The reproductive microbiome – clinical practice recommendations for fertility specialists

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RBMO

REVIEW

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BIOGRAPHY

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KEY MESSAGE

The reproductive microbiome is implicated in female reproductive health and is probably also implicated in pregnancy outcomes. Understanding how to assess and diagnose microbiome dysbiosis in the female reproductive tract could lead to improvements in reproductive outcomes.

ABSTRACT

The interest in and understanding of the human microbiome has grown remarkably over recent years. Advances in molecular techniques have allowed researchers to identify and study the microbiota and also use this information to develop therapeutic solutions for a spectrum of conditions. Alongside the growing interest in the microbiome, societal changes have resulted in many couples looking to start families later in life, therefore increasing the demand for assisted reproductive technologies. Combining these trends, it makes sense that clinicians are eager to understand and exploit the microbiome of their patients, i.e. the reproductive microbiome, in order to help them achieve their goal of becoming parents. This paper aims to provide an overview of the current and future research into the reproductive microbiome in relation to fertility and also share clinical practice recommendations for physicians who are new to this field or unsure about how they can utilise what is known to help their patients.

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INTRODUCTION

Since the first IVF baby was born in 1978, assisted reproductive technologies (ART) have become safer, more successful and more accessible; couples are choosing to have children later in life and the demand for ART is rapidly increasing (*Faddy et al., 2018*). However, some women are unable to become pregnant, or experience a pregnancy loss or a preterm delivery. A new perspective on fertility is emerging as a result of increasing knowledge about the microbiome – and its role in reproductive health.

The first observation of non-pathogenic bacteria inhabiting the human body was of *Escherichia coli* in the intestine of healthy children in 1885. Since then, other commensal bacteria (bacteria that live in harmony with the host) inhabiting different parts of the human body, such as nasal and oral cavities, skin and the urogenital tract, have been discovered throughout the 20th century. Their role has been underestimated and in general, microbes were considered a threat to human health. Due to advances in sequencing technology, the literature has started to highlight the role of commensal bacteria (the microbiota) in normal physiology. In the mutualistic relationship between microbial communities and the human host, the host provides the nutrients to support bacterial growth, while the microbial communities provide defence against pathogens, shape the development and maturation of the immune system, help to digest food and fibre, produce vitamins and metabolize xenobiotics (*Puebla-Barragan and Reid, 2019*). Of note, the term microbiota refers to all of the micro-organisms in an environment, including bacteria, archaea and single-cell eukaryotes (and sometimes viruses), and the term microbiome refers to the collection of genomes of the microbiota.

At present, there are five distinct microbiomes according to body site – the oral cavity, nasal cavity, skin, genitourinary tract and gastrointestinal tract (*Kumar and Chordia, 2017*). Within these distinct anatomical locations there are further microbial habitats, such as the tongue, cheek and lip of the oral cavity (*Kumar and Chordia, 2017*). Whereas the gut microbiota has by far the most dense and diverse microbial community, in the lower reproductive tract, a healthy microbiota is dominated by *Lactobacillus* species. *Lactobacilli* are bacteria that produce lactic acid and protect the vagina by sustaining a low pH that is prohibitive to the growth of most bacteria. The upper reproductive tract, long considered sterile, also has its own specific microbiota, which is 100 to 1000 times less dense (*Baker et al., 2018*), but dominated by a greater variety of bacterial species and also different strains of *Lactobacillus* than the healthy vaginal microbiota (*Moreno and Franasiak, 2017*).

The aim of this work is to briefly discuss the current research on the reproductive microbiome of female fertility patients in order to provide expert opinion on how to utilise this knowledge in clinical practice of infertility diagnosis and treatment. Bacterial therapies, commonly known as probiotics, are also increasingly researched, a natural consequence of our expanding knowledge of the microbiome. Targeted probiotics can replenish depleted microbiota, restore healthy conditions and consequently help treat infections and illness (*Puebla-Barragan and Reid, 2019*).

MATERIALS AND METHODS

A panel of 37 fertility experts, including clinicians from across Europe, met to generate best practice recommendations for fertility experts interested in utilising the microbiome to improve their clinical practice. Small groups were formed for initial discussion of four key topics:

1 Clinical assessment: How is the reproductive microbial ecosystem being assessed in women? Which tools are being used and in which patients?
2 Endometrium and implantation: What is the role of the endometrial microbiome in endometrial receptivity, implantation and recurrent implantation failure?
3 Infertility: How should we evaluate and treat infertile patients and patients with bacterial vaginosis utilising the microbiome?
4 Birth: What techniques can be used to evaluate microbial structures and evaluate risk for preterm birth and pregnancy loss in pregnant women?

A full list of the discussion topics was compiled (see Supplementary Material). The entire group came together to discuss all topics and the agreed statements were used as a basis to produce a series of recommendations for clinical practice supported by the literature. These recommendations appear as section titles throughout the paper and have been organised into three sections. First, a brief overview of the current literature on the relationship between the reproductive microbiome and fertility (Part I); second, nine clinical practice recommendations for fertility experts (Part II); and third, seven statements about the future of research into the reproductive microbiome (Part III).

Part I: Current literature

There are strong indications that the vaginal and endometrial microbiomes are associated with female reproductive health.

