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Female reproduction and the microbiota in mammals: Where are we?

Rosa M. Garcia-Garcia [a, *], María Arias-Álvarez [b], Daniela Jordán-Rodríguez [a, b], Pilar G. Rebollar [c], Pedro L. Lorenzo [a], Carmen Herranz [d], Juan Miguel Rodríguez [d]

[a] Department of Physiology, Veterinary Faculty, Complutense University of Madrid, Madrid, Spain
[b] Department of Animal Production, Veterinary Faculty, Complutense University of Madrid, Madrid, Spain
[c] Department of Agrarian Production, Technical School of Agronomic, Alimentary and Biosystems Engineering, Polytechnical University of Madrid, Madrid, Spain
[d] Department of Nutrition and Food Science, Veterinary Faculty, Complutense University of Madrid, Madrid, Spain

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ABSTRACT

While it is generally accepted that the mammalian vagina contains a site-specific microbiota that plays relevant roles in genital and reproductive health, the existence of an extra-vaginal microbiota in the female reproductive tract (i.e. follicular fluid, oviduct, endometrium, and placenta) is, at least, a matter of controversy. Many conclusions in this field have failed to consider the technical limitations, biases, and confounding factors inherent to next-generation sequencing (NGS) approaches. While this creates uncertainty in the field, there is no doubt this subject is set to be the focus of new research efforts because of its scientific and practical connotations in female reproductive health. The current art state, its limitations, and gaps in our knowledge about the female reproductive tract's microbiota and, particularly, about the microbes of the extra-vaginal environment are presented in this review. Also are discussed possible relationships between the gut and oral microbiota and reproductive events.

1. Introduction

Animals are holobionts or “superorganisms” that comprise not only the cells of the respective host species but, also, those of the microorganisms that inhabit their skin and mucosal surfaces collectively referred to as the “microbiota”. “Microbiome” is a closely related term for which there is yet no consensus definition and some authors consider it a synonym of microbiota. As an example, The Human Microbiome Project (HMP) defines the human microbiome as the collection of all the microorganisms, including eukaryotes, archaea, bacteria, and viruses, living in association with the human body. However, other authors define this term as the total genomic material contained by the microbiota, while others include their metabolites [1]. Many other definitions of “microbiome” have been proposed on the basis of different ecological, host and methodological perspectives [2]. While both terms are often interchangeably used, hereafter we will only use "microbiome" to specifically refer to the collective genomes of the microorganisms that reside in a host. In the past few decades, it has become evident that the microbiota is essential for the host, with roles such as the programming and regulating of metabolism, and neuroendocrine and immune functions [3,4]. Hence, the correct acquisition of the microbiota in early life and its changes throughout life have pronounced, both short- and long-term, impacts on the host's health [5]. Further, any imbalance in the composition of the microbiota (known as dysbiosis) disrupts homeostasis, which induces an inflammatory response and alters the physiological metabolic profile of an organism [6].

The study of the microbiota has relied mainly on culture-based procedures, from classic techniques and methods to the use of new substrates and growth conditions as the so-called “culturomics” This term is defined as the diversification of culture conditions, combined with identification by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF) to increase the cultivable bacterial repertoire of a biological sample. In such approaches, the demonstration of microbial viability is essential. In contrast, the culture-independent procedures (including those using next-generation sequencing [NGS] platforms) used up until now are limited to detecting microbial nucleic acid sequences and, mostly applied to the prokaryotic components of the microbiota. In this context, microbial DNA has been identified in many mammalian host locations that were traditionally thought to be sterile,

* Corresponding author.
E-mail address: rosa.garcia@vet.ucm.es (R.M. Garcia-Garcia).

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including virtually all portions of the female and male reproductive tracts [7]. This has led to coining the term “reproductive microbiome”.

It has been well established that the mammalian vagina contains a site-specific microbiota that plays relevant roles in genital and reproductive health, but the existence of an extra-vaginal microbiota in the female reproductive tract is, at least, a subject of passionate debate. Many conclusions have been drawn without considering the technical limitations and biases inherent to the NGS approach. However, given its important implications, this topic is set to become a focus of growing research. In this review, we present the current art state, limitations and gaps in our knowledge of the female reproductive microbiota with special emphasis on the microorganisms of the extra-vaginal genital tract and discuss the relationship between reproductive events and the role of the microbiotas of other body regions.

