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MINIREVIEW

Can *Lactobacillus* spp. Be a Factor Reducing The Risk of Miscarriage?

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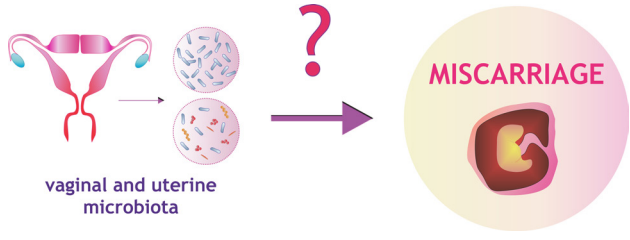
Abstract

Pregnancy loss is a common obstetric problem. Significant causes of miscarriage include genetic and epigenetic disorders of the embryo, immunological and endocrine factors, uterine malformations, improper embryo selection, and lifestyle. Perhaps a hitherto underappreciated cause of miscarriage may be an abnormal microbiota composition of the female reproductive system. *Lactobacillus* spp. is the most common bacteria within the reproductive tract. However, the protective role of *Lactobacilli* in the vagina has been well described in the literature, while it is still unknown what function *Lactobacilli* may have in the uterus. Moreover, new research shows that *Lactobacillus* spp. can have a role in miscarriage. However, both molecular and immunological mechanisms of host-*Lactobacillus* spp. interactions are not fully understood. Understanding these relationships will help address the importance and extent of the protective role of *Lactobacillus* spp. in miscarriage.



K e y w o r d s: *Lactobacillus*, miscarriage, pregnancy, uterine microbiota, vaginal microbiota

Introduction

Pregnancy loss is a common obstetric problem, affecting up to 25% of pregnancies worldwide (Larsen et al. 2013; Al-Memar et al. 2020). A miscarriage is the expulsion of a fetal egg from the uterus up to 22 weeks of gestation. Miscarriages can be divided into early miscarriages, up to 12 weeks of gestation, and late miscarriages, occurring between 12 and 22 weeks of gestation (Larsen et al. 2013). The European Society of Human Reproduction and Embryology (ESHRE) has introduced the additional term recurrent miscarriage (RM) when there are three or more consecutive pregnancy losses (Farquharson et al. 2005; Jauniaux et al. 2006; Christiansen et al. 2008). The occurrence of early miscarriage is dependent on the woman's age. Among women aged 20–24 years, it is 10% of pregnancies, while in women aged 40 to 44 years, it is 51% of pregnancies. It is related to the higher incidence of genetic aberrations in embryos of older women (Nybo Andersen et al. 2000). Late miscarriages occur less frequently and account for about 4% of all miscarriages (Ugwumadu et al. 2003).

Major causes of miscarriage include genetic (Franssen et al. 2006; Branch et al. 2010) and epigenetic disorders of the embryo (Daher et al. 2012; Yin et al. 2012), immunological (Holers et al. 2002; Calleja-Agius et al. 2012), and endocrine factors (Cocksedge et al. 2009), uterine malformations (Chan et al. 2011), improper embryo selection (Salker et al. 2010), and lifestyle (Larsen et al. 2013) (Fig. 1). Perhaps a hitherto underappreciated cause of miscarriage may be an abnormal microbiota composition of the female reproductive system. Currently, the normal state of vaginal and uterine microbiota that would promote a physiological pregnancy is being sought. So far, it has been shown that a normal pregnancy is characterized by a stable vaginal bacterial composition with a dominance of *Lactobacillus* spp. and low diversity of other bacteria (Ravel et al. 2011; MacIntyre et al. 2015) (Fig. 2). Numerous studies show

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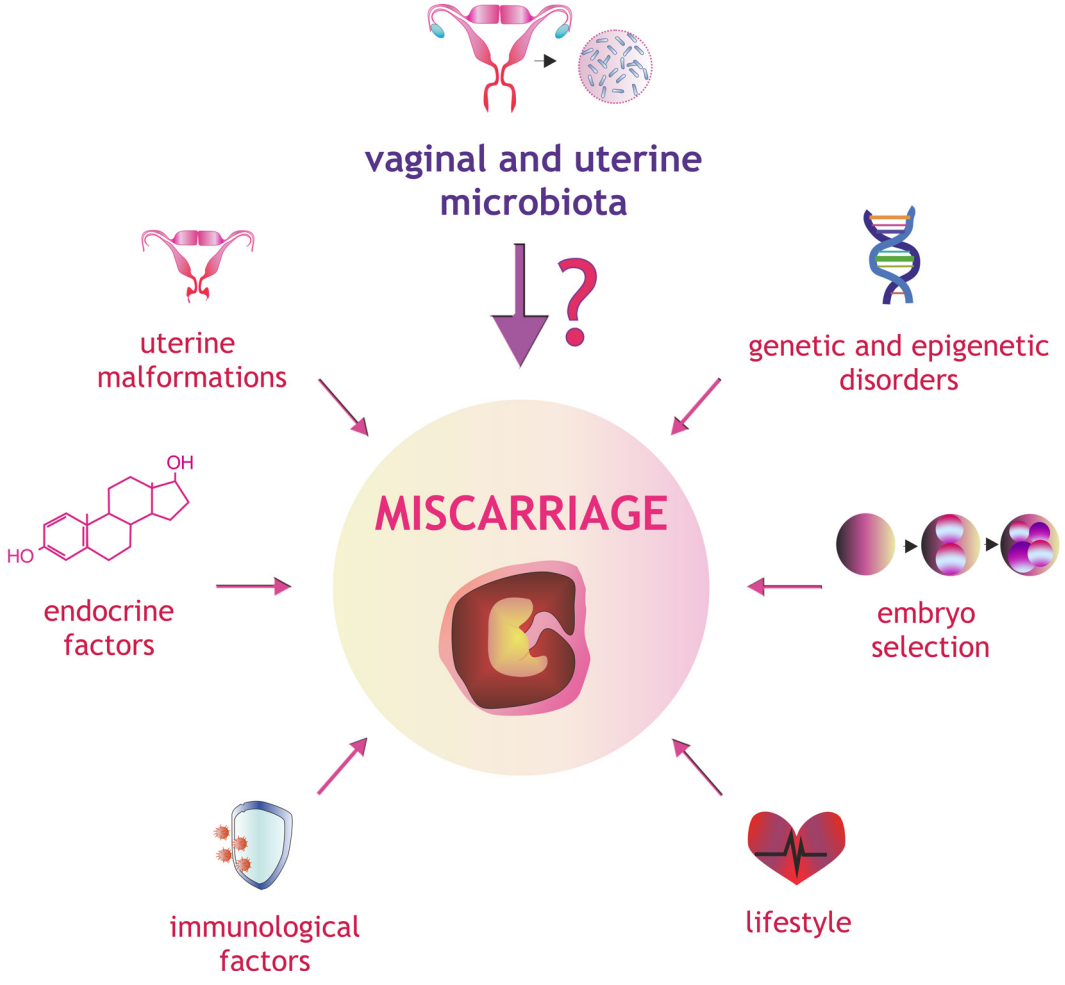

Fig. 1. Factors affecting the risk of miscarriage.

a possible relationship between preterm delivery, a decrease in *Lactobacillus* spp. and an increase in bacterial biodiversity in the vagina (Brown et al. 2018; Freitas et al. 2018; Al-Memar et al. 2020), bacterial vaginosis (BV), or aerobic vaginitis (AV). However, the relationship between miscarriage and the vaginal and uterine microbial composition is relatively poorly understood (Zhang et al. 2019; Al-Memar et al. 2020; Xu et al. 2020).