The vaginal microbiota is of great interest due to its complexity, the increasing knowledge of its role in women's health and its influence on reproductivity (*Moreno and Simon, 2019*). As changes in vaginal microbiota homeostasis or disrupted microbiota, referred to as dysbiosis, can be the cause of infertility and preterm birth, there is increasing interest in the characteristics of a healthy microbiota and in different ways that this can be re-established (*Bedaiwy, 2019*; *García-Velasco et al., 2017*; *Puca and Hoyne, 2017*). Dysbiosis is also associated with endometriosis, although it is as yet unclear whether dysbiosis causes endometriosis, or endometriosis induces dysbiosis. As of November 2019, there are 94 clinical trials registered on https://clinicaltrials.gov that include the search terms 'pregnancy' and 'microbiome'. Seventeen of these are specifically linked to the vaginal/endometrial microbiome.

The vaginal microbiome plays an important role in protecting the vagina – the first barrier from the external environment to the upper reproductive tract. A better understanding of the vaginal microbiome is therefore mandatory to further our understanding of natural fertility and reproductive technology.

As per our current knowledge, in non-pregnant, healthy women the vaginal microbiome is dominated by four *Lactobacillus* species: *L. crispatus*, *L. iners*, *L. jensenii* or *L. gasseri* (*Ravel et al., 2011*). The vaginal microbiota can be characterized by the presence or

absence of certain bacterial species. In 2011, *Ravel et al. (2011)* identified five vaginal microbial community state types (CST); four of them (I, II, III and V) are *Lactobacillus*-dominant and are more commonly found in European and Asian women. CST-IV, more frequent in Hispanic and African-American women, differs due to the increased abundance of strictly anaerobic bacteria (Gardnerella, Ureaplasma) and reduced presence of *Lactobacillaceae* (*Ravel et al., 2011*). *Lactobacillus* spp. produce lactic acid that helps to keep the vaginal pH below 4.5 and creates inhospitable conditions for pathogens to grow (*Graver and Wade, 2011*). The vaginal microbiota composition shows large ethnic differences and varies throughout the woman's lifetime depending on physiological events, like menstrual cycle and pregnancy, and external factors, like sexual activity, hygiene habits and medical treatments (*Gajer et al., 2012*; *Ravel et al., 2011*; *Zhou et al., 2007*).

The vaginal microbiota and its relation to infertility

Bacterial vaginosis is one example of an altered state of the vaginal microbiome, characterized by depletion of *Lactobacillus* and augmented diversity of anaerobic bacteria. Bacterial vaginosis affects 20–50% of reproductive-age women and it represents a risk factor for subfertility and infertility (*Mastromarino et al., 2014a*; *Sirota et al., 2014*). Increased presence of specific bacteria (*Atopobium vaginae*, *Ureaplasma vaginae*, *U. parvum*, *U. urealyticum* and *Gardnerella*), normally observed in asymptomatic bacterial vaginosis, together with higher abundance of *Candida* and reduced vaginal and cervical *Lactobacillus*, is often present in women with fertility problems (*Koedooder et al., 2019a*). Moreover, vaginal dysbiosis reduces the local defences against sexually transmitted pathogens (*Wiesenfeld, 2003*) and ascension of pathogens up the fallopian tubes can affect reproductive health (*Mastromarino et al., 2014a*).

The vaginal microbiota and its relation to IVF outcomes

The vaginal microbiota has lately become an important factor in IVF. The lack of consensus on its role comes from the lack of common criteria to cross-check data, small sample size, and the lack of data due to the novelty of the field. Altered vaginal microbiota and bacterial vaginosis may be related to poor pregnancy outcomes. Therefore, patients undergoing IVF should be screened and eventually treated to enhance the chances of success.

Several studies confirm that a vaginal microbiota rich in *Lactobacillus* spp. without bacterial vaginosis, either clinical or subclinical, leads to more positive outcomes with ART (*Babu et al., 2017*; *Eckert et al., 2003*; *Mangot-Bertrand et al., 2013*; *Moore et al., 2000*). *Haahr et al. (2016a)* studied the microbiota of 84 women undergoing IVF and found a strong relationship between microbiota composition and pregnancy. Of the 22 women who had an altered composition, only two women (9%) achieved a clinical pregnancy. Out of the 62 women with a normal microbiota composition, 29 women (47%) achieved a clinical pregnancy. *Koedooder et al. (2019b)* performed a prospective study of reproductive-age women undergoing IVF and found that embryo implantation was less successful in women with reduced *Lactobacillus* spp. in their vaginal microbiota. The authors proposed an algorithm, utilising the IS-pro sequencing method, to predict the IVF success rate based on the vaginal microbiota composition. They identified women with a low chance of becoming pregnant due to reduced *Lactobacillus* in the vaginal microbiota (18% of the dataset of 192 women). The model was 94% accurate (with high specificity [97%], but low sensitivity [26%]): 32 out of 34 women with an unfavourable profile did not achieve pregnancy. An external validation cohort study further supported the prediction specificity. According to high versus low *Lactobacillus* dominance the women were divided into two subgroups with favourable and unfavourable prediction, respectively. None of the women in the unfavourable group became pregnant (*Koedooder et al., 2019b*). In another study, *Lactobacillus crispatus*-dominant microbiomes were associated with higher live birth rate (*Haahr et al., 2019*; *Vergaro et al., 2019*). These findings suggest that a better knowledge about the vaginal microbiota prior to IVF may help both couples and healthcare professionals decide on the timing of their IVF cycles to maximize the results (*Moreno and Simon, 2019*).