Most recent microbiota studies of the female reproductive tract have focused on the human species. Many of these studies have provided a mere description of the main bacterial phyla and genera according to the DNA found in female genital tract samples. Although some reports considering other animal species have been published in the past decade [8–17], there is a need for more human and animal studies designed to gain insight into the changing composition and, particularly, the functional roles associated with such microbiota. This type of information will have considerable implications for improving female health and reproductive success.

2. Cervico-vaginal microbiota and reproductive health

It has been long known that the cervix and vaginal epithelium contains a site-specific microbiota [18], whose composition may vary depending on several factors (species, age, stage in the estrous or menstrual cycle, diseases, hormone treatments, stress, etc.) [18–23]. The human epithelial vaginal microbiota is dominated by bacteria of the genus *Lactobacillus* (with an abundance usually >70%). This dominance appears to be unique to humans, its relative abundance in other mammals (including non-human primates) is being much lower (abundance typically <1%) [21]. Consistently, the human vaginal pH is usually notably lower (median vaginal pH ~4.5) than that of non-human mammals (5.4–7.8). This pH reaches its lowest value during the period of highest estrogen levels [21], as estrogen stimulates glycogen production by exfoliated and lysed epithelial cells of the vagina [24]. Glycogen can be catabolized into smaller polymers and, subsequently metabolized by *Lactobacillus* spp. to lactic acid [25], which is responsible for the low vaginal pH [26]. Further, the adhesion of lactobacilli to the vaginal mucosa contributes to the physiology of the vaginal environment, through protection against invading pathogens [24].

However, the risks of suffering genital tract infections or obstetric problems in humans do not seem to be greater compared to other mammals [21]. In other words, vaginal pH and the relative abundance of lactobacilli across mammals do not seem to correlate with a higher or lower rate of such incidences. In non-human mammals, the protective functions of lactobacilli and a low pH seem to be provided by other bacteria and mechanisms, including species-specific interactions with the host immune system [27–29]. Such interactions between commensal vaginal bacteria and host immunity have yet to be comprehensively investigated in animals. For example, it has been suggested that differences in the composition of the vaginal and endometrial microbiota between healthy and endometritis-suffering donkeys may be associated with different immunological responses, yet this potential relationship was not investigated [30].

Overall, the common function of the vaginal microbiota in humans and other mammals seems to optimize reproductive outcomes, through protection against infections and the provision of immunological fitness, which are essential for endometrial health, fertility, embryo implantation and pregnancy success [18,31–36]. Some bacterial metabolites found in the human vagina, like glycerophospholipids and benzopyrene, have been positively correlated with lactobacillus abundance and associated with a lower rate of recurrent implantation failure [37]. Glycerophospholipids are precursors of arachidonic acid, which are important for the biosynthesis of prostaglandins (PGs) and, thus, participate in the implantation process [38,39]. Benzopyrene is a Cyclooxygenase 2 (COX-2)-inhibitor that regulates PG levels and may also positively correlate with embryo implantation [37]. In addition, it has been speculated that the mucus-associated vaginal microbiota may play a role in female-male chemical communication via pheromone production [15,16,40] involving the metabolism of endogenous organic compounds, like volatile acids, or the synthesis of odor compounds both in wild [41,42] and livestock animals [15,43].

Finally, the vagina is exposed to several external factors that presumably can affect the microbiota at this site, such as the sperm which harbors its specific microbiota [44–46]. In addition, some devices used frequently for managing reproduction may also transfer microorganisms like hormone intravaginal contraceptives in women [47], intravaginal progestagen devices for estrus synchronization in ruminants [13,48], tip catheters for artificial insemination [49], or the speculum or ultrasound probes. This transfer of microorganisms will serve to establish interactions between endogenous and exogenous microbiota and this may modify the vaginal microbiota [50]. The significance of this transfer of microorganisms for reproductive health, however, remains unclear.

As a conclusion, vaginal dysbiosis has been repeatedly associated with endometrial status, infertility or poor pregnancy outcomes in all mammalian species studied so far [33,37,51–56]. Therefore, identifying microorganisms on the cervico-vaginal mucosal surface may help to effectively improve insemination and pregnancy outcomes [15] as well as predict the success of assisted reproductive technologies (ART) [57–60].