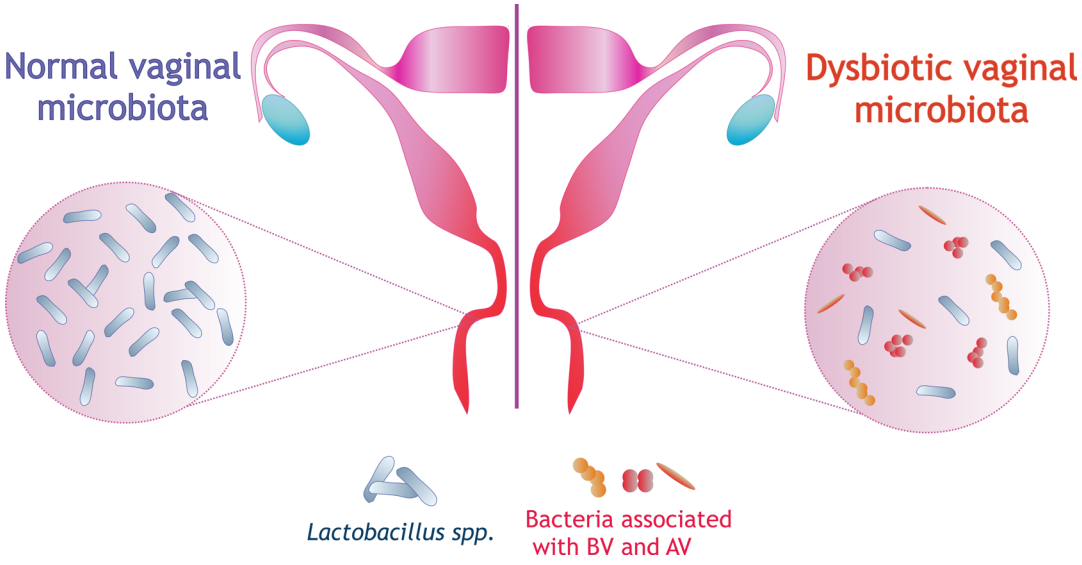

Fig. 2. Normal vaginal microbiota and vaginal dysbiosis.

The aim of this study is an attempt to answer the question of whether, in the light of available literature, the *Lactobacillus* spp. can be a factor reducing the risk of miscarriage.

Protective role of *Lactobacilli* in the vagina

The vaginal environment is a specific ecosystem with interactions between microorganisms, the host immune system, and vaginal epithelial cells. The vaginal microbiota is a particular example of microbiota in the human body due to the definite dominance of *Lactobacillus* spp. (up to 99%) and low bacterial diversity (Ravel et al. 2011; Gajer et al. 2012).

There are several types of vaginal microbiota (CST, from community state types). CST-I (45.4%), CST-II (8.2%), CST-III (26.8%), and CST-V (9.3%) are successively dominated by *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners*, and *Lactobacillus jensenii* (Ravel et al. 2011; Gajer et al. 2012). In the CST-IV group (10.3%), in which *Lactobacillus* is not a dominant genus, the following bacteria are present: *Gardnerella*, *Prevotella*, *Megasphaera*, *Sneathia*, *Atopobium*, *Streptococcus*, *Dialister*, *Lachnospira*, *Anaerococcus*, *Peptoniphilus*, *Eggerthella*, *Finegoldia*, *Rhodobaca*, *Anaerotruncus*, *Ureaplasma*, *Mycoplasma*, *Aerococcus*, *Parvimonas*, *Staphylococcus*, *Corynebacterium*, *Veillonella* (Ravel et al. 2011; Gajer et al. 2012; Kacerovsky et al. 2015).

The CST-I (dominated by *L. crispatus*) is a stable type; its transition to a pathological state has rarely been observed, provides a vaginal pH < 4.0, and may convert to the CST-III type or a microbiota consisting of different species of *Lactobacillus* genus. The CST-II (dominated by *L. gasseri*) is an unstable type; however, no transition to a pathological state has been observed, can temporarily convert to CST-I during pregnancy, provides a vaginal pH of approximately 4.4. The CST-III (dominated by *L. iners*) is a transitional type that facilitates transition to bacterial vaginitis (BV); this type is characterized by an increase in proinflammatory factors and a decrease in the level glycolysis enzymes. It is associated with an increase in vaginal pH > 4.5. Simplified metabolism of *L. iners* results in dependence on substances received from the host; thus, increasing sensitivity to environmental changes. In addition, the L-lactic acid produced insufficiently inhibits pathogens.

The CST-IV is the pathogenic type, dominated by anaerobic bacteria. There are CST-IVA with a small number of *L. iners* and CST-IVB dominated by such bacteria as *Atopobium*, *Gardnerella*, *Mobiluncus*, *Prevotella*, *Sneathia*. This type is most common among BV patients and healthy women of African descent. Low lactic acid levels damage to the mucin layer (which hinders *Lactobacillus* spp. adhesion), and the presence of a bacterial biofilm on the vaginal epithelial surface characterize the CST-IV type (Chee et al. 2020). The CST-V is a stable type (dominated by *L. jensenii*), is relatively poorly known, and provides a vaginal pH of approximately 4.2. There are reports that *L. jensenii* can affect the vaginal microenvironment by reducing lactate and succinate levels (Stafford et al. 2017). However, further studies are needed to characterize CST-V (Chee et al. 2020).

In the vagina, *Lactobacilli* are involved in protective functions by producing lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins (Boskey et al. 2001; Witkin and Linhares 2017; Amabebe and Anumba 2018; Bernabeu et al. 2019).