At this point, however, it is very difficult to evaluate and to compare different studies because there are many variables to be considered, such as the time of collection of the vaginal samples, hormonal stimulation, fresh or frozen embryo transfer, oocyte donation, and success rate, defined as implantation or live birth (*Fettweis et al., 2019*). Moreover, novel and more standardized molecular approaches have improved the ability to detect micro-organisms and will enhance future results.

CST-IV is most commonly associated with negative ART outcomes

Women with bacterial vaginosis and aerobic vaginitis often present with a microbiota comparable to CST-IV (*Smith and Ravel, 2017*). Bacterial vaginosis or a perturbed microbiota are also a common factor in frequent preterm delivery or miscarriage (*Buggio et al., 2019*; *García-Velasco et al., 2017*). CST-IV, with or without a diagnosed bacterial vaginosis, has been described as a common feature of preterm birth in Caucasian women (*DiGiulio et al., 2015*; *Donders et al., 2009*) and African-American women (*Nelson et al., 2016*). The vaginal microbiota is normally stable, *Lactobacillus*-dominated and less diverse during pregnancy (*Aagaard et al., 2012*). *DiGiulio et al. (2015)* observed that some women who presented with a CST-IV microbiota, depleted of *Lactobacillus* spp. throughout the gestational period, were more likely to deliver preterm. Another study found that a CST-IV vaginal microbiome was less frequent in women delivering at term even though it was not possible to define a correlation between this vaginal microbiota profile and preterm delivery (*Romero et al., 2014*).

The endometrial microbiota and its relation to IVF

Previously it was believed that the upper reproductive tract was sterile. After the discovery of a specific and independent endometrial microbiome (*Chen et al., 2017*), several studies concentrated on endometrial dysbiosis (*Benner et al., 2018*; *Moreno et al., 2016*). Endometrial dysbiosis can be the cause of implantation failure and lead to infertility (*Moreno and Simon, 2018*).

Infertility treatment is intrinsically complicated; however, the delicacy and importance of the implantation phase has proven to be critical for positive outcomes (*Diedrich et al., 2007*). A *Lactobacillus*-dominant endometrium is more receptive than an endometrium

with high bacterial diversity and a low proportion of *Lactobacilli*. The results of a study by Moreno et al. (2016) highlight that women with a *Lactobacillus*-dominant microbiota have a higher chance of success in implantation, 60% versus 23% in non-*Lactobacillus*-dominant microbiota; in pregnancy, 70% versus 33%; and in live birth, 60% versus 7%. In this study Moreno et al. (2016) also found that women with a receptive endometrium (*Lactobacillus*-dominant) have a significantly lower miscarriage rate, 17% versus 60% as compared with women with a non-*Lactobacillus*-dominant microbiota. These results differ from those obtained by Franasiak et al. (2016), who reported a comparable IVF success rate independent of the *Lactobacillus* dominance of the endometrial microbiota. Moreno et al. (2016) suggest this difference could be related to differences in the classification systems used in the two studies.

Hormonal fluctuations, especially in oestrogens, are implicated in the regulation of the vaginal microbiota and in the preparation of the endometrium for implantation and pregnancy (Wessels et al., 2018). It can be expected that hormones also influence the endometrial microbiota. Surprisingly, however, Moreno et al. (2016) found that the endometrial microbiota does not change under hormonal influence in the period preceding implantation. These results suggest that we should start to consider the endometrial microbiota and its health state prior to beginning IVF, in order to maximize the chance of positive outcomes.

The vaginal microbiota and its relation to preterm birth

The causes of preterm birth are very complex, making it challenging to prevent and also difficult to reduce the related infant mortality and the psychological and economic consequences of extended hospitalization of the newborn. As the vaginal microbiota is important for the health of the whole reproductive tract and for prevention of pathological infections, it can be expected that it affects not only pregnancy, but also delivery. The vaginal microbiome evolves during pregnancy in relation to gestational age and tends to lose the diversity observed in non-pregnant women (Freitas et al., 2017). Healthy pregnant women present a distinct vaginal microbiota profile dominated by *Lactobacilli*, with no differences depending on community type, ethnicity or body mass index (Aagaard et al., 2012).

A non-*Lactobacillus*-dominant vaginal microbiota and increased bacterial diversity during pregnancy are associated with higher risk of preterm birth (Brown et al., 2019). A group of 96 women were divided into three groups according to high (38), low (22) or no risk (36) of preterm birth and followed from week 6 of pregnancy. Augmented bacterial diversity and a concomitant reduced *Lactobacillus* spp. abundance were evident prior to premature rupture of membrane in 20% and 26% of high- and low-risk women, respectively. Only 3% of women that finished their pregnancy at term had an altered vaginal microbiota (Brown et al., 2019).