3. The “extra-vaginal” reproductive microbiota: a controversial issue

The notion that the human fetal environment is sterile under physiological conditions ("the sterile womb paradigm") has been accepted for decades. According to this theory, microbial colonization of the intestinal tract of healthy newborn begins during and after birth, both by vertical transmission (from the maternal microbiota) and horizontal transmission (from other sources). In contrast, many recent studies (mostly based on the use of culture-independent techniques) have challenged this traditional theory and proposed that microbiota acquisition begins in the uterus. However, while it is possible that not all healthy creatures are born sterile as previously assumed, it is also true that the findings of studies supporting the "*in utero* colonization hypothesis" should be considered with caution, as most of these studies have some methodological limitations.

Bacterial DNA can belong to living or dead organisms and few studies have confirmed the presence of viable microorganisms in the endometrial, placental or fetal environments. Even in such cases, only low concentrations of few bacterial species have been detected in a small percentage of samples. As an example, only 26% of samples from human placentas without signs of chorioamnionitis were found to carry viable bacteria and, again, only at low concentrations [61]. Hence, while some placental samples may not be completely sterile, this does not mean they contain a true well-organized site-specific microbiota.

It has also been argued that the use of molecular techniques for the study of low-abundance microbiotas is highly susceptible to false positives due to contamination. Accordingly, it is difficult to avoid contamination when collecting samples from the intrauterine environment within a clinical setting. Further, the presence of contaminating DNA in PCR reagents, DNA extraction kits, and molecular-grade water is a challenge when working with samples containing a low microbial biomass, such as those obtained from the placenta, amniotic fluid or meconium in healthy individuals [62,63]. This can lead to erroneous results and conclusions, which may quickly become “general knowledge” because of today's rapid spread of scientific findings, especially in a field as sensitive as human ART [64]. In effect, it has been shown that the lower the amount of bacterial DNA in a sample, the higher the proportion of sequences that can be attributed to reagent contamination. It should thus be noted, that only 0.002 mg of bacterial DNA can be extracted from 1 g of healthy placental tissue [65].

While the presence of contaminating DNA in molecular biology reagents has been reported in the scientific literature, its impact on the metataxonomic and metagenomic analysis of samples with a low microbial biomass has hardly been considered to date. Most publications on the human microbiota do not include negative controls and/or do not describe procedures for the removal or identification of contaminating DNA. Contaminant DNA sequences typically match water- and soil-associated bacterial genera, including *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Bradyrhizobium*, *Herbaspirillum*, *Legionella*, *Leifsonia*, *Mesorhizobium*, *Methylobacterium*, *Microbacterium*, *Novosphingobium*, *Pseudomonas*, *Ralstonia*, *Sphingomonas*, *Stenotrophomonas*, or *Xanthomonas*. Interestingly, a high percentage of the total taxa considered as "placental microbiota" in a highly publicized article [65] overlaps with those cited above. In fact, a metataxonomic study compared the microbiota of placental samples and that of the reagents used to extract microbial DNA, and no clear separation between the two types of samples was observed [66]. More recently, no scientific evidence could be found for a placental microbiota in terms of effects on human pregnancies [67] or complications during pregnancy [68].

The presence of microbes in meconium and amniotic fluid is often considered as evidence supporting the *in utero* colonization hypothesis. However, it has been argued that only a relatively small subset of such samples contains detectable (i.e. visualized or isolated) microorganisms, and that they could be, at least in part, the result of postnatal colonization in the case of meconium samples, or of the rupture of membranes prior to delivery in the case of amniotic fluid samples [69,70].

Finally, some authors propose that gnotobiology offers the most robust evidence against the presence of microbiotas in the fetal environment as, using this approach, it is possible to obtain "germ-free" animals by cesarean section and subsequently rear their offspring in a sterile environment [70]. Notwithstanding, it is also true that it is difficult -if not impossible-to transfer the results obtained in "germ-free" systems to the same events when they occur in a conventional host [7,71].

In conclusion, some authors have criticized the *in utero* colonization hypothesis as many of the studies examining this issue have used inadequate molecular approaches to study "low biomass" microbial populations, lacked appropriate controls and/or did not show bacterial viability [66,70]. However, as stated above, some studies have unambiguously shown the presence of live bacteria in fetal samples from healthy mothers [61]. Although skeptics of *in utero* colonization have attributed this to subclinical conditions, the results of such studies also indicate that fetal colonization can occur, at least occasionally, and that this issue and its relationship with reproductive health merits further investigation.

Understanding the role of still hypothetical extra-vaginal reproductive microbiotas, especially in the first stages of oocyte maturation, embryo and fetal development, and offspring colonization, is essential because of impacts on reproductive health, pregnancy outcomes, and health later in life. Here, we critically review the state of the art of extra-vaginal reproductive microbiota based on current data, although more robust research is needed to define the structure and roles of this reproductive microbiota in reproductive outcomes.