Lactic acid is a significant protective factor of the vagina. *Lactobacillus* spp. produce its two isomeric types, D-lactic acid, and L-lactic acid, with D-lactic acid showing a stronger protective effect (Boskey et al. 2001). L-lactic acid acidifies vaginal secretions (to about pH < 4), thus hindering the binding of other microorganisms to vaginal epithelial cells and inducing autophagy in epithelial cells to degrade microorganisms. Lactic acid has a blocking effect on histone deacetylase, stimulating gene transcription and DNA repair (Witkin and Linhares 2017; Amabebe and Anumba 2018; Bernabeu et al. 2019). In sterile cultures, *L. crispatus* and *L. gasseri* can produce both D- and L-lactic acid, while *L. iners* produces only the L-isomer, whereas *L. jensenii* produces only the D-isomer (Witkin et al. 2013).

Another protective mechanism relies on the production of hydrogen peroxide (H_2O_2). This compound, which has a broad antimicrobial activity, is produced by many lactic acid bacteria isolates. It has been shown that 94–95% of *L. crispatus* and *L. jensenii* strains produce hydrogen peroxide (Vallor et al. 2001). The H_2O_2 shows high *in vitro* activity against *Escherichia coli*, *Candida albicans*, or *Staphylococcus aureus* (Sgibnev and Kremleva 2015). It can inhibit the multiplication or destroy pathogenic strains of vaginal bacteria, especially those with the limited expression of hydrogen peroxide-degrading enzymes, including *Prevotella*, *Peptostreptococcus*, and *Gardnerella anaerobes*, among others. The lack of this compound in the vagina increases catalase-negative bacteria, associated with an increased risk of genitourinary infections, including BV and Human Immunodeficiency Virus infection (HIV) (Aroutcheva et al. 2001; Amabebe and Anumba 2018; Tachedjian et al. 2018).

Vaginal *Lactobacilli* also produce bacteriocins. These are proteins or protein complexes that show potent bactericidal activity. Bacteriocins kill pathogens such as *Gardnerella vaginalis*, *Escherichia coli* or *Candida albicans*, by inhibiting DNA synthesis. Bacteriocins resemble antibiotics in their action (Aroutcheva et al. 2001; Alpay

Karaoğlu et al. 2003; Deplanche et al. 2019). *Lactobacillus* spp. also shows strong adhesion to the nonkeratinized vaginal epithelium, displacing pathogenic microorganisms such as *C. albicans*, *G. vaginalis*, *E. coli*, *Streptococcus agalactiae*, or *S. aureus* from the epithelial surface. *Lactobacillus* spp. and *G. vaginalis* may bind to the same receptors on the surface of vaginal epithelial cells. However, *Lactobacillus* spp. has a higher affinity for vaginal epithelial cells and displaces *G. vaginalis* (Kovachev 2018). Studies show that the combination of *L. crispatus* UBLCp01, *L. gasseri* UBLG36 and *L. johnsonii* UBLJ01 may be helpful in preventing/treating vaginal dysbiosis and maintaining a healthy vaginal ecosystem when used as vaginal probiotics. Features such as adherence and antimicrobial potential, exopolysaccharide production, and biofilm-forming ability of strains are essential characteristics that influence their potential against pathogens (Ahire et al. 2021).

The composition of vaginal microbiota fluctuates under the influence of many factors such as environmental conditions (antibiotics, pre- and probiotics, contraception), lifestyle (hygiene and sexual habits), individual characteristics (genetic, immunological factors, age, hormonal status, ethnicity, socioeconomic status) and general health (Macklaim et al. 2015; Brooks et al. 2017; Kervinen et al. 2019). There are cases when certain changes in the composition of vaginal microbiota occur during pregnancy. During pregnancy, the levels of various hormones change dynamically, produced primarily by the placenta, which becomes a gland for the secretion of many biologically active substances. Such hormones include estrogen and progesterone. Estrogens increase the synthesis of glycogen in the vaginal epithelium, which provides a substrate for lactic acid bacteria (Taddei et al. 2018; Heil et al. 2019; Serrano et al. 2019). Also, the absence of menstruation or changes in cervical and vaginal secretions affects the different states of pregnancy microbiota (Walther-António et al. 2014). In addition, the precise reciprocal interaction between the microbiota and locally acting immune cells are responsible for the inhibition of pathogen growth, but also for the tolerance to paternal antigens that are present in the semen and embryo (Agostinis et al. 2019; Kervinen et al. 2019; Bardos et al. 2020; Monin et al. 2020).

Studies have also been conducted on the relationship between ethnicity and vaginal microbiota composition. Experiments conducted by Walther-António et al. (2014) on pregnant Caucasian women showed that *L. crispatus* was the dominant genus; for a smaller number of subjects, it was *L. iners*, and a small proportion of patients showed the transition from *L. crispatus* to *L. iners*. Afro-American populations have greater interindividual diversity in the vaginal microbiota than Caucasians. It is particularly important because gynecologic-obstetric complications are more common among these populations (Walther-António et al. 2014). Caucasian women have a predominantly *Lactobacillus* spp. dominated microbiota (approximately 90%), Asian and Hispanic women have microbiota percentages of approximately 80% and 60%, respectively, and African women only 37% (Anahtar et al. 2015). Studies show that Asian women have a different composition of the vaginal microbiota, with a higher prevalence of *L. iners*. The reason for these differences remains unclear. It may be related to genetic and environmental factors, including geographic location, diet, age, BMI, drug exposure, physical activity, and availability of resources such as access to medical care. However, studies show that the presence of *L. iners* in the vagina of Asian women does not increase the risk of abnormal pregnancy (Serrano et al. 2019; Kumar et al. 2021).

***Lactobacillus iners*: friend or foe?** Standard culture and microscopic methods, used for many years to determine the presence of lactic acid bacteria in the vagina, could not detect *L. iners* in vaginal samples. Employing molecular biology methods helped to detect the presence of seven strains of *L. iners* in the vagina and urinary tract in 1999 (Falsen et al. 1999).

Current knowledge indicates that *L. iners* is the predominant genus in the vaginal microbiota among older women, pregnant women, and women of Afro-American descent (Srinivasan et al. 2010; Mls et al. 2019). The *L. iners* is also frequently isolated from the vagina of women diagnosed with BV, shortly after BV treatment, and during menstruation (Lopes dos Santos Santiago et al. 2011, Gajer et al. 2012). It is suggested that this genus is very flexible and can quickly adapt to changing conditions prevailing in the vaginal niche. The function analysis of proteins encoded by the *L. iners* genome revealed that this genus could show both commensal and pathogenic properties. The *L. iners* genome encodes proteins predicted to be involved in optimal adaptation to the vaginal niche, such as iron-sulfur proteins and the σ factor. Several genes have also been identified in the *L. iners* genome suggesting that it may be an opportunistic pathogen (Petrova et al. 2017). For example, the genome of *L. iners* strains encodes the toxin inerolysin (Rampersaud et al. 2011), related to the vaginolysin of *G. vaginalis* (Srinivasan et al. 2012). Furthermore, the genome size of *L. iners* is also unique among *Lactobacillus* genus. The AB-1 strain of *L. iners* has by far the smallest genome yet known among *Lactobacillus* spp., consisting of a single chromosome of approximately 1.3 Mbp, in contrast to other lactic acid bacteria in which the genome size is approximately 3–4 Mbp (Macklaim et al. 2011; 2013). It appears that there may be some clonal variants within the *L. iners* genus that show commensal properties in some cases and pathogenic properties in others (Petrova et al. 2017).