Black women are more likely to deliver preterm, yet the vaginal microbiota of pregnant women shows minor to no diversity across different ethnicities (Brown et al., 2019; Fettweis et al., 2019). The National Institutes of Health's Integrative Human Microbiome Project includes samples of vaginal microbiome from more than 1500 pregnant women of diverse ancestries and omics data generated from a subset of about 600 pregnancies. Fettweis et al. (2019) used these data to provide evidence of the role of *Lactobacillus crispatus* in lowering the risk of preterm birth. American women of European ancestry have a vaginal microbiota rich in *L. crispatus* and a significantly lower risk of preterm birth when compared with American women of African ancestry, who are more likely to have a vaginal microbiome poor in *Lactobacillus* spp. Additionally, bacterial vaginosis-associated bacterium 1, a bacterium that is positively associated with preterm birth, is more common in Black women (Fettweis et al., 2019). These results correspond to those obtained in British women with Caucasian, Asian and Black ethnicities. All Caucasian and Asian British women who delivered at term had a *Lactobacilli*-dominant vaginal microbiome, but 12% of Black women who delivered at term were *Lactobacilli*-depleted (Brown et al., 2019; MacIntyre et al., 2015).

Some women with pre-pregnancy dysbiosis show a shift toward a healthy *Lactobacillus*-dominant bacterial community around weeks 15 to 20 of pregnancy. This normalization happens in about 50% of women (Hay et al., 1994). However, there is also a strong association between pre-pregnancy bacterial vaginosis and preterm birth because ascending vaginal infections cause intrauterine inflammation, which is responsible for one-third of preterm birth (Buggio et al., 2019; Leitich et al., 2003).

To identify an increased risk of preterm birth early in pregnancy, despite the natural evolution of the vaginal microbiome during gestation, would lead to earlier diagnostics and possible therapeutic interventions. Based on thorough meta-analysis of vaginal microbiome datasets, a correlation between the vaginal microbiome in the first trimester and preterm delivery has been established. This correlation and related different microbiota profiles have been exploited to propose a 'Taxonomic Composition Skew' metric as a possible diagnostic tool to predict preterm delivery with high accuracy (Haque et al., 2017).

Part II: Recommendations for clinical assessment and clinical practice1

For all infertility patients, ask if they have clinical symptoms (smell, discharge, urinary tract infection, Candida) currently and during their cycle

Our understanding of healthy and unhealthy vaginal microbiome is rapidly developing. When a woman is evaluated for vaginal dysbiosis, it is important to identify the cause in order to prescribe the proper treatment. Assessment of symptoms combined with microscopic and pH evaluation of the vaginal smear can help distinguish between bacterial vaginosis, trichomoniasis, candidiasis and lactobacillosis (Hainer and Gibson, 2011). However, almost 30% of women with a vaginal disorder do not receive a proper diagnosis (Sha et al., 2005; van de Wijgert and Jespers, 2017).

Bacterial vaginosis is the most common vaginal disorder in reproductive-age women, although 50% of women are asymptomatic (Haahr et al., 2016a). Bacterial vaginosis is characterized by vaginal dysbiosis (altered microbiome composition) presenting as a depletion of *Lactobacillus* and a higher diversity of other anaerobic bacteria, such as *Gardnerella*, *Mycoplasma* and *Prevotella* (Turovskiy et al., 2011). Bacterial vaginosis

is positively associated with infertility, miscarriage and preterm birth (*Donders et al., 2009*). To confirm diagnosis and provide proper treatment, all women reporting vaginal symptoms (unusual discharge, burning or itching, unusual smell) should have their vaginal secretions tested (*van de Wijgert and Jespers, 2017*).

Urinary tract infections and yeast infections can be the cause of reproductive tract damage, i.e. tubal damage or pelvic inflammatory disease, and result in infertility (*Mastromarino et al., 2014b*). The main clinical symptoms of these conditions are malodourous vaginal discharge and itching/burning sensations (*Klebanoff et al., 2004*). Good clinical practice requires that women consulting for infertility treatment report whether they have suffered from one of these symptoms in the past, during their cycle, or at the moment of consultation.

For women with no symptoms there is currently no further testing recommended in clinical practice

Bacterial vaginosis is a deviation from a healthy *Lactobacillus*-dominant microbiota and several studies correlate infertility problems with vaginal dysbiosis (*van de Wijgert et al., 2014*; *Weström, 1994*). Based on a cross-sectional study of 84 healthy women and 116 women with infertility problems, *Babu et al. (2017)* suggest adding a routine vaginal microbiota screening for all women undergoing infertility treatment. At present, however, this evidence is too limited to recommend a routine screening of all fertility patients. Moreover, molecular diagnostic testing is expensive and there is no supporting literature to recommend it in asymptomatic women (*van der Veer et al., 2018*).

If vaginal pH is below 4.5 this could indicate lactobacillosis

The pH of vaginal secretions is an important parameter for an accurate diagnosis in women presenting with vaginal symptoms such as burning or malodourous discharge. Many vaginal microbiota pathologies are characterized by *Lactobacillus* depletion, lower lactic acid production and a consequent increase in pH. However, lactobacillosis, a less common vaginal condition, presents with normal or lower pH values (*Ventolini et al., 2014*). Lactobacillosis presents comparable symptoms – itching/burning, and abundant vaginal discharge – to those of bacterial vaginosis and is characterized by the higher abundance of long segmented *Lactobacilli* chains (*Ventolini et al., 2014*).

