

# The conundrum of the uterine microbiome



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## Abstract

Our purpose is to review current information describing the reproductive tract microbiome in the mare. Defining the reproductive tract microflora in any species, including the mare, lies in the need to be able to determine the physiologic state of the microbiome in healthy patients and thereby develop a deeper understanding of the impact dysbiosis can have on reproductive health in breeding animals and overall health in all patients. It is likewise important to understand the impact of the general health of the animal on causing dysbiosis within the reproductive tract. With the background information we have, and the advancement of sequencing techniques at our disposal we continue to challenge the veterinary dogma of a 'sterile uterus' and shed light on several factors that impact the reproductive tract microbiome so that clinical practices can be adjusted accordingly.

**Keywords:** Mare, paradigm shift, endometrial microbiota, 16S ribosomal RNA, dysbiosis

## Introduction

It has been standard dogma in both human and veterinary medicine that although the vagina is colonized with commensal bacteria, the uterus is maintained as a sterile environment.<sup>1</sup> However, some studies based on in vitro culturing indicate that a variety of microorganisms may be present in the uterus of clinically healthy mares,<sup>2</sup> the clinical veterinary literature most often describes the uterus of a normal (healthy) mare as a site that does not host a resident microbial community.<sup>3</sup> Therefore, failure to remove pathogenic bacteria, sperm and other inflammatory substances may cause postbreeding endometritis in mares.<sup>4</sup> Furthermore, several bacterial genera (e.g., *Streptococcus*, *Escherichia*, and *Staphylococcus*) working as opportunistic pathogens in the equine uterus are known to cause pregnancy loss and infertility in mares due to infectious bacterial endometritis.<sup>5,6</sup>

Although culture-based studies laid the foundation of our understanding of the reproductive tract microbiota, as far back as 3 decades ago there was an indication that culture-based methodologies may underestimate diversity and overestimate the role of culturable bacteria in aiming to identifying microbial populations.<sup>7</sup> Focusing on culturable bacteria that are often the minority members of microbial communities, enhances the risk of missing and therapeutically disrupting those microbes that are more abundant.<sup>8-10</sup> Furthermore, the current general consensus is that culture-based technologies detect < 10% of the resident microbial community in a sample.<sup>11</sup>

With the advent of 16S ribosomal RNA (16S rRNA) gene-based bacterial detection and identification techniques, the sterile womb paradigm has been challenged through fluores-

cence in situ hybridization with 16S rRNA targeted probes in pregnant and nonpregnant women.<sup>12</sup> Several microbiological communities with variable richness and diversity were identified among other species in distinct areas of the reproductive tract.<sup>13</sup> Canine vagina (cranial) and endometrium are home to a diverse microbiome; more specifically, a distinct dissimilarity exists in the structure and diversity of the endometrial microbiome in comparison to the microbiome of the cranial vagina.<sup>14</sup> Use of the 16S rRNA gene amplicon sequencing identified bovine and ovine vaginal microbiomes differently compared to earlier identification via culture-based techniques.<sup>15</sup> Therefore, it is clear that the traditional culture-based techniques lack ability to capture the inherent diversity of the native microbial ecosystems that prevail within the mammalian reproductive tract.

The importance of having a comprehensive understanding of the resident microbiota of the reproductive tract of a healthy mare or any domestic animal species, lies in the need to understand the role of these commensal microorganisms, and how dysbiosis may impact fertility and the overall health of the animal. Furthermore, decoding the composition of the uterine microbiome has a vital role in comprehending the effect of broad-spectrum antibiotics on shifting the overall composition of the resident microbiota possibly having important roles in the reproductive process and fertility.<sup>16</sup>

The Human Microbiome Project<sup>17</sup> via 16S rRNA gene sequencing gathered much information regarding microbiota in various body sites. Consequently, human microbiome was recently described as 'the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space.'<sup>18</sup> The mammalian vagina has been well-de-

scribed and known to harbor a rich microbial ecosystem,<sup>19-25</sup> yet only a few nonhuman vaginal microbiomes have been defined to date.<sup>15,26-28</sup> Conversely, a group of different microbial communities inside the human uterine cavity have been described, but not without controversy,<sup>29</sup> and yet it is unclear where these originate. For instance, uterine colonization is hypothesized to occur from gut, oral cavity, bloodstream, and vaginal ascension.<sup>29,30</sup> Regardless, there is a strong indication that the health of the vaginal microbiome impacts reproductive health and that variations in vaginal microbial composition, particularly the loss of key genera, are implicated in gynecologic and obstetric diseases.<sup>31</sup>