Presence of *Lactobacilli* in the vagina and maintenance of pregnancy

There are reports that *Lactobacilli*, due to their protective role, may contribute to the normal course of pregnancy (Szubert et al. 2021). Data show that their absence is observed in pre-eclampsia (Gomez et al. 2016), gestational diabetes (Dunn et al. 2019), and preterm labor (Elovitz et al. 2019), among others. Numerous studies have confirmed that women with a vaginal microbiota dominated by *Lactobacillus* spp. bear a lower risk of preterm birth (Ansari et al. 2020; Aslam et al. 2020; Gerson et al. 2020; Kosti et al. 2020).

Far fewer studies have examined the effects of *Lactobacillus* spp. on fertilization success, implantation, and early embryonic development as well as recurrent implantation failure (RIF). Based on 16S rRNA gene sequencing of the vaginal microbiota, the vaginal *Lactobacillus* spp. showed a significant positive correlation with the pregnancy rate and the RIF group, all of the genera were significantly increased, especially the aerobic bacteria (8.5% for the RIF group and 2.3% for the control group, $p < 0.05$) (Fu et al. 2020).

The relationship between miscarriage and vaginal bacterial composition has been studied by Nelson et al. (2007; 2015). They analyzed the effect of changes in the vaginal microbiota, in the first trimester of pregnancy, on the risk of miscarriage in the second trimester. It has been shown that a lack of *Lactobacillus* spp. in the vagina during the first trimester of pregnancy may be related to the risk of miscarriage in the second trimester (HR: 1.32; 95% CI: 1.10–1.64) (Nelson et al. 2007). Similarly, Xu et al. (2020) showed that a lack of *Lactobacillus* spp. may be a contributing factor for pregnancy loss. In 80% of women included in this study who had a miscarriage, the number of *Lactobacillus* spp. in the vagina was lower than the control group (Xu et al. 2020).

Interestingly, other reports comparing vaginal microbiota at the genus level in women with confirmed miscarriage correlated with a reduction in the number of *Lactobacillus* spp. in the first or second trimester of pregnancy (Al-Memar et al. 2020). It also proved that the risk of pregnancy loss in the second trimester among women with confirmed BV diagnosed in the first trimester was increased but not statistically significant. However, women with the most severe BV changes in the vaginal microbiota had a twofold increase in the risk of pregnancy loss in the second trimester compared to women with normal vaginal microbiota (HR: 2.49, 95% CI: 1.13 to 5.48). Similarly, Bretelle et al. (2015) reported a correlation between pathogenic bacteria in the vagina, including *Chlamydia trachomatis*, *Atopobium vaginae*, and *G. vaginalis*, and late miscarriage and high-risk pregnancies. Also, genital tract infections primarily characterized by anaerobic bacteria such as *Gardnerella*, *Prevotella*, *Megastrobila*, and *Cyclospora* increase the risk of miscarriage (Xu et al. 2020).

Interesting studies investigate the relationship between recurrent miscarriage (RM) and *Lactobacillus* spp. and the growth of pathogenic bacteria in the vagina. Non-pregnant women with three or more consecutive miscarriages were selected for recurrent miscarriage studies (Llahi-Camp et al. 1996; Işik et al. 2016; Kuon et al. 2017; Zhang et al. 2019). Llahi-Camp et al. (1996) microscopically evaluated Gram-stained vaginal smears of women with one miscarriage and women diagnosed with RM. Results showed that BV was significantly more common among women with a history of one second-trimester miscarriage (27/130; 21%) than among women with RM (31/370; 8%) (Llahi-Camp et al. 1996). Similarly, in a study conducted by Işik et al. (2016), the presence of BV was statistically associated with the occurrence of one miscarriage in the last six months ($p < 0.05$), while no significant association was found between BV and recurrent miscarriages ($p > 0.05$). Thus, it was concluded that there is no direct relationship between BV and RM. However, a recent study based on 16S rRNA gene sequencing of vaginal microbiota shows that women with RM have a higher genus richness ($p = 0.037$) in the vagina than healthy women and bacteria such as *Atopobium*, *Prevotella* and *Streptococcus* are identified (Zhang et al. 2019). Women with RM also showed a reduced amount of *Lactobacillus* spp. (Zhang et al. 2019; Fan et al. 2020). Kuon et al. (2017) found that women whose vagina is colonized by *G. vaginalis*, and Gram-negative anaerobes more often have RM. Almost 20% of patients with RM showed vaginal colonization by *G. vaginalis* and 15% by *Enterobacteriaceae*. In addition, vaginal *Lactobacilli* have been reported less frequently in women with RM (Kuon et al. 2017). Other studies also suggest that BV may contribute to chronic endometritis, which correlates with the occurrence of RM (Bardos et al. 2020). The effect of vaginal microbiota on miscarriage and recurrent miscarriage is summarized in Table I.

However, many uncertainties arise, including whether BV diagnosed in women with RM results from subsequent miscarriages or has developed independently. There is also no clear answer to whether the presence of *Lactobacilli* significantly prevents pregnancy loss.

***Lactobacillus iners* – a new suspect.** The already described dual nature of *L. iners* is why a vaginal microbiota dominated by *L. iners* provides only limited protection against vaginal dysbiosis and sexually transmitted diseases.

Verstraelen et al. (2009) showed that a vaginal microbiota dominated in the first trimester of pregnancy by *L. iners* is associated with a tenfold increase in the risk of developing vaginal dysbiosis in the third trimester of

Table I
Effect of vaginal microbiota on miscarriage and recurrent miscarriage.