3.1. Follicular fluid (FF) microbiota

The environment in which the oocyte develops is crucial to its subsequent quality and ability to be fertilized. Data regarding the existence of a microbiota at this site have been conflictive. Not only have some authors been able to detect both bacterial nucleic acids (*Lactobacillus* spp., *Actinomyces* spp., and *Cutibacterium* spp.) and cells in human FF but also described have been differences between the right and left ovaries of the same host [72–75]. In contrast, a recent well-controlled study failed to detect specific microbiotas in ovarian cystic fluid regardless of the presence or absence of endometriosis and the type of cyst [76].

Although the actual existence of an FF-related microbiota needs to be confirmed, its composition has been effectively linked to pregnancy outcomes. Positive correlation between the presence of *Lactobacillus* spp. in the FF and pregnancy rate after *in vitro* fertilization (IVF) and embryo transfer was found both in healthy women and in those with fertility problems [72]. This effect might be due to the production of flavonoids and inhibition of host eicosanoid secretions by lactobacilli, which reduces oxidative stress and inflammation, and stimulates oocyte competence [77]. However, the presence of certain microorganisms in the FF can compromise oocyte quality [73]. Indeed, the *in vitro* co-culture of mouse oocytes with low levels of *Cutibacterium acnes* or *Lactobacillus gasseri* previously isolated from FF caused oocyte DNA fragmentation, which could be a reason for the poor success of IVF [72,78]. Previously, it was observed that the bacteria present in porcine FF were also detrimental for oocyte maturation owing to the secretion of a metabolite interfering the binding of follicle-stimulating hormone (FSH) to its receptor [79]. However, such bacteria were not identified so additional studies are required to confirm this observation and determine which bacterial species may be involved in this negative outcome.

Oocyte development is impaired when uterine infection occurs because of activation of intracellular signaling cascades such as the mitogen-activated protein kinase (MAPK) pathway, stimulating Toll-like receptor 4 (TLR4) and other cytokines like tumor necrosis factor α (TNF-α), interleukins IL-1β, and IL-6 via the extracellular signal-regulated kinase/nuclear factor-κB (ERK/NF-κB) pathway [80,81]. Excessive stimulation of these pathways can initiate an inflammatory response by inducing the release of inflammatory mediators in FF, and increasing oxidative stress in the follicular environment [82], which impairs oocyte development. Lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria have been also associated with other infections of the female genital tract such as endometritis [80,83].

The presence of LPS in the FF induces an increase in follicular apoptosis and atresia [84], probably because LPS inhibits follicular activity by suppressing the transcription of steroidogenic enzymes, such as P450 and CYP17 [85], and beta-catenin [86]. When LPS is administered to cows by subcutaneous injection, the FF level of estradiol increases, and in parallel, the FF concentration of LPS increases in the dominant follicles [86]. However, LPS reduces the progesterone response to follicle stimulant hormone (FSH) in rats [84]. In addition, LPS *per se* affects oocyte maturation competence [83,87], disturbing events like meiotic resumption and cytoplasmic

maturation, cytoskeletal dynamics, and oxidative stress [83], and produces modifications in epigenetic marks of oocytes in cows [88] and sheep [89]. Embryo development is also affected, and blastocyst rates and the number of cells at the trophoblast stage are diminished [83], even in oocytes obtained from heifers challenged *in vivo* with LPS and *in vitro* matured (IVM), IVF, and *in vitro* cultured (IVC) [87].

Some speculations could be made about the effects of metabolites released by the bacteria found in the FF. As an example, high FF concentrations of hyaluronan have been associated with high levels of apoptosis in granulosa cells [90]. Some of the bacteria isolated from human FF, like *Cutibacterium* spp., *Streptococcus* spp., and *Escherichia coli*, are hyaluronidase-producing species and have been associated with a poor quality of cumulus cells [91]. Another hypothesis is that Gram-negative bacteria might release extracellular vesicles (EVs) containing immunomodulatory compounds involved in cell-to-cell communication [92]. If these EVs are released in the FF, they might act as a signal for communication with follicular cells. Indeed, it is known that exosomes containing bioactive molecules that potentially improve follicular and embryo development are present in FF [93,94]. More studies focusing on FF-related microbes are required to shed light on their relationship with follicle development, oocyte maturation and reproductive outcomes.