As with equine, the healthy bacterial composition of the human uterus has yet to be well defined. However, compared to veterinary patients, great strides have been made towards understanding the human reproductive tract microbiome. We do know that similar to equine and canine, the human uterine microbiome is highly diverse, though not as heavily populated as the vagina,<sup>14,32-34</sup> but their composition remains to be fully unraveled. In humans, similar to what we reported in canine,<sup>14</sup> when bacterial communities from paired endometrial fluid and vaginal samples from the same subjects were analyzed, different bacterial communities were detected between the uterine cavity and vagina.<sup>35</sup> Furthermore, this study and others reported the existence of an endometrial microbiota that is highly stable during embryo implantation, and that pathological modification of its profile was associated with poor reproductive outcomes.<sup>36</sup>

In a recent extensive review of human endometrial microbiomes, a wide range of impacting factors were discussed.<sup>37</sup> The reproductive tract microbiome of women of reproductive age is often dominated by *Lactobacillus* species, whereas multiple other bacteria, including *Anaerococcus*, *Atopobium*, *Bifidobacterium*, and *Gardnerella* and others, were also isolated in lower proportions. Additional work detected bacterial networks wherein *Lactobacillus* was negatively correlated with *Gardnerella*, *Bifidobacterium*, and *Atopobium* and positively associated with the commensals *Clostridium* and *Streptomyces*.<sup>38</sup> This group behavior was also reported in vaginal samples during the menstrual cycle and demonstrates a certain degree of dependence between some species inside the female reproductive tract.<sup>39</sup> However, there are multiple other studies indicating that *Lactobacillus* was not the most abundant genus of the endometrial microbiome. Instead, there was a predominance of *Acinetobacter*, *Pseudomonas*, *Sphingobium*, and *Vagococcus*,<sup>32</sup> and *Moraxellaceae*, *Propionibacteriaceae*, *Pseudomonadaceae*, and *Streptococcaceae*, accounted for a large fraction of the uterine microbiota.<sup>40</sup> A main difference between these studies was the geographical location and ethnicity of the patients that ranged from Chinese, Italian, and of European decent.<sup>41-44</sup>

There are substantial fluctuations in bacterial communities in the female genital tract.<sup>37</sup> These shifts correlate with parameters (e.g., age, hormonal changes, and ethnicity) and the use of intrauterine devices. Although most data come from vaginal samples given the site's easy accessibility and lower risk of sample contamination, there is increasing knowledge about changes in the endometrial microbiome. They also stated that these changes and natural swings within the endometrial microbiome should be considered before therapeutic approach-

es (e.g., antibiotics or probiotics), because a 'suboptimal microbial taxonomic composition' might spontaneously shift to a 'healthy and eubiotic state' in a short interval. It is also important to consider that increasing maternal age is 1 of the main reasons why patients pursue assisted reproductive techniques for reasons that are unlikely to be related to the microbiome of their reproductive tract.

As noted above, in the human there are recognized natural changes within the vaginal and endometrial microbiomes, but there are also indications that changes of these bacterial communities could lead to infertility and other obstetric conditions.<sup>45</sup> Conversely, distinct bacterial communities are able to thrive through virulence mechanisms such as mucin degradation, biofilm formation, and antimicrobial resistance, leading to dysbiotic states.<sup>46</sup> Although there is no consensus on the endometrial microbiota, commensal bacteria could also help maintain an eubiotic state. This has been borne out in studies of the human genital tract metabolome and proteome which detected substantial differences among healthy and infertile patients. Protein expression patterns in endometrial fluid samples indicated processes related to immune response, inflammation, and cell-cell adhesion in nonpregnant women.<sup>47</sup> Further studies detected naturally occurring peptides with antimicrobial activity,<sup>48</sup> known for their protection against a myriad of pathogens, including bacteria, viruses, and fungi.<sup>49,50</sup> Therefore, influences on general health that induce variations in the reproductive tract environment may impact the balance of the microbiome and could induce a change in fertility.

Gestational diabetes mellitus (GDM) is a major concern in humans, and it is also reported in canine.<sup>51</sup> A review of the scientific literature of human studies involving GDM revealed the interrelationship between maternal gut microbiome and overall health.<sup>52</sup> In particular, changes in gut microbiota composition in the second and third trimesters of pregnancy, and that gut microbiota might be a potential diagnostic biomarker for the diagnosis, prevention, and treatment of GDM.<sup>53</sup>

An interesting insight on general health influences that impact the gut microbiome and thereby on reproductive health have been identified in laboratory animal studies. Studies using rats investigating impacts of diet and exercise demonstrated that maternal diet influenced metabolic and microbiome dysfunction such that they improved both metabolic function and the microbiome in rats with gestational diabetes, indicating an important link to long-term maternal and offspring health.<sup>54,55</sup>

