the HPV-positive patients was more diverse, and the presence of *Sneathia* and *Megasphaera* was strongly associated with the HPV infection [66]. In another study, the presence of other anaerobic bacteria, *Bacteroides plebeius*, *Acinetobacter lwoffii*, and *Prevotella buccae*, was also found to be associated with the HPV infection [67], while the *L. gasseri*-dominated community (CST-II) was associated with the increased clearance of detectable HPV [68].

Interestingly, in another study, the presence of *L. iners* was associated with a further clearance of HPV infection, while the dominance of *Gardnerella* was a marker of HPV progression [69]. High microbiome diversity was associated with the HPV infection progression to cervical intraepithelial neoplasia grade 2 and 3 (CIN2+) [69].

Therefore, the above-mentioned studies demonstrated the protective effects of *L. iners* and *L. gasseri* against HPV infection.

Using the VEC culture, it was shown that communities with a high abundance of *S. epidermidis* (>10^4 genomic copies), *A. vaginae*, *G. vaginalis*, and *Bacterial vaginosis*-associated bacteria (*BVAB2*) are associated with decreased Zika virus titers. At the same time, the Zika virus does not affect the microbiome composition [57]. It can be explained by the increased secretion of pro-inflammatory cytokines in the presence of *S. epidermidis* and BV-associated bacteria, which might have a protective effect [47]. Average herpes simplex virus 2 (HSV-2) titers were lower in the VEC cultures colonized by the microbiomes dominated by *Lactobacillus* spp. [57].

Patients with highly diverse microbiomes have a higher risk of HIV infection [70]. Recently, it was shown that patients with CST-IV have a 4-fold increased risk of HIV infection compared to those with the *L. crispatus* communities (CST-I) [50]. The presence of *Prevotella melaninogenica*, *Veillonella montpellierensis*, *Mycoplasma*, *P. bivia*, and *Sneathia sanguinegens* in CST-IV was positively associated with HIV infection. In another study, *Parvimonas*, *Eggerthella*, *Gemella*, *Sneathia*,

Megasphaera, and *Mycoplasma* were found to be strongly associated with the HIV infection [71]. The *L. iners*-dominated microbiome (CST-III) is also a risk factor in the HIV infection [50].

HIV-positive patients with BV have a more diverse composition of the microbiome compared to women with BV in the absence of HIV [72].

Microbiome, antiphospholipid syndrome, and congenital thrombophilia

In patients with antiphospholipid syndrome and congenital thrombophilia, an unfavorable microbiome can trigger blood clot formation and thrombosis of the chorionic bed (the so-called "two-hit theory" [73,74]). This occurs mostly due to the TLR4 stimulation by the lipopolysaccharides of Gram-negative bacteria [75]. So far, the data on the role of the reproductive tract microbiome in this process is absent.

Microbiome and menstrual cycle disorders

Steroid sex hormones can affect the composition of the reproductive tract microbiome. Thus, the microbiome before menarche and in post-menopause is different from the one in reproductive-age women because of the lack of estradiol and progesterone production by the ovaries [76–78]. The microbiome can change depending on the menstrual cycle phase under the influence of estrogens and progesterone [79–83]. Menstrual cycle disorders, for example, polycystic ovary syndrome (PCOS), can lead to changes in the vaginal microbiome composition due to changes in the estradiol and progesterone levels [84,85]. Changes in the microbiome in patients with menstrual cycle disorders appear before pregnancy but might also persist in early pregnancy. Patients with PCOS have a lower abundance of *Lactobacillus* spp. with the prevalence of *G. vaginalis*, *C. trachomatis*, *Mycoplasma*, and *Prevotella* [86,87]. Alterations in the microbiome composition in PCOS patients can be explained by the absence of ovulation and, therefore, progesterone-secreting corpus luteum. It is still unclear whether the reproductive tract microbiome itself can impact the development of PCOS [84]. Since patients with PCOS in the anamnesis have a higher rate of recurrent miscarriages [88], it is possible that the PCOS-related changes in the pre-existing microbiome can be associated with miscarriages.