3.2. The oviductal microbiota

Important events such as sperm capacitation, fertilization, and early embryo development take place in the oviduct within a complex signaling cascade involving Janus kinase/signal transducer and activator of transcription (JAK/STAT) and ERK pathways [95,96]. The information available about the microorganisms that may inhabit or transit the oviduct is scarce, although interesting interactions between gametes and non-pathogenic oviductal bacteria have been proposed. Although this review does not deal with the microbiota of the male reproductive system, we should mention that semen usually contains a rich and diverse microbiota [45,97–99]. Consequently, fertilization may not occur under sterile conditions [100]. In fact, the bacterial communities that have been described in the human oviduct seem to reflect those that are particularly abundant in human semen (e.g., *Staphylococcus* spp., *Enterococcus* spp., *Cutibacterium* spp.) or in the human vagina (*Lactobacillus* spp.) [101]. In addition, bacterial profiles appear to change between the fimbria and the proximal oviduct [102], the isthmus and ampulla, and right and left oviduct [101], but no correlation with ovarian follicular or luteal status has been detected so far [101]. Additionally, the oviductal microbiota can be affected by factors such as hormone treatments or menopause [101,102]. Further studies are required to establish the reproductive roles of oviductal microorganisms in humans and other mammalian species.

3.3. The endometrial microbiota

The uterus plays a key role in implantation and fetal development, two functions in which the involvement of the endometrial immune system is particularly relevant. Recently, several authors have claimed that the uterus harbors a site-specific microbiota, which seems to be different from that existing in the vagina [29,53,58,103]. Overall, the endometrial microbiota displays a higher bacterial diversity and richness compared to the vagina and cervix in a variety of animal species, including domestic cattle, dogs, giant pandas, horses and humans [28,53,58]. It should be underscored that the bacterial DNA load decreases from the vagina upwards [104]. In humans, this load is approximately 10,000 times lower in uterine or oviduct samples than in vaginal samples [105].

Most of these studies, however, have not provided data showing the presence of viable microbes in the endometrial tissue of healthy hosts [28]. In most, endometrial-related samples were collected through the vagina, which facilitates cross-contamination even when using devices specifically designed for endometrial sampling. As a result, many studies have found a high abundance of lactobacilli sequences, which seems difficult to explain when considering the endometrial mucosa's characteristics (e.g., pH).

Several routes and sources have been postulated so far to explain the origin of the bacteria that hypothetically reach the uterine environment. First, it has been suggested that some of them may arise from the bloodstream after the physiological translocation of commensal bacteria from the oral cavity or the gastrointestinal tract [106]. The bloodstream has also been described as a route of transmission of uterine pathogens from the gut to the uterus in cows [107]. Other bacteria may arise from the vagina and could reach the endometrial mucosa by ascending through the cervix. In effect, a recent study has suggested that the translocation of vaginal microbes may be involved in either impairment or protection of the uterine environment, depending on the microorganisms translocated [103]. These two ways have also been proposed for the presence of microorganisms in the oviduct [106]. Contaminations arising from any device, such as those used in ART (i.e. catheter tips for embryo transfer) or intravaginal contraceptives, have also been incriminated as minor or occasional sources of microorganisms. However, the lack of studies investigating the role of semen as a major source of microbes for the female reproductive tract is surprising, despite the fact that, as stated above, this biological fluid contains a rich microbiota [44–46] and has a high potential to influence female genital health [108].

A hypothetical uterine microbiota has recently attracted the attention of scientists, not only because of its potential role in human fertility but, also, because of its possible role as a driver of metritis and other uterine diseases, leading to infertility mainly in cows and to serious consequences for the dairy industry [8,9,11,23]. Endometrial microbiota may play a role in modulating the immune response when the developing embryo implants [109], and in pregnancy maintenance through participation in maternal immune and metabolic responses [50]. However, the association between endometrial microbiota and reproductive outcomes is still controversial because of the current lack of solid scientific evidence, the low number of recruited subjects in the studies published so far, and/or the publication of studies showing conflicting results. As an example, while differences in the uterine microbiota between women who became pregnant and those who did not after embryo transfer have not been found in several studies [110,111], others have suggested the existence of specific uterine microbiota profiles associated with a better reproductive response in terms of implantation and pregnancy rates [112]. Further, metritis is a frequent postpartum disease and significant changes in the profile of the uterine microbiota have been detected between healthy females and those with endometritis in several species, including humans [113], cattle [8] and equines, including donkeys [30] and horses [114]. It seems that uterine bacterial diversity declines with time postpartum in healthy cows while it is lower in cows with metritis [11,115], and is characterized by increases in the relative abundance of the genera *Porphyromonas*, *Bacteroides,* and *Fusobacterium*, and a decrease in the abundance of *Clostridium sensu stricto* 1 [23].