Vaginal dysbiosis can be measured in multiple ways, there are no gold standards in assessment. For women with symptoms/suspicions of dysbiosis, it is recommended to perform a speculum exam and pH test. If available also perform a wet smear, Gram Nugent score, and quantitative polymerase chain reaction (qPCR)

Several techniques have been developed to evaluate and confirm a diagnosis of bacterial vaginosis: the Amsel criteria, Nugent score and more recently qPCR. The Amsel criteria and Nugent score are inexpensive tests but they cannot adequately measure microbial complexity and thus may give inaccurate results (*Mendling, 2016*).

In 1983, Amsel *et al.* proposed four practical diagnostic criteria: (i) thin, white-greyish homogenous vaginal discharge; (ii) vaginal pH >4.5; (iii) a 'fishy' odour after adding 10% potassium hydroxide to vaginal secretions; (iv) presence of epithelial cells coated with bacteria on wet mount microscopy. Bacterial vaginosis is confirmed if three out of four of these criteria are met. The Amsel criteria is an inexpensive technique that may provide the first evidence of dysbiosis. However, the evaluation of the results relies on microscopy, direct observation and experienced personnel (*Kusters et al., 2015*; *Sha et al., 2005*). Another limitation of the Amsel criteria is that the increased bacterial diversity causing dysbiosis is consistent with an altered pH, but might not alter the other Amsel criteria, thus resulting in a negative test. In different cohort studies, only about 40% of women with diagnosed bacterial vaginosis have been found positive according to the Amsel criteria (*Sha et al., 2005*; *van der Wijgert et al., 2014*).

The Nugent scoring system, proposed in 1991, is a Gram-stained scoring system of vaginal swabs. It is a 0–10 standardized scale evaluating vaginal microbiota based on the presence/absence of *Lactobacilli* spp., *Gardnerella vaginalis* and *Mobiluncus* spp. bacterial morphotypes. A lower abundance of *Lactobacilli* and an increased presence of other bacteria result in a higher Nugent score (*Nugent et al., 1991*). A Nugent score of 0–3 represents a healthy microbiota, 4–6 an intermediate microbiota, and 7–10 confirms bacterial vaginosis. The test is inexpensive and more sensitive than the Amsel criteria, but it takes a long time and requires an experienced microscopist to correctly evaluate results that remain subjective (*van der Wijgert et al., 2014*).

New molecular biology techniques based on DNA and RNA analysis, including qPCR, allow a more objective analysis. Even in the midst of great microbial complexity, the characterization of DNA/RNA sequences allows the identification of bacteria present in minor amounts or which are difficult to cultivate (*Cartwright et al., 2012*). By utilising qPCR, a larger number of bacteria can be detected compared with bacteria culturing techniques (*Kusters et al., 2015*). The Amsel criteria and Nugent score can miss a diagnosis of bacterial vaginosis because they can underestimate the complexity of microbiota alterations (*Cartwright et al., 2012*). Recently, the performance of a novel diagnostic tool, the AmpliSens assay, was compared with the classical methods, and proposed for use in clinical practice as a more rapid, objective and accurate diagnosis (*van den Munckhof et al., 2019*).

There is limited evidence on whether uterocervical colonization should be routinely assessed

The discovery of a distinct uterine microbiota is recent and there is little consensus on the composition of a core healthy uterine microbiota (*Baker et al., 2018*). Nevertheless, authors agree on the hypothesis that uterine microbiota, independent of the vaginal microbiota, might play a role in infertility (*Moreno and Franasiak, 2017*). Further research is needed to better understand the composition and the role of uterine colonization, keeping in mind that low-density microbiota are extremely sensitive to microbial and DNA contamination. To date there is no evidence supporting clinical testing to evaluate the uterine microbiome (*Baker et al., 2018*).

Antibiotics should not be used on a routine basis to change vaginal typology

Antibiotics are not free from secondary effects, varying from skin rash to

anaphylaxis, and overuse can increase the chance of developing antibiotic resistance. The efficacy of using antibiotics in conjunction with IVF treatment is controversial (*Haahr et al., 2016b*; *Kaye et al., 2017*; *Kroon et al., 2011*; *Moore et al., 2000*). Use of antibiotics before embryo transfer could impact the normal healthy colonization of the endometrium and reduce the efficacy of the procedure (*Kroon et al., 2011*). On the other hand, the risk of contamination of the upper reproductive tract by vaginal bacteria is higher during transcervical and transvaginal procedures and antibiotic prophylaxis could be preventive (*García-Velasco et al., 2017*). Based on a systematic review, the *American Society for Reproductive Medicine (2017)* strongly recommends against the use of antibiotics before embryo transfer. There is an ongoing randomized clinical trial of IVF patients at Washington University to evaluate the clinical pregnancy rate when withholding routine prophylactic antibiotic therapy during IVF (NCT03386227).