MICROBIOME AND EARLY MISCARRIAGE

Materials and methods

Here, we analyzed the articles on the role of the microbiome in the pathogenesis of early miscarriages published over the past 20 years from the EBSCO, PubMed, Scopus, Google Academy, ResearchGate, and Elibrary databases.

Keywords and inclusion and exclusion criteria for searching are shown in Table 1.

Research with less than 10 samples in each group articles with unavailable full text conference abstracts and studies with no control group were excluded from analysis. Culture-based studies metabolomics research studies of male reproductive tract microbiome and animal studies were also excluded

RESULTS

The existing studies of the microbiome by high-throughput sequencing can be divided into two groups (Fig. 2) depending on sampling and the presence of pregnancy.

The main results of the studies are shown in Table 2.

Table 1. Selection strategy for the review on the female reproductive tract microbiome and the early miscarriages

Databases	EBSCO, PubMed, Scopus, Google academy, ResearchGate, Elibrary
Search keywords	• [vaginal microbiome] AND [miscarriage OR early pregnancy loss OR missed abortion OR spontaneous abortion] • [uterine microbiome OR endometrial microbiome] AND [miscarriage OR early pregnancy loss OR missed abortion OR spontaneous abortion] • [cervical microbiome OR microbiome of the cervix] AND [miscarriage OR early pregnancy loss OR missed abortion OR spontaneous abortion] • [microbiome] AND [vagina OR vaginal] • [microbiome] AND [cervix OR cervical] • [microbiome] AND [endometrium OR uterus OR uterine]
Other sources	Additional studies were identified in references of found articles and included in the review
Inclusion criteria	• Published in peer-reviewed journals over the past 20 years • Studies focused on early miscarriages only • Studies on female humans
Exclusion criteria	• Research with less than 10 samples in each group • Studies with no control group • Full-text articles unavailable, including conference abstracts • Culture-based studies • Metabolomics research • Studies of male reproductive tract microbiome • Animal studies

Microbiome in non-pregnant patients with a history of miscarriage or before IVF

In the first group of analyzed studies, the microbiome has been assessed in non-pregnant women with a history of recurrent miscarriages or in patients who underwent *in vitro* fertilization (IVF). In the case of IVF patients, most authors assessed the implantation rate, rather than the rate of miscarriages and live births [34,89–91]. The rate of live births after IVF with regard to the vaginal microbiome was estimated in one study only [92].

Zhang et al. [93] showed that the vaginal microbiome of non-pregnant women with a history of recurrent miscarriages (n = 10) had a higher abundance of *Firmicutes* and lower abundance of *Actinobacteria* and *Bacteroidetes* compared to the healthy women (n = 10) (samples were obtained by vaginal swabs). Three taxa (*Atopobium*, *Prevotella*, and *Streptococcus*) were significantly more abundant in the patients with recurrent miscarriages, while in the control group, the most abundant taxa were *Lactobacillus* and *Gardnerella*. The limitation of the study was a small number of patients in each group. The stage of the cycle was not taken into account during sampling. No exclusion criteria, such as the history of immune and endocrine disorders, as well as chromosomal abnormalities of the fetus, were applied to the studied groups.

In another study of the vaginal microbiome, non-pregnant women with a history of recurrent miscarriages (n = 16) were found to have a higher abundance of *Atopobium* compared to healthy women (n = 20), while *Lactobacillus* and *Gardnerella* were more abundant in the healthy patients [94]. Samples were taken by vaginal scraping. The advantage of this research was that vaginal scraping allows the detection of intracellular microorganisms. The limitation of the study was the same as for the previously described one.

Moreno et al. [35] studied the uterine microbiome obtained by aspiration from the uterine cavities of 35 infertile women with receptive endometrium before the IVF and investigated its effect on the rates of embryo implantation, miscarriage, and live birth. The contamination of the samples by the vaginal and cervical microbiota was avoided by using an outer sheath for the uterine catheter and additional removal of cervical mucus before the catheter extraction. It was found that patients with the non-*Lactobacillus*-dominated (n = 15) communities have fewer implantations, ongoing pregnancies, and live births, than patients with *Lactobacillus*-dominated communities (n = 17). The occurrence of miscarriages was not significantly different between the two groups. The strong point of the research was preimplantation genetic testing provided for all transplanted embryos, while its limitations were the absence of exclusion criteria, such as immune and endocrine disorders, and a small number of patients with miscarriages (n = 5).

