



Final report summary

The uterine microbiome
– key to infertility?



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Abstract

The objective of this project was to characterise genetic material from the community of all bacterial organisms (microbiome) residing in the uterus of mares and on the penis of stallions at several time points. Very little is known about the relevance of the microbiome and how it relates to fertility in horses.

The study showed that barren mares that fail to conceive during the breeding season harbour more genetic bacterial material in their uterus than mares that do conceive. Furthermore, conventional identification techniques only isolate a small number of bacteria in the equine uterus, many of them known pathogens; the microbiome identification technique used in this project found more bacterial species than would have been found using conventional methods. This may be relevant for diagnostic testing of infertile mares and suggests there is a need for more sensitive detection methods.

The study also found that the stallion's microbiome is influenced by the environment and can change significantly during the breeding season. This highlights the need for good hygiene practices at the time of mating in order to minimise biosecurity risks.

Background

Endometritis, an inflammation of the uterine lining, is an important cause of infertility in mares (Traub-Dagartz et al., 1991). However, the disease process is complex and still poorly understood. Currently, limited information on the bacterial population in the equine uterus is available beyond the detection of specific disease-causing organisms in some subfertile mares. Infertility not only leads to increased costs but also to foals being born later in the season, which achieve lower prices at yearling sales (Cui, 2016) and also perform poorer in races throughout their lives (Langlois and Blouin, 1997).

This explains why subfertility, in particular endometritis, has been the focus of intense research efforts for many years. Given that less than 1% of bacteria in the environment can be grown on media using conventional techniques (Handelsman 2004), and based on the results of molecular identification techniques in other species, it is likely that these cultured bacterial species represent the vast minority of the resident population in the equine uterus. The in-depth analysis of the uterine microbiome in fertile and subfertile mares is an essential step towards improved reproductive health in horses.

The role of the microbiome on the stallion's penis in endometritis has not yet been investigated. Again, while routine culture methods are performed, it is highly likely that only a subset of bacteria present can be identified. This project provided an improved understanding of the resident microbiota (community of microorganisms) and the role they play in equine infertility.

Objectives

The objectives of this project were to:

1. Identify differences in microflora between fertile and infertile mares to determine whether microbiome analysis is a useful tool in the diagnosis of endometritis.
2. Compare traditional culture-dependent methods of assessing the presence of bacteria in the equine uterus with novel culture-independent techniques to assess whether there is a need for new diagnostic procedures.
3. Assess the microbiome in fertile and infertile mares at several time points to assess whether the bacterial population in the equine uterus is stable over time.
4. Characterise the microbiome of the stallion's penis to elucidate whether the stallion has a role in the transfer of microorganisms between mares. If this is found to be the case, changes to management recommendations might result.

Research

Uterine cytobrush samples were collected from 157 maiden mares aged eight years or less and 116 barren mares aged four to 15 years at the beginning of the 2018 and 2019 breeding seasons. An additional 23 samples were collected from mares that were not pregnant at the end of the season. Furthermore, 20 mares, 10 previously maiden and 10 previously barren, were sampled after foaling in the following year.

Swab samples from 16 stallions' penises (from the urethra, urethral fossa, shaft and glans of the penis) were taken at the beginning of the 2018 breeding season, once (n=8) or twice (n=8) during the season,

again at the beginning of the 2019 breeding season (n=10), and once (n=7) or twice (n=3) during the season. Uterine swabs were assessed for bacterial growth by conventional culture methods.

DNA extraction was undertaken using routine methods and the genomic DNA recovered was used as a template for PCR amplification of the V3–V4 variable region of the bacterial 16S rRNA gene. Amplicons were sequenced on an Illumina MiSeq instrument. DNA sequence data was then processed to assess the equine microbiome composition.

The richness (number of bacterial species) and evenness (distribution of bacterial numbers) of bacterial gene sequences were compared between maiden, barren and wet mares and correlated with reproductive success, as well as between stallions and time points.