The use of antibiotics can however be of relevance for certain repeated implantation failure (RIF) patients

Chronic endometritis is an inflammatory state of the uterine endometrium that is normally asymptomatic. The inflammatory state is the result of an imbalance between the microbiome and the host immune system, possibly due to micro-organisms, viruses or parasites, but the aetiology remains unclear (*Park et al., 2016*; *Wu et al., 2017*). The role of chronic endometritis in fertility has long been underestimated, but it is now recognized as a possible cause of not only infertility but also early miscarriage (*Johnston-MacAnanny et al., 2010*). Chronic endometritis leads to a non-receptive endometrium which is a major cause of RIF (*Margalioth et al., 2006*). The fact that the upper reproductive tract is not sterile (*Mitchell et al., 2015*), and the latest data showing that both placenta and amniotic fluid may present bacteria of their own, have generated a new awareness to the role of the microbiota in pregnancy and newborn health (*Schoenmakers et al., 2018*). The efficacy of antibiotic therapy in treating women with certified chronic endometritis and RIF has been established. Women with no infection as compared with women with persistent infection after antibiotic therapy had higher rates of implantation, 37% versus 17% ($P = 0.069$); clinical pregnancy, 65% versus 33% ($P = 0.039$); and live birth, 61% versus 13% ($P = 0.02$) (*Cicinelli et al., 2015*). Although data do not exist for their use presently, probiotics as a method of treating chronic endometritis represents a promising approach and requires further study.

Pre-conception counselling is useful and lifestyle habits can impact reproductive outcomes (possibly by modulation of the vaginal microbiota), therefore weight loss, physical activity and lifestyle changes should be discussed and encouraged in infertile patients

A woman's health is fundamental to a healthy pregnancy and for the future health of the baby (*Stephenson et al., 2018*). Women who wish to conceive are likely to change bad habits, however advice is often inaccurate or incomplete (*Bookari et al., 2017*). Pre-conception diet and lifestyle changes can not only lead to better general health but also increase the chances of a positive reproductive outcome.

Obesity represents a major health threat with many related pathologies, including metabolic syndrome, diabetes, cardiovascular disease and cancer. The estimated reduction in fertility is 18% for obese women (*Pantasri and Norman, 2014*). Weight loss is strongly related to improved ovulation, hormonal rebalance and increased pregnancy rate in obese women (*Clark et al., 1995*; *Sim et al., 2014*). Diet and mild-to-moderate daily physical activity should be the first step in infertility treatments.

Smoking is another major health threat for society as a whole and in particular for infertile women. The pregnancy rate of heavy smokers (>10 cigarettes) is significantly lower when compared with that of non-heavy smokers (<10 cigarettes) (34.1% versus 52.2%, respectively), and overall women who smoke are more likely to be subfertile (*Bolumar et al., 1996*). However, for women who smoke, pregnancy outcomes can be quickly changed as women who quit smoking have a time to first pregnancy that is comparable to that of non-smokers (*Bolumar et al., 1996*).

Diet and smoking are directly involved in bacterial vaginosis, and bacterial vaginosis is associated with infertility, pregnancy loss and preterm birth. Overweight and obese women are more likely than healthy-weight women to have a higher Nugent score and bacterial vaginosis (*Brookheart et al., 2019*). Women who smoke are more likely than non-smokers to have a CST-IV vaginal microbiota depleted in *Lactobacillus* spp., and a high Nugent score indicating a risk for bacterial vaginosis (*Brotman et al., 2014*).

Lifestyle changes, especially those that affect nutrition, can lead to changes in the gastrointestinal microbiome and could have a positive impact in infertile patients

Diet has a major impact on the gut microbiome, by far the densest and most metabolically active human-associated microbial community (*Lozupone et al., 2012*; *Vieira-Silva et al., 2016*; *Wu et al., 2016*), and so nutritional interventions may improve reproductive outcome partly through modulating the gut microbiome composition. A disturbed gut microbiota has been implicated in many diseases – inflammation, metabolic disorders, obesity and cancer (*Cani et al., 2007*; *Hildebrandt et al., 2009*; *Le Chatelier et al., 2013*; *Ridlon et al., 2014*) – most of which can also affect fertility. Therefore, the importance of microbial communities at body sites other than the reproductive tract have to be considered as potential actors in human fertility.

Part III: Future studies and research techniques to consider

Tobacco, alcohol, cannabis, stress, physical activity, infections, lack of sleep, body mass index, antibiotic exposure, drug exposure, sexual activity, diabetes/metabolic disorders and hygiene habits might impact microbiome composition; modification of these factors should be assessed in every study in order to assess their impact on the microbiota

The recent discovery of the non-sterility of the upper reproductive tract (*Mitchell et al., 2015*) and recent debate over whether even the placenta and amniotic fluid may present bacteria of their own, have brought a new awareness to the role of the microbiota in pregnancy and newborn health (*Schoenmakers et al., 2018*). Moreover, a disturbed vaginal microbiota has proven to be a major factor in infertility, miscarriage and preterm birth (*Moreno and Simon, 2019*).

The human microbiota evolves naturally; the vaginal microbiota changes during pregnancy (*MacIntyre et al., 2015*)

and the gut microbiota evolves from childhood to old age (*Woodmansey, 2007*) and can be altered due to exogenous pressures including poor nutrition, smoking, stress, drug exposure and antibiotic exposure (*Gohir et al., 2015*; *Falony et al., 2016*; *Wen and Duffy, 2017*). Consequently, lifestyle adjustment and maybe dietary supplements could be proposed to help women with infertility problems, to promote both pregnancy outcomes and newborn health. Future research should concentrate on the role of these external factors to further understand their impact on the microbiota.