The same authors analyzed a larger cohort of patients (n = 342) and showed a higher abundance of *Haemophilus* and *Staphylococcus* and a lower abundance of *Lactobacillus* spp. in the endometrial fluid before IVF in patients with further clinical miscarriages (n = 22) compared to those with live births [95]. The advantage of the study was a large number of samples; the limitations were the absence of patient selection with the exclusion of those with immune and endocrine disorders, as well as the absence of preimplantation genetic testing of embryos. A multicenter study using the samples obtained in Asia, America, and Europe might have its strong and weak points. It is known that the normal microbiome in the first trimester of pregnancy can differ depending on the country [29–31], which makes study groups more heterogeneous.

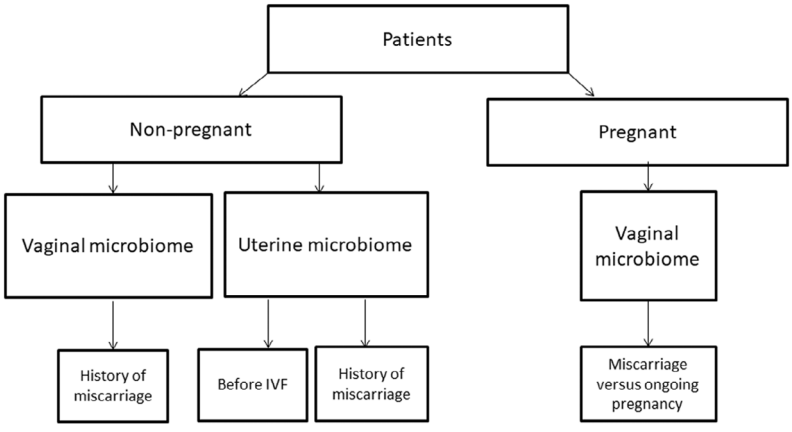

Fig. 2. Microbiome study design.

Table 2. Studies on the female reproductive tract microbiome and early miscarriages

Article ID	Study groups and number of patients	Sampling	Detection method	Results	
				Increased	Reduced
Non-pregnant, vaginal microbiome					
Zhang et al. (2019)	Women with the history of recurrent miscarriages (n = 10)	Vaginal swab	16 S rRNA gene V3-V4 region sequencing (Illumina MiSeq)	*Firmicutes* *Atopobium* *Prevotella* *Streptococcus*	*Actinobacteria* *Bacteroidetes*
	Healthy patients (n = 10)			*Lactobacillus* *Gardnerella*	
Jiao et al. (2021)	Women with the history of recurrent miscarriages (n = 16)	Vaginal scraping	16 S rRNA gene V3-V4 region sequencing (Illumina MiSeq)	*Atopobium*	
	Healthy patients (n = 20)			*Lactobacillus* *Gardnerella*	
Non-pregnant, uterine microbiome					
Moreno et al. (2016)	*Lactobacillus*-dominated (n = 17) Non-*Lactobacillus*-dominated (n = 15)	Aspiration by intrauterine catheter	16 S rRNA gene V3-V5 region sequencing (454 Life Sciences GS FLX+ Roche)	Patients with non-*Lactobacillus*-dominated communities had fewer implantations, ongoing pregnancies, and live birth, but the same rate of miscarriages compared to *Lactobacillus*-dominated communities	
Moreno et al. (2022)	Patients prior to IVF (n = 342), including patients with further miscarriages (n = 22)	Aspiration by intrauterine catheter	16 S rRNA gene V2-4-8, V3-6, V7-9 region sequencing (Ion S5 XL system)	*Haemophylus* and *Staphylococcus* in miscarried group	*Lactobacillus* in miscarried group
Kyono et al. (2019)	*Lactobacillus*-dominated (n = 56) Non-*Lactobacillus*-dominated (n = 36)	Aspiration by intrauterine catheter	16 S rRNA sequencing, but the method and equipment are not described	The rate of miscarriages did not differ between *Lactobacillus*-dominated and non-*Lactobacillus*-dominated communities	
Vomstein et al. (2022)	Women with the history of recurrent miscarriages (n = 20)	Aspiration by intrauterine catheter	16 S rRNA gene V3-V4 region sequencing (Illumina MiSeq)	*Firmicutes*, *with further increase after ovulation* *Proteobacteria*, *with increase after ovulation*	
	Healthy patients (n = 10)			*Firmicutes* (*mostly Lactobacillus* spp.), *with further increase after ovulation* *Proteobacteria*, *with further decrease after ovulation*	
Pregnant, vaginal microbiome					
Nelson et al. (2015)	Women with miscarriages (n = 74) Women with ongoing pregnancies (n = 344)	Vaginal swab (self-collection by patients)	qPCR for seven BV-associated species (*BVAB1*, *BVAB2*, *and BVAB3*, *Leptotrichia*/*Sneathia* spp., *G. vaginalis*, *Mobiluncus* spp., *Megasphaera phylotype 1-like* spp., and *Atopobium* spp.) (equipment is not described)	*BVAB3* *Leptotrichia*/*Sneathia* spp. *Megasphaera*	
		Vaginal swab		CST IV	*Lactobacillus* spp.