We and others have previously established that the uteri of clinically healthy mares host a resident microbiome.<sup>56-58</sup> The importance in understanding this potential shift in accepted dogma is underscored by the fact that many pregnancy-related complications in humans,<sup>59</sup> and other mammals, especially those in important livestock species,<sup>60,61</sup> are well known to be of bacterial origin. Equine endometritis occurs in 25 - 60% of breeding mares resulting in infertility,<sup>62,63</sup> postpartum metritis, septicemia, and fatality in newborn foals.<sup>64</sup> Historically, genera such as *Staphylococcus*, *Escherichia*, *Pseudomonas*, and *Klebsiella* were considered invasive pathogens indicating dysbiosis in mares.<sup>65</sup> However, our current and previous studies

indicate that these 4 genera are common members of healthy mare uterine microbiomes, underscoring the importance of understanding the structure and diversity of a healthy uterine microbiome of the mare.

In addition to reviewing the current information on the microbiome of the female reproductive tract, we want to share what we have learned in our efforts to characterize the equine microbiome in the nonpregnant mare. As mentioned above, we began our metagenomics investigations in the mare to document the underestimation of microbial diversity with common culture methods relative to the presence of microbial DNA, using the 16S rRNA gene approach over 11 years ago.<sup>56</sup> Subsequently, via 'next generation sequencing' (MicroGenDX, formally PathoGenius Laboratory, Lubbock, TX)<sup>66</sup> samples from 10 clinically normal mares were compared to results obtained via traditional methods of diagnosing infectious endometritis. The main problem with that analyses is that the DNA sequences were compared against a bank of known human and equine pathogens and therefore they did not consider all possible normal nonculturable bacteria that have heretofore been undocumented in the normal microbiota of the mare. At the same time a second abstract<sup>67</sup> was presented, very similar to that we published earlier, wherein uterine fluid samples obtained from mares were subjected to metagenomic DNA sequencing of the 16S rRNA gene. These mares were followed around the period of ovulation/artificial insemination (n = 10) and during early pregnancy (n = 10). The metagenomic sequencing identified over 200 bacterial species in both culture negative and culture positive samples, demonstrating as we have that the uterus is not a sterile site at any point during and after estrus. *Proteobacteria* and *Bacteroidetes* phyla were statistically associated with culture positive samples, according to the Bonferroni correction. Their pilot study strongly correlated with ours in evidencing of the presence of a complex bacterial microbiome of organisms that fail to grow using routine uterine culture methods. Finally, from the same proceedings, a third abstract was published describing the metagenomic analysis of the equine placental microbiome.<sup>68</sup> Fecal, oral, and vaginal samples were taken from pregnant mares within 30 days of foaling, as well as the gravid and nongravid regions of the chorioallantois at foaling (n = 4). Genomic DNA was isolated from all samples, and the bacterial 16S RNA gene was amplified by PCR. Similarly, they reported a relative abundance of bacterial species within the chorioallantois. The 3 phyla represented in the gravid horn were *Firmicutes* (now *Bacillota*), *Proteobacteria*, *Bacteroidetes*, with the same 3 phyla plus *Actinobacteria* in the nongravid uterine horn. The most abundant phyla within the oral, fecal, and vaginal samples *Firmicutes* and *Proteobacteria* were also detected in the chorioallantois. The authors noted that the most abundant bacterial phyla in gravid and nongravid chorioallantois shared substantial overlap, suggesting similar, but not identical, environments within various compartments of the chorioallantois. They also reported that phyla of relatively high abundance in oral and vaginal samples corresponded to those in chorioallantois, indicating possible associations between placental and extra-placental microbiota. However, there was substantial differences between the gravid uterine horn and fecal samples.

As we reviewed above, there have been indications that the reproductive tract can be affected by exercise, ethnic influences that may include dietary differences, and geographical location. We have comprehensively analyzed the resident endometrial microbiome of clinically healthy mares from 4 geographical locations on 2 continents in an attempt to establish a stringent core microbiome of the healthy equine uterus.<sup>56</sup> To the best of our knowledge, this is the first extensive study based on 16S rRNA gene amplicon sequencing, conducted to gain a comprehensive understanding about the uterine microbiome of the healthy mare.

Clearly, this is a fast moving and clinically relevant area of investigation within the field of equine reproductive medicine and health. Further controlled studies are underway in determining the viability of this diagnostic tool in identifying clinical and subclinical cases of infectious endometritis and the role of specific components of the microbiome in the promotion of fertility and maintenance of pregnancy. In this review we have provided insights into the dynamic nature of reproductive tract microbiota and highlighted the need to be aware of the various influences on them.

## Conflict of interest

None to declare.

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