	References	Conclusions
MISCARRIAGE	Nelson et al. (2007)	Lack of *Lactobacillus* spp. in the vagina during the first trimester of pregnancy may be associated with a risk of miscarriage in the second trimester
	Bretelle et al. (2015)	The presence of pathogenic bacteria such as *Chlamydia trachomatis*, *Atopobium vaginae*, and *Gardnella vaginalis* in the vagina is associated with high-risk pregnancies and may contribute to miscarriage
	Nelson et al. (2015)	BV correlates with miscarriageAl-Memar et al. (2020) Decreased vaginal *Lactobacillus* spp. during the first or second trimester of pregnancy correlates with risk of miscarriage
	Chang et al. (2020)	The presence of *Lactobacillus iners* in the vagina increases the risk of miscarriage
	Xu et al. (2020)	The presence of *Gardnerella, Prevotella* as well as *Megastrobila*, and *Cyclospora* and the lack of *Lactobacillus* spp. in the vagina may contribute to pregnancy loss
RECURRENT MISCARRIAGE (RM)	Llahi-Camp et al. (1996)	The BV is significantly more common among women who have had a second-trimester miscarriage than among women with RM
	Işik et al. (2016)	The BV is associated with the occurrence of one miscarriage in the past six months. The BV does not affect the occurrence of recurrent miscarriage
	Kuon et al. (2017)	*Lactobacillus* spp. is not present in the vagina of women with RM
	Zhang et al. (2019)	Pathogenic bacteria in the vagina include *Prevotella*, *Atopobium*, and *Streptococcus* and reduced *Lactobacillus* spp. correlate with RM

pregnancy, which may subsequently result in obstetric complications (RR 10.41, 95% CI 1.39–78.12, $p = 0.008$). Kindinger et al. (2017) reported that the dominance of *L. iners* in the vagina of women around 16 weeks of gestation is a risk factor for preterm birth ($p < 0.01$). In contrast, the dominance of *L. crispatus* is a strong predictor of term birth (Kindinger et al. 2017). Similarly, Petricevic et al. (2014) emphasize that dominance of *L. iners* in the vagina of pregnant women may increase the risk of preterm birth. A recent study conducted by Chang et al. (2020) characterized the vaginal microbiota profiles of pregnant women. They collected vaginal swabs at 16–20 weeks of gestation and then observed the pregnancy course of the study participants. It was found that women whose vagina was dominated by *L. iners* were more prone to miscarriage (Chang et al. 2020).

The *L. iners* is a very intriguing bacterial genus. Although it has been recognized as a common commensal, it is vital to assess the probability of different clonal variants of *L. iners*, which in some cases promote vaginal health and in others are associated with dysbiosis and gynecological complications in pregnancy.

Do *Lactobacilli* have protective functions in the uterus?

For many years, there was a prevailing belief that the uterus of healthy women is a sterile organ. However, this view is currently being challenged. The results of previous studies have not clearly defined the normal uterine microbiota composition (Bardos et al. 2020). Unlike the vaginal microbiota, the uterine microbiota is characterized by lower bacterial abundance and higher bacterial biodiversity. It has been estimated that the endometrial microbiome can contain four orders of magnitude fewer bacteria than the vaginal microbiome and therefore has low biomass. Low biomass microbiomes present in the endometrium, urine, or blood may have an important role in homeostasis and physiology. However, their study might be burdened by potential contamination from bacterial DNA present in the air, on laboratory equipment, and in reagents (Elnashar 2021). Additionally, determining the endometrial microbiota composition is not simple due to the difficulty in collecting the material and the possibility of contamination from vaginal bacteria. Examination of the uterine microbiota of women undergoing hysterectomy revealed the presence of both *Lactobacillus* spp. and other bacteria in the endometrium (Salim et al. 2002). In 2017, Chen et al. (2017) identified the following bacteria in the endometrium: *Lactobacillus* (30.6%), *Pseudomonas* (9.0%), *Acinetobacter* (9.0%), *Vagococcus* (7.2%), *Sphingobium* (5.0%), and other microorganisms. Koedooder et al. (2019) in a review paper identified bacteria from the families *Lactobacillaceae*, *Streptococcaceae*, *Bifidobacteriaceae* as major components of the endometrial microbiota. Even greater experimental difficulties are related to assessing the endometrial microbiota composition during pregnancy. One possibility is to collect an endometrial sample immediately after a cesarean section. Collection of endometrial samples from physiologically pregnant women showed six types of bacteria present in most samples: *Cutibacterium*, *Escherichia*, *Staphylococcus*, *Acinetobacter*, *Streptococcus*, *Corynebacterium*. The presence of *Lactobacillus* spp. was characterized by high quantitative variability in the samples of studied women (Leoni et al. 2019).

In the study of uterine microbiota, *Lactobacilli* are identified, but despite their protective role in the vagina has been well described in the literature (Boskey et al. 2001; Witkin and Linhares 2017; Amabebe and Anumba 2018; Valenti et al. 2018; Bernabeu et al. 2019; Ansari et al. 2020; Aslam et al. 2020; Gerson et al. 2020; Kosti et al. 2020), it is still unknown what function *Lactobacilli* may have in the uterus.

Studies conducted by Shiroda and Manning (2020) showed that *Lactobacillus* spp. strains are not detrimental to *in vitro* culture of human endometrial stromal cells (dT-HESCs). The dT-HESC cells were used as a model to represent the outermost layer of fetal membranes. This study showed that *Lactobacillus* spp. strains do not induce dT-HESC cell death. Thus, a hypothesis was made that these strains could theoretically prevent invading pathogens, similar to that in the vagina (Shiroda and Manning 2020). To test this thesis, the same team conducted another study using dT-HESC cells and a strain of *Streptococcus group B* (GBS), the presence of which is unfavorable in pregnant women. As biofilms are generally considered to facilitate tissue colonization by pathogenic bacteria, the researchers sought to determine whether culturing dT-HESC cells infected with GBS and then treating them with *Lactobacillus* spp. would affect the ability of GBS to form a biofilm. However, the results for *Lactobacillus* spp. inhibition of biofilm formation by GBS were not statistically significant. Given that *Lactobacillus* spp. secretes multiple inhibitory compounds, the effect of supernatants of different *Lactobacillus* spp. strains on GBS growth was also examined. It was shown that the tested supernatants inhibited GBS growth, biofilm formation, and host cell invasion. Supernatants from *L. reuteri* 6475, *L. gasseri* 33323 and *L. reuteri* 17938 decreased the AUC of colonizing GBS strain from 1.4 to 0.4, 0.2 and 0.5 ($p < 0.00005$), respectively. Additionally, supernatants from *L. reuteri* 6475, *L. gasseri*, *L. reuteri* 17938 and *L. crispatus* culture inhibited GBS biofilm formation from 1.7 to 0.1, 0.3, 0.2 and 0.7, respectively ($p < 0.0005$). Future studies should evaluate the role of factors such as bacteriocins isolated from lactic acid bacteria on pathogenic bacteria infection using dT-HESC cells (Shiroda et al. 2020).