Table 2 *(continued)*

Article ID	Study groups and number of patients	Sampling	Detection method	Results	
				Increased	Reduced
Al-Memar et al. (2019)	Women with early miscarriages (n = 64) Women with ongoing pregnancies and further full-term labor (n = 83)		16 S rRNA gene V1-V2 hypervariable region sequencing (Illumina MiSeq)		
Xu et al. (2020)	Women with missed abortion (n = 25) Women with ongoing pregnancy (n = 25)	Vaginal swab	Microscopy and 16 S rRNA gene V4 region sequencing (Illumina, HiSeq3000/4000)	Microscopy: patients with miscarriages had a lower amount of *Lactobacillus* spp. and a higher bacterial diversity compared to the control group 16 S rDNA sequencing: no differences in the alfa and beta diversity. A significant difference was found in the relative abundance of species of the *Erysipelotrichia* and *Fusobacteriia* classes, *Erysipelotrichaceae* family, and genera *Finegoldia*, *Coprococcus*, and *Roseburia*	
Liu et al. (2021)	Women with missed abortion (n = 22)	Vaginal swab	16 S rRNA gene V4 region sequencing (Illumina, MiSeq)	*Staphylococcus*, *Escherichia/Shigella*, *Bacteroides*, *Halomonas*, *Crenarchaeota*, *Bacillus*, *and Acetobacter*, Higher diversity. Presence of phylum *Thaumarchaeota* (*Archaea*)	*Lactobacillus*
	Women with anembryonic pregnancies (n = 15) Women with ongoing pregnancies (n = 15)				
Sun et al. (2022)	Women with missed abortion (n = 50)	Vaginal swab	16 S rRNA gene V1-9 region sequencing (no data about sequencer)	*Proteobacteria*, *Actinobacteria*, *Chlamydiae*, *and Fusobacteria*	Firmicutes and Saccharibacteria *Mycoplasma genitalium* *Ureaplasma* spp.
	Women with ongoing pregnancies (n = 54)			*L. crispatus*, *L. jensenii*, *L. gasseri*	

The uterine microbiome of 92 infertile patients before IVF was comprehensively studied by Kyono et al. [96]. The endometrial fluid was collected using an IVF catheter with a special shield. The cervix was rinsed with an antiseptic solution to avoid bacterial contamination by the cervical microbiome. Only half of the patients had *Lactobacillus*-dominated uterine microbiomes; other abundant bacteria were *Atopobium*, *Bifidobacterium*, *Gardnerella*, *Megasphaera*, *Sneathia*, *Prevotella*, *Staphylococcus*, and *Streptococcus*. The rate of miscarriage after a single vitrified-warmed blastocyst transfer was not different between the *Lactobacillus*-dominated (n = 56) and non-*Lactobacillus*-dominated (n = 36) groups, including communities that became *Lactobacillus*-dominated after the treatment with probiotics and prebiotics. The limitations of the study were a small number of samples in the miscarriage group (n = 8) and comparing *Lactobacillus*-dominated group versus non-*Lactobacillus*-dominated group instead of miscarriage *versus* ongoing pregnancy group. Other causes of miscarriage were not excluded. The methods of microbiome analysis were not described.