The microbiota of different body locations (oral cavity, gut) changes during pregnancy and the post-partum period [116]. However, to our knowledge and because of ethical and technical constraints, to date no study has assessed possible shifts in the endometrial microbiota throughout pregnancy. The microbiota may, nevertheless, play an important role in local interactions

between the embryo and endometrium during implantation, placentation and embryonic growth [117]. Such interactions could be driven by hormonal changes [40,50] and the immune environment [116] during this critical period. For example, it has been proposed that the commensal microbiota may contribute to remodeling events (i.e. angiogenesis, cell differentiation, etc.) that the endometrium undergoes to reach an embryo-receptive state [49]. In addition, the vaginal microbiota changes from the first to the third trimester of pregnancy, when it attains its lowest bacterial diversity and richness [118], suggesting a similar shift in the endometrial microbiota. In effect, reports already exist of changes in the relative abundance of some phyla from days 2 to 4–14 postpartum in endometrial samples from cattle [11].

The bacterial-induced production of endometrial inflammatory cytokines, such as IL6, at the implantation time, is thought to be an important event for pregnancy success in cows [77]. Further, some bacterial metabolites affect implantation and early embryonic development in the uterus. LPS has a negative impact on the implantation rate and placentation in mice through DNA damage and modification of the local immune environment [119] causing embryo loss [120]. In contrast, indole-3-lactic acid is an aryl hydrocarbon receptor (AHR) agonist, which beneficially modulates the immune response and reduces inflammation, promoting pregnancy in cows [77]. The production of low concentrations of H_2O_2 by bacterial species of the genera *Lactobacillus*, *Streptococcus* or *Enterococcus* at the site of placental attachment in dairy cattle has been proposed to increase the production of transcription factors, promoting angiogenesis and increasing the likelihood of a successful pregnancy [77]. However, it must be remembered that the healthy uterus is an anaerobic environment, and, under such conditions, bacteria cannot produce H_2O_2. Nevertheless, characterizing the uterine microbiota and their metabolites in healthy hosts is a key strategy in the search for biomarkers of implantation success and favorable pregnancy outcomes.

4. The reproductive microbiota: beyond the female genital tract

While the existence and relevance of an extra-vaginal microbiota in the female reproductive tract still needs to be clarified, it is generally recognized that the digestive microbiota (including the oral and gut microbiota) has a considerable impact on pregnancy outcomes and offspring development through a variety of mechanisms that span from immune modulation to hormone metabolism [42,121–123].

The gut microbiota is essential for the regulation of host metabolism, immunomodulation and neuroendocrine modulation, and also for the control of gut pathogens that may colonize the female genital tract. These functions are themselves closely interrelated and interconnected with reproductive physiology (Fig. 1).

The intestinal microbiota regulates several hormones such as ghrelin, leptin, glucagon-like peptide 1 or peptide tyrosine tyrosine (PYY), which play relevant metabolic roles in the organism [124–126]. These hormones modulate appetite and food intake, body fat storage, lipogenesis, fatty acid oxidation and body weight [124,127,128]. These processes are essential during gestation and thus may impact conception and pregnancy success.

In the past few years it has become evident that the gut microbiota is one of the principal regulators of circulating estrogens through the secretion of β-glucuronidase and other enzymes (the so-called “estrobolome”) [129]. Dysbiosis of the gut microbiota impairs this process, reducing the deconjugation of estrogens and their circulating levels. Gut dysbiosis contributes to the development of several conditions including endometrial hyperplasia, endometriosis, polycystic ovary syndrome (PCOS), and infertility [130,131]. The gut microbiota also regulates the production of circulating progesterone, serotonin and glucocorticoids. However, while the gut–ovary axis emerges as a new concept, so far few studies have attempted to link reproductive hormones to the gut microbiota [129,132]. Such studies have linked the gut microbiota: to fluctuating levels of progestogens and glucocorticoids during estrous stages and pregnancy in the rhinoceros [133]; to increasing serotonin and melatonin levels in the presence of dietary fiber diminishing ovarian follicular atresia in the pig [132]; and to secretion of the enzyme β-glucuronidase which metabolizes estrogens via their conjugation and deconjugation in humans [129,134].