Researchers' attention is currently focused on explaining possible relationships between the presence of specific bacteria in the uterus and pathological gynecological conditions such as uterine cancer or endometriosis (Wei et al. 2020; Lu et al. 2021). In addition, recently, investigators have attempted to determine the correct uterine microbiota composition that would promote pregnancy and normal pregnancy course (Kyono et al. 2018; Garcia-Grau et al. 2019; Leoni 2019; Brandão and Gonçalves-Henriques 2020; Kong et al. 2020; Riganelli et al. 2020; Schoenmakers and Laven 2020; Moreno et al. 2021). Determination of normal uterine microbiota composition would allow studying the correlation between specific bacteria and different gynecological conditions, including miscarriage.

Lactobacillus dominated uterine microbiota and miscarriage

The potential influence of uterine microbiota on the risk of miscarriage is an interesting field of research. Some of these studies identify the presence of *Lactobacilli* in the uterus as beneficial (Kyono et al. 2018; Moreno et al. 2021) or not affecting pregnancy (Leoni et al. 2019) and others as a contaminant from the vagina (Riganelli et al.2020). There are also studies where the presence of *Lactobacilli* in the uterus is defined as an adverse condition for the body (Fang et al. 2016).

The study conducted by Kyono et al. (2018) showed that in pregnant women, the endometrial microbiota could be divided into *Lactobacillus* dominated microbiota (LDM) or non-*Lactobacillus* dominated microbiota (NLDM). After single vitrified-warmed blastocyst transfers, the rates of pregnancies obtained were higher in the LDM group (58.9%) than the NLDM group (47.2%), but they were not statistically significantly different. The results of this study do not prove the benefit of *Lactobacillus* spp. dominance in the endometrium concerning the number of pregnancies obtained. However, it was suggested that endometrium that is dominated by *Lactobacillus* spp. may benefit implantation (Kyono et al. 2018). Subsequent studies have found that lack of *Lactobacillus* spp. dominance and overgrowth of *Gardnerella*, *Atopobium*, and *Prevotella* strongly associated with implantation failure using assisted reproductive techniques. Restoration of a favorable endometrial microbiota composition dominated by *Lactobacillus* spp. may improve reproductive results (Garcia-Grau et al. 2019; Schoenmakers et al. 2019; Brandão and Gonçalves-Henriques 2020; Kong et al. 2020). Moreno et al. (2021) defined dysbiotic endometrium, which correlated with abnormal pregnancy course, as dominated by: *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus*, and *Streptococcus*. The presence of *Lactobacillus* spp. has been described as beneficial for normal endometrial function (Moreno and Simon 2018; Moreno et al. 2021).

The literature provides a case report of one patient in whom the microbiological composition of uterine fluid was examined twice: before spontaneous miscarriage and during physiological pregnancy. In the first case, the uterine fluid contained less *Lactobacillus* spp. and showed higher biodiversity than in the case of a pregnancy resulting in childbirth. The adverse effect on pregnancy was more pronounced when *Gardnerella*

Table II
Effect of uterine microbiota on fertility and pregnancy maintenance.

References	Conclusions
Kyono et al. (2018)	Endometrium dominated by *Lactobacillus* spp. favors embryo implantation
Moreno and Simon (2018)	Dysbiotic endometrium characterized by *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus*, and *Streptococcus* correlates with abnormal pregnancy
Leoni et al. (2019)	The presence of mixed bacterial microbiota, not always *Lactobacillus* spp., is associated with a normal pregnancy
Moreno et al. (2020)	The endometrium before miscarriage is characterized by a greater diversity of bacteria and fewer *Lactobacillus* spp.
Moreno et al. (2021)	Types of bacteria such as *Enterococcus*, *Enterobacteriaceae*, *Streptococcus*, *Staphylococcus*, *Gardnerella*, *Mycoplasma*, *Ureaplasma*, *Chlamydia*, and *Neisseria* are responsible for chronic endometritis and suspected to have adverse effects on implantation as well as may contribute to miscarriage

and *Streptococcus* dominated the endometrium. It was also shown that bacterial types such as *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Gardnerella*, *Mycoplasma*, *Ureaplasma*, *Chlamydia*, and *Neisseria* are responsible for chronic endometritis and are suspected of having an adverse effect on implantation and may contribute to miscarriage (Moreno et al. 2021).

However, the role of *Lactobacillus* spp. is not shown by all studies to be necessary for normal reproductive processes. In a study conducted by Leoni et al. (2019) endometrial samples collected during planned cesarean section showed the presence of *Lactobacillus* spp. bacteria, but their number was characterized by quantitative variation within the studied group of women. The authors suggest that the presence of these bacteria during pregnancy does not appear to be a prerequisite for normal pregnancy (Leoni et al. 2019). In contrast, Riganelli et al. (2020) show that the presence of *Lactobacilli* in the uterus is the translocation of these bacteria from the vagina. Some reports show that *Lactobacillus* spp. is prevalent among women with endometrial polyps or chronic endometritis (Fang et al. 2016). The effect of uterine microbiota on fertility and pregnancy maintenance is summarized in Table II.

In conclusion, while some studies (Moreno and Simon 2018; Moreno et al. 2021) show that *Lactobacillus* spp. is commonly present in the uterus and is a marker of reproductive health, others bring the presence of *Lactobacillus* spp. in the uterus to contamination of test samples with vaginal bacteria or to a pathological condition of the body (Baker et al. 2018; Kyono et al. 2018; Leoni et al. 2019; Riganelli et al. 2020; Moreno et al. 2020).

Immunomodulatory properties of *Lactobacilli* in miscarriage

The vagina contains various cells and receptors associated with the immune system that recognize and respond to the presence of microorganisms (Wira et al. 2005). Both commensal and pathogenic bacteria are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), dectin-1 receptor, and nucleotide-binding oligomerization domain (NOD). Those receptors are present on both the squamous epithelial cells lining the vagina and the columnar cells lining the upper section of the female genitalia (Villa et al. 2020).

The decidua cells such as T lymphocytes, macrophages and natural killer cells (NK) under stimulation produce specific cytokines. Cell-to-cell communication at the mother-embryo interface leads to changes in the expression of the type and amount of cytokines. Immune tolerance or immune stimulation may be related to modifications in the cytokine pattern of T lymphocytes. How disruption of the vaginal microbial ecosystem and the endometrium may adversely affect implantation and miscarriage is not fully understood. Al-Nasiry et al. (2020) proposed possible mechanisms that may contribute to the impact of bacteria on the implantation process. First, the dominance of non-commensal bacteria may weaken the integrity of the endometrial mucosal barrier by affecting the tight junctions of the epithelium. It, in turn, may further weaken host defense mechanisms and allow pathogens to penetrate the endometrial stroma and induce an immune response by antigen-presenting cells (APCs) and other immune cells expressing PRRs. Abnormal stimulation of T lymphocytes, either directly by invading pathogens breaching the mucosal barrier or indirectly by absorbed bacterial products, results in an imbalance in cytokine production in favor of pro-inflammatory T helper 1 cells (Th1), dominated by tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-2 (IL-2) (Al-Nasiry et al. 2020).