In addition, the limitations of all three above-mentioned IVF studies were the absence of comparison with the healthy controls (for example, with the male factor of infertility).

In the recent study by Vomstein et al. [97], the endometrial microbiome was obtained by uterine flushing through a sterile catheter in non-pregnant patients with a history of recurrent pregnancy loss (n = 20) and healthy controls, who had never been pregnant (n = 10). The absence of the vaginal contamination was proved by comparing the vaginal and endometrial samples. In the control group at a taxa level, the authors found a high abundance of *Firmicutes* during the follicular phase with a further increase along the next stages of the menstrual cycle. *Proteobacteria* were also detected in high abundance at the follicular phase but significantly decreased after ovulation. In the recurrent miscarriages group, a high abundance of *Firmicutes* during the follicular phase and its significant increase during the luteal phase were observed. This was accompanied by the expansion of *Proteobacteria* during the luteal phase. At a lower taxonomic level, the *Lactobacilalles* family was more abundant in the control group compared to the recurrent miscarriages group. The limitation of the study was a small number of samples.

Microbiome in patients with ongoing pregnancies and miscarriages

The second group were the studies on the female reproductive tract microbiome in early pregnancy and on the possibility of using the obtained data for predicting early pregnancy loss with the help of high-throughput sequencing and quantitative polymerase chain reaction (qPCR) as non-culturing methods.

Nelson et al. [98] used qPCR to estimate the presence and quantity of seven BV-associated bacteria (BVAB1, BVAB2, and BVAB3, *Leptotrichia*/*Sneathia* spp., *G. vaginalis*, *Mobiluncus* spp., *Megasphaera* phylotype 1-like spp., and *Atopobium* spp.) in the vaginal microbiome during ongoing pregnancy under 14 weeks of gestation. In total, 418 pregnant women were included in this study, and 74 experienced miscarriages. The presence and the quantity of *Lactobacillus* spp. were not estimated in this research. The vaginal samples were self-collected by the patients using vaginal swabs. The patients were divided into two groups: women with miscarriages and women with ongoing pregnancies. The authors found that the high concentration of BVAB3 in the vagina increased the risk of miscarriages by 20%. The presence of *Leptotrichia*/*Sneathia* or *Megasphaera* phylotype 1-like species had a protective effect and decreased the risk of early pregnancy loss. A 10-time increase in the content of these bacteria decreased the risk of miscarriages by 20% and 19% accordingly. The authors created the multivariate models that included maternal age, the content of BVAB3, and the content of *Leptotrichia*/*Sneathia* or *Megasphaera* phylotype 1-like species, for miscarriage prediction. No sensitivity or specificity for this prediction model was provided. The limitation of the study was using the PCR method with a small number of detected species and self-collection of samples, which therefore can be taken from different parts of the vagina or even contaminated by the microbiota of the external genitalia. Another disadvantage was that patients with immune, endocrine, and genetic disorders were not excluded. Chromosomal abnormalities of the fetus were not detected. Primers and kits for amplification, as well as the equipment used in the study, were not described.