The use of probiotics to treat atypical vaginal microbiota is being studied

In recent years many probiotic oral supplements have appeared on the market, with increased evidence of their efficacy, mostly to treat gastrointestinal conditions (*Ford et al., 2014*). Several oral and vaginal probiotics have been developed to help women restore healthy vaginal microbiota.

Back in 1992, a pioneering study reported that a daily oral dose of yogurt enriched with *Lactobacillus acidophilus* was effective in treating recurrent vulvovaginal candidal infections after 6 months of treatment (*Hilton et al., 1992*). Since then, many studies have investigated the role of *Lactobacillus* spp. supplementation in restoring the vaginal microbiota (*Falagas et al., 2007*). Products are mostly formulated with various combinations of *Lactobacillaceae*, including *L. crispatus*, *L. gasseri*, *L. plantarum*, *L. reuteri* and *L. rhamnosus*. The presence of *Lactobacillus* spp. is a recognized characteristic of a healthy vaginal microbiota (*García-Velasco et al., 2017*), while a decrease in their abundance is often associated with altered pH and dysbiosis, bacterial vaginosis or vulnerability to pathogens (*Ravel et al., 2011*). Several studies confirm the beneficial role of oral or vaginal probiotic supplementation in achieving and maintaining a healthy microbiota (*Anukam et al., 2006*; *Homayouni et al., 2014*; *Reid et al., 2001*, *2003*). Others question probiotic efficacy, albeit confirming the absence of any adverse effect related to their use (*Barrons and Tassone, 2008*; *Buggio et al., 2019*).

There is consensus on the efficacy of vaginal probiotics when used in addition to antibiotic therapy. *Lactobacillus* spp. supplementation has proven effective in the prevention of recurrent infections after standard metronidazole therapy (*Menard, 2011*). Antibiotics are not species-specific, so while they fight pathogens, they also decrease *Lactobacilli* and other commensal bacteria, thus resulting in a depletion of healthy bacteria in the vaginal microbiota (*Macklaim et al., 2015*). The concomitant use of antibiotics and probiotics might be the optimal therapy to fight pathogens and correctly repopulate the vaginal microbiota (*Moreno and Simon, 2019*).

As previously discussed, bacterial vaginosis and unbalanced microbiota can reduce success in fertility treatment. Research on the efficacy of supplements in restoring vaginal health is quite new and has evolved quickly over the past few years as a result of next-generation techniques (*Campisciano et al., 2017*). There is yet to be consensus on the efficacy of probiotics (*Anukam et al., 2006*; *Barrons and Tassone, 2008*; *Buggio et al., 2019*; *Homayouni et al., 2014*; *Reid et al., 2001*, *2003*), and conflicting results may be due to probiotic strain choice, or the posology adopted to treat specific conditions. To date, available data on probiotics to support infertility treatments are insufficient but promising (*García-Velasco et al., 2017*). A future challenge is to confirm probiotic activity and to precisely characterize the specific strains with the aim of creating therapies effective in infertile women.

The use of probiotics to prevent preterm birth is supported by a large body of evidence (*Kirihara et al., 2018*; *Vitali et al., 2012*). *Kirihara et al. (2018)* reported that a combination of probiotics (*Streptococcus faecalis*, *Clostridium butyricum* and *Bacillus mesentericus*) successfully improved perinatal outcome. A total of 121 women with high risk of preterm delivery were divided into two groups, one of which received the probiotics. The women receiving probiotics (n = 45) had longer gestations: only one woman (2%) delivered before 32 weeks, while in the control group 19 women (25%) delivered before 32 weeks. Overall, gestation was 36 weeks on average in the probiotic group and 34 weeks on average in the control group. This work shows the beneficial effects of probiotics despite the intrinsic limits of retrospective studies and potential confounding factors. In another study, women receiving an oral, eight-strain probiotic containing a mixture of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* strains in the last 3 months of pregnancy had modulated vaginal microbiota and an increase in anti-inflammatory cytokines (*Vitali et al., 2012*). Given that inflammation is a major cause of preterm birth (*Romero et al., 2014*), it is possible that reducing vaginal inflammation could have implications in preventing preterm birth.

Microbiota profiling should be used in a standardized manner (collection, analysis, statistics)

The boundaries of the healthy reproductive tract microbiota must be clearly defined before the relationship with fertility can be established. To date we have some important insights, but data are insufficient to definitively characterize healthy versus dysbiotic status. The first step towards this aim is to standardize protocols, sampling methods and sizes, sequencing techniques and bioinformatic pipelines (*Koedooder et al., 2019a*). This will provide comparable data and possibly lead to general conclusions that can be used as a basis for effective therapies (*Koedooder et al., 2019a*; *Watson and Reid, 2018*).

Prospective studies (randomized, controlled, with large patient numbers) need to be standardized in collection, laboratory techniques and statistical analyses in order to study the microbiota and its relation to fertility outcomes

A healthy reproductive tract microbiota is correlated to fertility and to positive fertility treatment outcomes. The research is rapidly growing, but many issues remain unexplored or controversial. Systematic reviews show that studies are difficult to compare because of varying cohort demographics, inclusion criteria, sample size, laboratory techniques, etc. (*Fettweis et al., 2019*; *Peelen et al., 2019*). *Peelen et al. (2019)* propose a database of essential and desirable items relating to quality, method and topic to overcome the low quality of information relating the vaginal microbiota. Improved study design and sampling strategies are needed to standardize research and establish causal relationships between microbiota and adverse outcomes.