Lactobacillus spp. is one of the most dominant genera of the healthy vaginal microbiota. Nevertheless, the interactions between this commensal bacterium and the immune system are largely unknown (Keelan 2011). Interactions between the microbiota of the reproductive

tract and components of the immune system, located in the vagina and uterus, may influence the production of a specific environment, favorable or unfavorable for the development of pregnancy.

It is known that lactic acid bacteria can interact with mucosal immune cells or epithelial cells lining the mucosa to modulate specific immune system functions (Wells 2011). Lactic acid, produced by *Lactobacillus* spp., shows immunomodulatory properties by inducing an anti-inflammatory response in vaginal and cervical epithelial cells. The balance of cytokines secreted by Th1 and Th2 cells is a critical component of a normal immune response (Szekeres-Bartho and Wegmann 1996; Wells 2011; Valenti et al. 2018).

Cytokines. Microbial stimulation of PRR initiates signaling cascades, leading to the activation of specialized cells, including NK cells, macrophages, $CD4^+$ and $CD8^+$ T cells, and cytotoxic T lymphocytes, and the secretion of specific cytokines (Genc et al. 2004a; 2004b). It has been shown that specific bacterial species in the vagina can affect the pattern of secreted cytokines. The lactic acid and hydrogen peroxide produced by *Lactobacillus* spp. bacteria can modulate cytokine production. Women whose vaginal microbiota is classified as CST-IV have been shown to have increased levels of cytokines such as TNF-α, IFN-γ, interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-4 (IL-4), and interleukin-8 (IL-8). Similarly, diagnosed BV has been shown to increase levels of immune mediators such as IL-1β, IL-8, TNF-α, IFN-γ, IL-2, interleukin-6 (IL-6) (Beghini et al. 2015), and AV has been shown to increase levels of IL-1β and IL-6 in vaginal secretions, which may promote obstetric complications (Amabebe and Anumba 2018; Donders et al. 2020). Anahtar et al. (2015) showed that *Prevotella amnii*, *Mobiluncus mulieris*, *Sneathia amnii*, and *Sneathia sanguinegens* (CST-IV) induce upregulation of IL-1α, IL-1β, and IL-8 secretion. Additionally, women whose vaginal microbiota is classified as CST-III showed significantly higher IFN-γ and IL-8 levels compared to CST-I. During the transition from CST-I to CST-III and CST-IV, significant increases in IL-1α, IL-1β, and TNF-α were observed (Anahtar et al. 2015).

Both in humans and a mouse model, increases in pro-inflammatory cytokines were found to be associated with an increased risk of pregnancy loss (Clark et al. 1998; Raghupathy et al. 1999). Production of specific cytokines can affect fertility and pregnancy maintenance (Marzi et al. 1996; Garzia et al. 2013). Increased production of IL-2 and decreased interleukin-10 (IL-10) has been observed in reproductive disorders (Marzi et al. 1996; Garzia et al. 2013). In a study conducted by Xu et al. (2020) low number of *Lactobacillus* spp. in the vagina correlated with increased IL-2 in women who had a miscarriage early in pregnancy. The presence of H_2O_2 produced by lactic acid bacteria appeared to be associated with lower levels of certain vaginal pro-inflammatory cytokines. Additionally, increased amounts of *L. crispatus* were related to the inhibition of IL-1β production (Xu et al. 2020). In a mouse model of *Lactobacillus rhamnosus* HN001, *Lactobacillus acidophilus* LA-14 inhibited *G. vaginalis*-induced expression of IL-1β, TNF-α, and interleukin-17 (IL-17). In contrast, IL-10 expression increased due to *L. rhamnosus* HN001 and *L. acidophilus* LA-14 treatment (Jang et al. 2017).

The IL-2, TNF-α and IFN-γ cytokines have been shown to significantly increase in the serum of infertile patients (An et al. 2015). It has also been proven that vulvar and vaginal candidiasis can contribute to reproductive disorders by increasing the production of certain cytokines. Another study shows that the vaginal mucosa has a potential function in local immune responses against pathogens, not only bacterial but also fungal, which may result in obstetric complications (Niu et al. 2017; Abdul-Aziz et al. 2019).

Natural killer cells. Natural killer (NK) cells are present in peripheral blood (pNK) and uterine tissue (uNK) (Moffett et al. 2004). It has been shown that uNK and pNK cells may be associated with reproductive processes (Thum et al. 2007; Kuon et al. 2017). The uNK cells play a key role in the initiation and maintenance of pregnancy. The uNK cells are not cytotoxic, secrete pro-angiogenic factors, and regulate trophoblast invasion. They are involved in the remodeling of spiral arteries thus have a beneficial effect on pregnancy. After successful implantation, the uNK cells reach a peak and constitute about 70% of all uterine lymphocytes in the first trimester of pregnancy but decrease in the second half of pregnancy (Bulmer et al. 1991; Dons'koi et al. 2014). A different role in reproductive processes has been attributed to pNK cells. There are reports that increased levels of pNK cells may have a negative effect on reproduction (Thum et al. 2007). It can be hypothesized that the presence of a potentially pathogenic microorganism can stimulate an inflammatory response leading to systemic changes in immune parameters revealed by pNK elevation. It is well known that lipopolysaccharides from Gram-negative bacteria are potent immunostimulators. Indeed, LPS is a potent activator of NK cell activity (Lindemann 1988; Kuon et al. 2017).

Some studies indicate that women with RM have altered peripheral blood NK parameters (increased numbers and/or activation levels) compared to women without diagnosed RM (King et al. 2010). The role of *Lactobacillus* spp. in pNK regulation may be related to its function in maintaining proper vaginal pH. Fluctuations in vaginal pH due to changes in the vaginal microbiota have increased susceptibility to infections, which may indirectly affect fertility. Patients with

unexplained infertility reported an association of abnormal vaginal flora with increased levels of TNF-α and IFN-γ in cervical mucus, which was related to increased numbers of pNK cells (Nakano et al. 2015). Recent research showed a significantly higher percentage of pNKs correlated with the presence of *G. vaginalis* in the vagina of women with RM (Seshadri and Sunkara 2014), but no association between the presence of *G. vaginalis* in the vagina and the amount of uNKs. In addition, the lack of *Lactobacillus* spp. has been shown to correlate with a decreased number of pNK cells (Kuon et al. 2017). However, there is still no pathophysiological explanation as to why pNK is elevated in a group of women with RM (Park et al. 2010; Kuon et al. 2017; Fu et al. 2021).