Xu et al. [99] compared the patients admitted to an outpatient clinic with a missed abortion (n = 25) with the patients with an ongoing pregnancy (n = 25). The samples of the vaginal microbiome were collected using vaginal swabs. The authors estimated the diversity and amount of *Lactobacillus* spp. by microscopy of the vaginal smears and compared the obtained data with the results of 16 S rRNA sequencing. According to the microscopy, the patients with miscarriages had a lower amount of *Lactobacillus* spp. and a higher bacterial diversity compared to the control group. According to the 16 S rRNA sequencing, no differences in the

alfa and beta diversity (alpha diversity represents the richness and diversity of the microbial community; beta diversity represents the similarity of the microbial composition between samples). A significant difference was found in the relative abundance of species of the *Erysipelotrichia* and *Fusobacteriia* classes, *Erysipelotrichaceae* family, and genera *Finegoldia*, *Coprococcus*, and *Roseburia*. The concentrations of IL-2 and IL-10 in the vaginal lavage fluid were determined by ELISA to estimate the Th1/Th2 ratio. The level of IL-2 (produced by Th1 lymphocytes) in the vaginal fluid and the IL2/IL10 ratio were increased, while the level of IL-10 (produced by Th2 lymphocytes) was decreased in the miscarriage group compared to the patients with ongoing pregnancies. However, the authors did not assess the correlation between the levels of these cytokines and the presence and abundance of bacterial taxa or species. The limitation of the study was a relatively small number of samples and the absence of detection of chromosomal abnormalities in the fetuses. The case group included only patients with no adverse pregnancy history, which also might have affected the results. The taxonomic resolution by the V4 region of the 16 S rRNA gene, used in this research, was poor. It was not mentioned, which group had a higher abundance of these microorganisms.

Liu et al. [100] examined the patients with missed abortions (n = 22), anembryonic pregnancies (n = 13), as well as women with ongoing pregnancies (n = 15) of the same gestational age. The vaginal microbiome of women with missed abortions was more diverse and had a higher content of *Staphylococcus*, *Escherichia/Shigella*, *Bacteroides*, *Halomonas*, *Crenarchaeota*, *Bacillus*, and *Acetobacter* and a lower content of *Lactobacillus* than the microbiomes from the other two groups. Representatives of the phylum Thaumarchaeota (Archaea) were found in a number of patients with miscarriages. The CSTs were significantly different between the miscarriage group and the other two groups, but not between the anembryonic pregnancy and ongoing pregnancy groups. The authors developed a set of markers containing 12 operational taxonomic units that can be used for predicting missed abortion. The advantage of the study was comparison of the patients with missed abortions and anembryonic pregnancy. The limitations were a small number of patients in the groups, no detection of fetal chromosomal abnormalities, and the necessity of further larger studies for defining the markers' sensitivity and specificity. The V4 region of the 16 S rRNA gene, which has poor resolution, was used in this research.

In their comprehensive research, Al-Memar et al. [101] analyzed vaginal microbiomes of 161 pregnant women several times during the first trimester (5–8, 8–10, 10–14, and >14 weeks of gestation) using 16 S rRNA sequencing. Among these pregnancies, 64 resulted in the first trimester miscarriages, 14 – in the second-trimester miscarriages, and 83 – in the full-term labor. The samples were collected by vaginal swabs. Patients with miscarriages in the first trimester lacked the *Lactobacillus* spp.-dominated microbiomes and had a higher proportion of CST IV communities. This was independent of vaginal bleeding and was reported before the miscarriage. The patients with complete and incomplete miscarriages had a lower proportion of *Lactobacillus* spp.-dominated communities and a higher proportion of CST IV compared to the patients with missed abortions. The strength of this research was a large cohort of patients and multiple testing before the miscarriage. The limitations were the absence of exclusive criteria, such as endocrine, immune and genetic disorders, as well as no testing for chromosomal abnormalities in the fetus.

In a recent study by Sun et al. [102], the vaginal microbiomes of 50 patients with missed abortions and 54 patients with ongoing pregnancies were analyzed. It was found that the vaginal microbiome in the case group was more diverse than in the control group. On the phylum level, the abundance of *Firmicutes* and *Saccharibacteria* was decreased, while the abundance of *Proteobacteria*, *Actinobacteria*, *Chlamydiae*, and *Fusobacteria* was increased in the patients with missed abortions. On the species level, the relative abundance of *L. crispatus*, *L. jensenii*, *L. gasseri*, but not *L. iners*, was significantly lower in the case group. Interestingly, *Mycoplasma genitalium* and *Ureaplasma* spp. were significantly lower in the case group, while the content of *Mycoplasma hominis* did not differ significantly from the control group. In total, the authors found significant differences between 345 species, but the majority did not exceed 1%. The limitation of the study was that other causes of missed abortion, such as chromosomal abnormalities of the fetus, endocrine abnormalities, and autoimmune diseases, could not be excluded completely.