Interestingly, changes also occur in the gut microbiota during pregnancy [135]. In women, these changes have been detected between the first and last trimester. Such a modification of the gut microbial profile is one of the main driving forces of the physiological metabolic and immunological changes that occur in pregnancy, preparing the mother for the energy and nutrient demands required for a successful pregnancy and lactation [136]. The transfer of feces obtained from pregnant mice in their last trimester of gestation to normoweight germ-free mice leads to increased adiposity, inflammation, and hyperglycemia, which are characteristic features of late pregnancy [136]. In contrast, the transfer of feces obtained from pregnant mice in early pregnancy had no impact on recipient mice. In turn, the high availability of glucose during late pregnancy is able to readjust the expression of carbohydrate-related functions by gut microbiota [137].

More recently, differences have been described in the gut microbiota between healthy and infertile women [138], although the implications of such changes remain unknown. Some studies have also shown a potential link between the pathophysiology of PCOS and gut dysbiosis [139,140].

Several studies have also confirmed significant changes in oral microbiota composition and structure between non-pregnant and pregnant fertile women and, also, throughout pregnancy [135,141–143]. These changes seem to be hormone-dependent and have been associated with the high susceptibility of pregnant women to gingivitis [144]. Gingivitis in pregnant women with good oral health before becoming pregnant is usually a transient estrogen-driven problem unrelated to an increased risk of an adverse pregnancy outcome.

On the contrary, periodontal disease is usually unrelated to the hormone changes that characterize pregnancy and leads to an increase in the abundance of some bacterial pathobionts (e.g., *Porphyromonas gingivalis*, *Fusobacterium nucleatum)* [145–147]. Eventually, such microbes may translocate through ulcerated periodontal pockets in the gum epithelium, reach the bloodstream and then spread to distant organs (liver, placenta) where they may lead to a wide range of adverse pregnancy outcomes, including preeclampsia, pre-term birth, low birth weight, stillbirth or miscarriage [148–152]. The first evidence of oral-uterine translocation was provided by Han et al. [153], who found identical bacterial clones in the amniotic fluid of a woman undergoing a preterm delivery and her subgingival plaque. These findings have been confirmed in other cases of preterm delivery or neonatal sepsis [146,147]. In animal models, the negative influence of oral dysbiosis caused by periodontal pathogens was found to induce a maternal immunoinflammatory response. This involved the exacerbated expression of cytokines in the placenta leading to preterm birth and low birth rates in mice [154] and rats [155]. However, so far no investigation has linked the oral microbiota to reproductive parameters in farm animals.

As a conclusion, the possibility of modulating the oral and gut microbiota before or during pregnancy represents an unexplored new line of research in mammalian reproduction. Findings to date