Outcomes/key findings

In general, the microbial diversity was higher on the stallions' penises than in the mares' uteri. In both, the microbiome was highly dynamic and changed significantly between seasons and, with regard to the stallions, within the season. However, a more stable 'core microbiome' was detected in stallions.

Conventional identification techniques failed to identify known equine genital pathogens in some mares, while more were identified by microbiome analysis. Bacteria that have been linked with infertility in the human were found in mares and stallions, e.g. *Moraxella*, which were only found in some stallions in the low-fertility group.

While the richness and evenness of the microbiome was comparable in maiden, barren and wet mares, the barren mares that failed to fall pregnant in the study season harboured a higher number of bacterial species in their uterus than successfully bred barren and maiden mares (Figure 1). Exploring this phenomenon further may lead to the design of a prognostic test that allows prediction of fertility success. Additionally, whether fertility of those mares can be improved by 'modulating' their microbiome, e.g. by transferring microbiota from fertile to infertile mares, should be investigated.

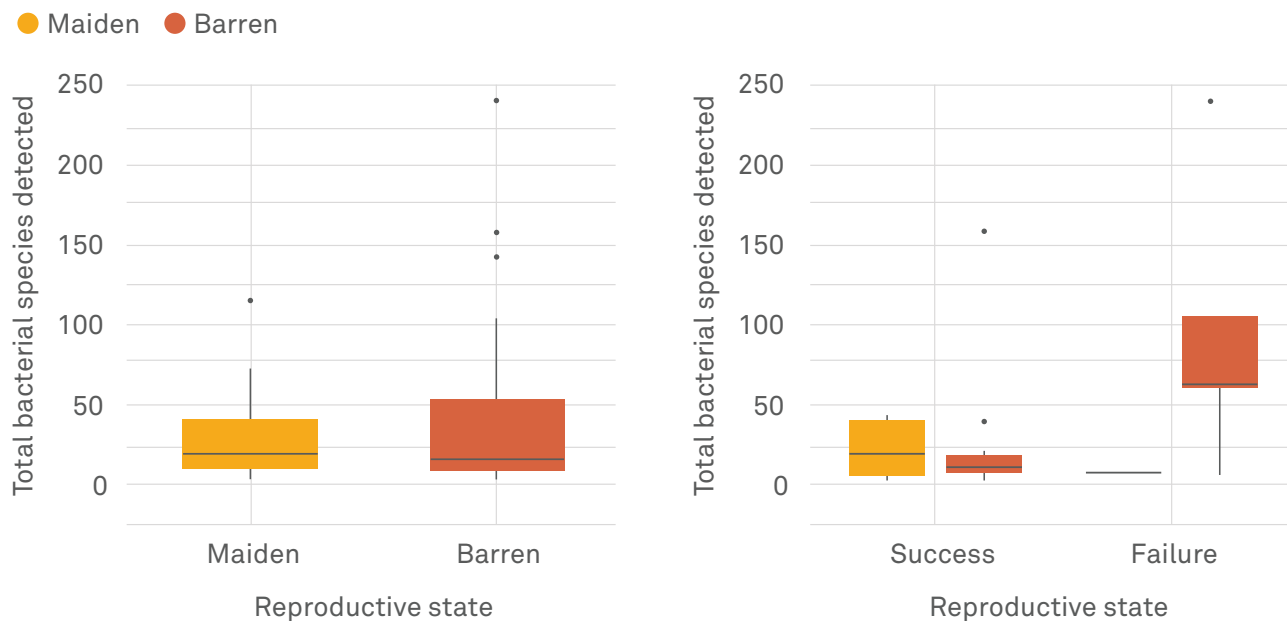


Figure 1. Total bacterial species detected in maiden and barren mares, and in mares with reproductive success (pregnant after one breeding) and reproductive failure (four or more times bred unsuccessfully in a season). While species richness is comparable for maiden and barren mares, the bacterial richness is higher in the reproductive failure group in barren mares.