To our knowledge, so far there is no comprehensive research on the uterine microbiome in patients with miscarriages compared to patients with ongoing pregnancies, admitted for legal abortions.

DISCUSSION

Most studies on early miscarriages have been performed using cell cultures [103,104]. However, it has been proved that working with cell cultures does not allow to fully assess the composition of

the microbiome of the lower and upper parts of the female reproductive tract [105], which requires the use of other research methods, *for example*, high-throughput sequencing of the 16 S rRNA.

The number of studies on the female reproductive tract microbiome in patients with miscarriages is limited; moreover, these studies have different designs. For example, studies on the vaginal microbiome use different sampling methods (vaginal swab, vaginal scrapping) and different groups (miscarriages vs. ongoing pregnancies or patients with *Lactobacillus*-dominated microbiome vs. non-*Lactobacillus*-dominated). Some studies excluded patients with immune and endocrine disorders and fetal chromosomal abnormalities, while others did not, which prevents the use of meta-analysis.

According to the results, ongoing pregnancy is associated with the *Lactobacillus*-dominated vaginal and uterine microbiome. Meanwhile, on a species level, CSTs in normal early pregnancy can depend on the ethnicity. Therefore, in each research, the use of the control group is essential. In multicenter studies, if the differences between control groups from various regions of the world are significant, such groups cannot be united into one control group.

To our knowledge, the studies on the microbiome of cervical canal in patients with miscarriages are absent. It is known that certain bacterial species (*Chlamydia*, *Mycoplasma*, *Ureaplasma*, *N. gonorrhoeae*, *etc*.) can live only in the columnar epithelium, and some of them persist only inside the cells. The vagina is lined with the stratified squamous epithelium. In our opinion, the microbiome of the columnar epithelium of the cervical canal better reflects the condition of the endometrial columnar epithelium. It was shown earlier that 13 bacterial taxa are constantly present in the endometrial tissue, but not in the endometrial fluid [106].

Another issue is that some studies do not mention the medium used for the sample collection and the time when the samples were frozen. If the samples are collected in a special transport medium used for microbial cultivation and not frozen immediately, this may increase a relative abundance of aerobic microorganisms (which are capable to grow in such a medium) and decrease the abundance of anaerobic microbes. Therefore, the microbial material must be collected in special solutions for DNA and RNA storage.

Moreover, none of the studies have assessed the correlation between the clinical data (age, parity, height, weight, body mass index, gestational age, gynecological history), microbiome composition, and local immunity.

CONCLUSION

The microbiome composition might influence many aspects of miscarriage pathogenesis. Changes in the microbiome can affect the local immune status, lead to chronic endometritis due to the abundance of non-*Lactobacillus* bacteria, and are associated with thrombosis in patients with antiphospholipid syndrome and congenital thrombophilia.

There is a lack of clinical research on the role of the reproductive tract microbiome in the pathogenesis of early miscarriages. However, the most common observation in the majority of such studies, irrespectively whether pregnant or non-pregnant patients have been examined, is a low abundance of *Lactobacillus* spp. and the prevalence of non-*Lactobacillus* species in both the vagina and the endometrium.

Studies of the cervical microbiome, including the differences between the microbiomes of the cervical mucus and cervical epithelium, are required. Unlike the uterine microbiome samples, cervical microbiome can be sampled at any time, including ongoing pregnancy, and therefore can be used for the prognosis of miscarriages.

We also failed to find publications on the uterine microbiome of patients with ongoing pregnancies (e.g., admitted for abortion) and patients with miscarriages (missed abortions).

Further studies are needed to develop methods for prognosis and prophylaxis of early miscarriages.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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