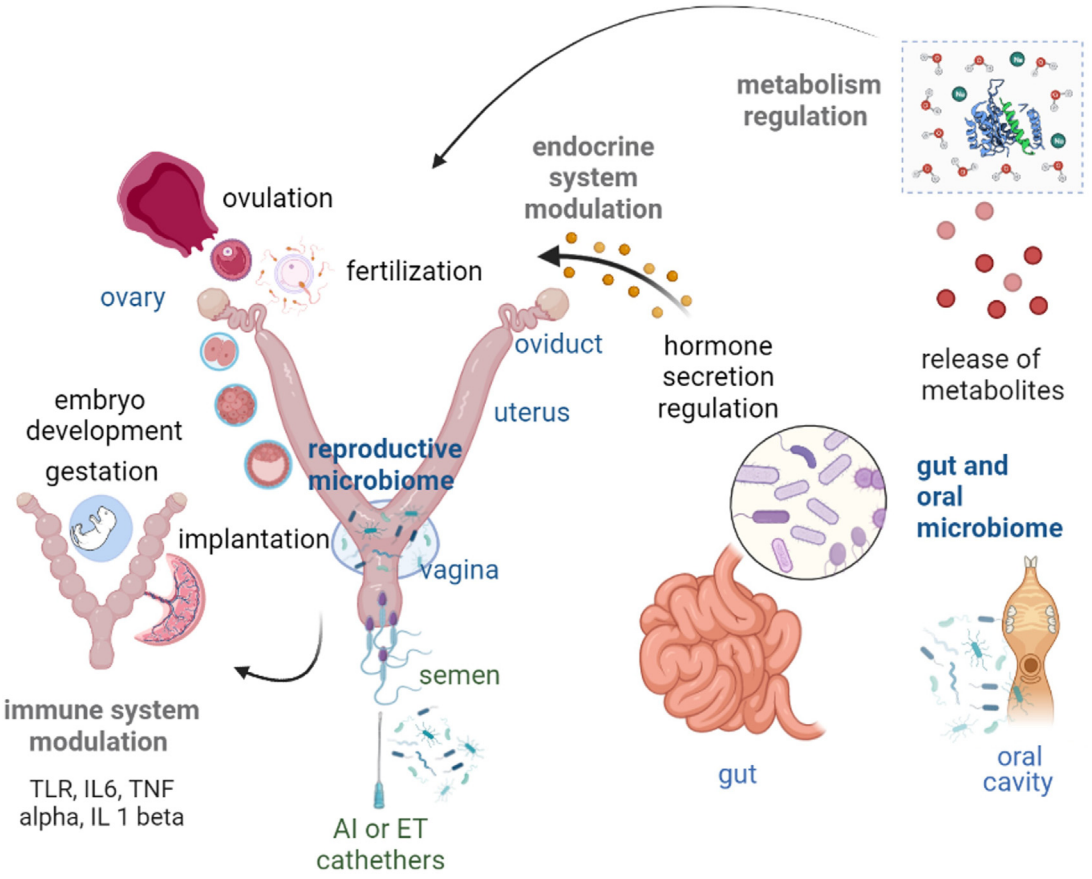

Fig. 1. Reproduction involves a series of well-orchestrated metabolic, neuroendocrine and immune events, from ovulation, fertilization, embryo development and gestation to delivery, that may be modulated by the microbiotas of the host's reproductive and digestive (gut and oral microbiotas) systems. Exogenous sources of microorganisms from semen or ART devices might also participate in the regulation of reproductive processes. AI: artificial insemination, ET: embryo transfer, TLR: Toll-like receptor, IL: interleukin, TNF: tumor necrosis factor.

provide direction for future studies designed to improve reproductive outcomes by establishing mechanisms of the gut microbiota underlying inflammation and oxidative stress, which compromise ovarian function and oocyte quality.

5. Concluding remarks

The study of the reproductive microbiota is an emerging field of biomedicine and veterinary sciences. While we are only at the start of this fascinating era of research, the importance of the microbiota as a regulator and/or driver of many physiological and pathological processes, including those of reproduction, means that some unsolved questions could be revisited from a perspective of microbiota roles. For example, some diseases (e.g., endometriosis, PCOS, etc.) that are not well understood at present might be explained, at least in part, by considering microbiota-associated functions.

Understanding the role of still hypothetical extra-vaginal reproductive microbiotas, especially in the first stages of oocyte maturation, embryo and fetal development, and offspring colonization is essential because of its impacts on reproductive health, pregnancy outcomes, and health later in life. We should underscore the need to strictly control studies examining the role of live microbial cells (or their nucleic acids) to avoid or detect possible contamination during sample collection and processing. Otherwise, the reliability and accuracy of the results obtained cannot be guaranteed. In this context, many studies focusing on animal and human microbiotas have been based on low numbers of individuals and microbiota-related techniques usually face many technical limitations, confounding factors, and potential biases. In other words, it is necessary to take some precautions when drawing general conclusions.

The terms “eubiosis” and “dysbiosis” are a matter of discussion. While the gut has been the focus of most studies in the microbiota/microbiome field, to date, scientists have not provided a clear answer to the question “What is a normal gut microbiota/microbiome?” Our knowledge of the microbiota beyond the gut and/or beyond the human species is much more limited. Methodological issues should be resolved to improve this. "Omics technologies" such as metagenomics, metatranscriptomics and metaproteomics combined with statistical modeling can provide insight into the relationship between specific phenotypic traits and the abundance of microbiotas and their functions [156]. By characterizing metabolic output, metabolomics can unveil functional interactions between the microbiota and the host [156]. We should endorse the use of rigorous decontamination software packages to remove potential contaminants during data analysis.

According to the available data, it is clear that the host microbiota is both directly and indirectly involved in mammalian reproduction. In the coming years, the search for microbiota-related markers of reproductive success or failure is set to increase the efficacy of ART procedures and/or improve pregnancy outcomes.

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Author contributions

RMGG conceived and planned the review article. JMR drafted, provide critical feedback and helped shape the manuscript. MAA, DJ, PGR, PLL and CH drafted the manuscript and edited subsequent versions. All authors contributed ideas and substantively revised it. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare they have no competing interests that could be perceived as compromising the impartiality of this review.

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