For research purposes one might consider assessing the microbiome in all fertility patients to gain more insight into pathophysiology and outcomes

Distinguishing a commensal microbiota from a pathophysiologic microbiota without altering standard clinical practice is an important step to better understand the reproductive microbiota. *Franasiak et al. (2016)* characterized the endometrial microbiota without altering standard clinical practice. At time of embryo transfer, the 5 mm distal part of the catheter was sterilely placed in a PCR tube and analysed by next-generation sequencing (NGS). The results of this study were not statistically significant, but they suggest a direction for future studies and propose a way to analyse the microbiota with very limited starting material and without changes to standard clinical practice. These techniques could be applied to all fertility patients and provide insight into pathophysiology.

NGS and other genetic testing methods are useful in research. More evidence is required to support routine use in diagnosing infertility, preterm birth and recurrent pregnancy loss

As the microbiota plays a role in infertility, recurrent pregnancy loss and preterm birth it is logical to consider it as a counterpart in infertility treatment. The questions are multiple. How do we define a healthy microbiome? Which analyses should we use? How do we correctly interpret the results? NGS has increased the resolution and accelerated the research, allowing full complex microbiota communities to be monitored. Recent studies show that small changes in vaginal microbiota can greatly affect fertility, therefore we need more specific tests in order to create accurate therapeutic protocols for infertility treatments (*Campisciano et al., 2017*). NGS has detected a new bacterium, *Atopobium vagina*, highly represented in the vaginal microbiota of women suffering from idiopathic infertility. With a less specific analysis, the Nugent score, no difference was detected between fertile and infertile women (*Campisciano et al., 2017*). This result demonstrates that we have only touched the surface and we must fully exploit new technologies to deepen the knowledge. Moreover, as we continue to move from research to clinical practice, this analysis is required in order to improve ART success rates (*Haahr et al., 2019*; *Koedooder et al., 2019a*; *Moreno et al., 2016*).

Other genetic testing methods such as qPCR, phylogenetic microarrays (e.g. V-Chip) (*Paily and Agans, 2011*) and molecular fingerprinting (e.g. IS-pro™) (*Koedooder et al., 2019b*), have proved useful in microbiome profiling. There is an inherent trade-off to these techniques, forcing a choice between the broadness of the profiling or the resolution and sensitivity of the profiling. Metagenomic sequencing offers broad profiling, with very high definition in the case of shotgun metagenomic sequencing, however low-abundance species will be missed. IS-pro™ is, similarly, a broad-spectrum molecular technique that depicts the relative composition of the dominant fraction of the microbiota. As discussed in Part 1, an algorithm using the IS-pro™ technique was successfully used for IVF failure prediction (*Koedooder et al., 2019b*). Phylogenetic microarrays contain probes that target specific species. It therefore offers fast results and high resolution but for a predetermined panel of micro-organisms of interest. qPCR is the technique most appropriate for low biomass specimens or to detect low-abundance species in a community (*Haahr et al., 2019*). qPCR assays are cumbersome if the panel of interest is extensive, but allow assessment of quantitatively specific elements of the microbiota at any level of resolution (from strain to full bacterial content) and will even detect bacteria that are present in very small amounts and that are difficult to cultivate (*Cartwright et al., 2012*), which could be important for the early detection of microbiota perturbations.

Perhaps in the future, NGS and other molecular techniques could be used prognostically for counselling to optimize the time of embryo transfer

Embryo transfer is a crucial step in fertility treatments and can fail for many reasons including uterine anatomy, immunological factors or embryo genetics. The reproductive tract microbiota is one variable that has recently been associated with embryo transfer failure. Several studies concentrate on the vaginal and endometrial microbiota to confirm their role in embryo transfer, and to better characterize the bacteria involved (*Cicinelli et al., 2015*; *Koedooder et al., 2019b*). The introduction of NGS and molecular techniques allows the characterization of previously non-culturable bacteria in altered microbiota (*Lamont et al., 2011*). These findings can boost our knowledge about the microbiota and provide a new perspective on which bacterial strains are involved in embryo transfer failure. Some preliminary studies show that molecular tests have a high sensitivity in detecting bacterial vaginosis: ATRiDA® (sensitivity 96.9%, specificity 70.2%) and AmpliSens® (sensitivity 80.6%, specificity >90%) (*van den Munckhof et al., 2019*; *van der Veer et al., 2018*). On the basis of new findings, future researchers should aim to incorporate diagnostic tools into clinical practice with the goal of optimizing the time of embryo transfer to maximize the success rate.

DISCUSSION

A more complete understanding of the role of the reproductive microbiome promises improvements to fertility treatments and a more nuanced understanding of reproductive health as a whole. The microbiome is the new frontier; the currently accumulated knowledge will propel the discovery of new treatment solutions and protocols for superior fertility outcomes. Although the field is new, there are already ways to utilise what is known to improve clinical practice and achieve better reproductive outcomes. Current and future research in large clinical cohorts should uncover more ways in which the microbiota can be modulated pre-conception and during pregnancy in order to minimize pregnancy loss and preterm birth and maximize implantation, pregnancy rate and at-term delivery of healthy babies.

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