NK cells are innate lymphocytes with a $CD3^{-}CD56^{+}$ phenotype. Studies indicate that $CD56^{+bright}$ NK cells, which have a high affinity for IL-2 and produce various cytokines, are predominantly present in the uterus during pregnancy (Vince and Johnson 2000; Koopman et al. 2003), while the presence of $CD56^{+dim}$ NK cells, with moderate affinity for IL-2, having cytotoxic activity in pregnancy is related to the risk of miscarriage (King et al. 2010).

The relationship between peripheral and uterine NK cells is still unclear. It is commonly argued that blood and uterine NK cells have different phenotypes and that uNK cells are benign, produce cytokines, and are likely essential for normal pregnancy (Moffett-King 2002). However, there is some evidence for the transfer of pNKs to the uterus and their differentiation to uNK-like phenotypes in pregnancy (King et al. 2010; Cerdeira et al. 2013). The pNKs are increasingly implemented as a useful diagnostic tool to initiate immunomodulatory therapies in patients with RM.

Extracellular trap. In 2004, Brinkmann et al. first described a new protective mechanism of neutrophils, known as a formation of neutrophil extracellular traps (NETs). The NETs, which are composed of DNA strands, histones, neutrophil elastase, myeloperoxidase, other peptides, enzymes such as lactoferrin, lysozyme C, neutrophil defensins, cathepsin G, gelatinase, cathelicidins, leukocyte proteinase 3, and calprotectin are shed under the influence of pathogens (Nija et al. 2020). The NET formation is one of the mechanisms to fight pathogens (Brinkmann and Zychlinsky 2012). Subsequent studies have shown that extracellular traps can be produced not only by neutrophils but also by macrophages (Aulik et al. 2012; Hellenbrand et al. 2013), monocytes (Muñoz-Caro et al. 2015), eosinophils (Yousefi et al. 2008), as well as basophils (Morshed et al. 2014). The formation of neutrophil extracellular traps is not always beneficial to health. NETosis is an effective antimicrobial mechanism that protects the host from several infectious diseases.

At the same time, it is a double-edged sword of the innate immune system in the sense that if neutrophil extracellular traps are produced in excess or if they are not removed promptly, it can induce many diseases, including autoimmune disorders, coagulation disorders, and even cancer metastasis (Nija et al. 2020). It has been suggested that neutrophils have a protective role at the maternal-fetal tissue interface. In the case of infection or other stimuli not yet studied, neutrophils become over-activated and cause damage to the placenta and fetal membranes (Tong and Abrahams 2020). Preliminary studies show that overproduction of NETs in pregnancy is a detrimental phenomenon to pregnancy and can cause, among others, pre-eclampsia at the end of pregnancy. It is hypothesized that NETs occupy space within the trophoblast villi, reduce blood flow in the placental vessels, and ultimately cause fetal hypoxia. NETosis, aided by activated vascular endothelial cells, can destroy maternal endothelial cells (Brinkmann and Zychlinsky 2012; Niedźwiedzka-Rystwej et al. 2019).

The study conducted by Omeljaniuk et al. (2020) evaluated neutrophil extracellular traps in women who had a miscarriage during the first trimester of pregnancy. The study material consisted of the woman's blood serum and trophoblast fragments after miscarriage. The presence of essential structural elements of NET was observed in the trophoblast fragments. According to the author, the presence of NET structural elements in the placenta correlated with their presence in the mother's peripheral blood suggests a relationship between NETosis and miscarriage (Omeljaniuk et al. 2020). The study conducted by Doster et al. (2018) examined whether *ex vivo* infection of fetal membrane fragments with GBS could affect the formation of macrophage extracellular traps (METs). Extracellular trap-associated structures were found in fetal membrane fragments, confirming MET formation after GBS stimulation. Thus, infection with pathogenic bacteria can cause extracellular traps in the placenta and thus affects the fetus (Doster et al. 2018).

Can *Lactobacilli* regulate extracellular trap formation? A report by Vong et al. (2014) indicates that in a mouse model using bone marrow-derived cells, the probiotic strain *L. rhamnosus* GG inhibits *S. aureus*-induced NET for neutrophil motion. Moreover, LGG suppressed reactive forms of oxygen production and phagocytic capacity of neutrophils, thus possibly providing some level of hyporeactivity (Vong et al. 2014; Mutua and Gershwin 2021). The ability of LGG to inhibit *S. aureus*-induced NETs also translates into protection against cellular cytotoxicity. The *S. aureus* secretes pore-forming toxins that cause lysis of neutrophils, including leukocidin, which has been shown previously to induce NET formation (Pilsczek et al. 2010). It

remains to be determined whether LGG secretes bacteriocins that have antimicrobial activity against *S. aureus* or whether it directly interferes with the production of toxins secreted by *S. aureus* (Vong et al. 2014).

It is not clear whether *Lactobacillus* spp. can exert beneficial effects on pregnancy by inhibiting neutrophil extracellular traps formation. Further studies are needed to address whether a similar mechanism to Vong et al. (2014) may occur in placental membranes. These studies could provide evidence of new immunomodulatory properties of *Lactobacillus* spp. in pregnancy by regulating the formation of extracellular traps.

Conclusions

Miscarriage is one of the most common obstetric complications. The abnormal vaginal and uterine microbial composition may be one of the factors that increase the risk of miscarriage. *Lactobacillus* spp. is the most common bacteria within the reproductive tract. Microbiological tests before conception and in early pregnancy to determine the vaginal microbial composition may be important to understand the mechanisms that promote proper embryo implantation, placenta formation, and reduce the incidence of miscarriage. Although the presence of *Lactobacilli* in the vagina has long been confirmed, its presence in the uterus continues to raise some doubts.

Future research should focus on determining whether BV diagnosed among women who have had a miscarriage is its result or has developed independently. It is important to determine whether the presence of *Lactobacilli* significantly prevents pregnancy loss. Furthermore, it is necessary to assess the prevalence of different clonal variants of *L. iners*, which in some cases promote vaginal health and in others are associated with dysbiosis and gynecologic complications in pregnancy. In addition, a thorough understanding of both the molecular and immunological mechanisms of host-*Lactobacillus* spp. interaction is required. Only by considering these relationships will it be possible to answer the question of the importance and extent of the protective role of *Lactobacillus* spp. in miscarriage.

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Conflict of interest

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