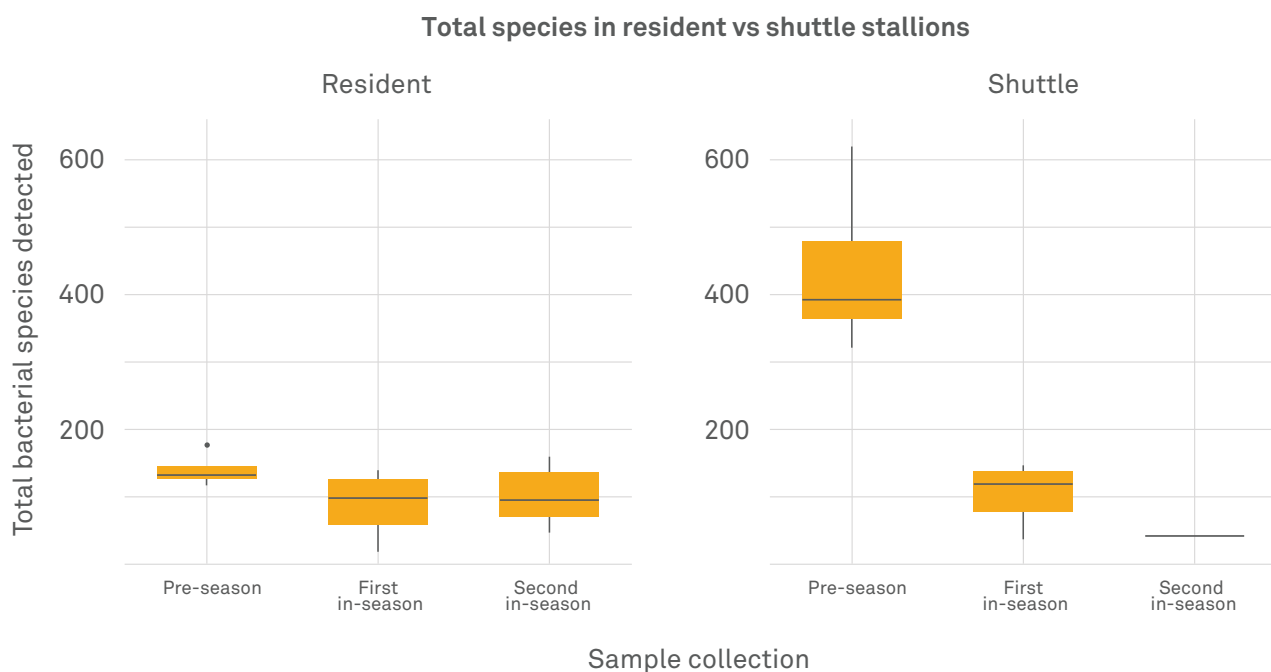


Figure 2. Total bacterial species detected in five 'resident' versus five 'shuttle' stallions. While shuttle stallions have a significantly higher abundance of bacterial species sequences at the beginning of the breeding season (pre-season swab), this is comparable between resident and shuttle stallions in-season.

The abundance of 'shuttle' stallions' microbiome at the beginning of the breeding season was highly influenced by traveling to the Northern Hemisphere in the preceding season. The differences disappeared during the season, suggesting that the microbiome is dynamic and highly influenced by environmental

factors (Figure 2). The findings highlight the need for good hygiene practices to minimise potential biosecurity risks. While this study couldn't demonstrate a clear transfer of bacteria between mares and stallions, this can't be excluded at this point and should be explored in more targeted studies.

Implications for industry

This work found that culture-independent methods allow the identification of a higher number of microorganisms, and therefore more potential candidate pathogens that can be linked to equine infertility. These includes known equine genital pathogens and also novel pathogens that have been found to correlate with infertility in other species. While they have not been found to be exclusively associated with infertile mares or stallions with low fertility, their potential contribution to decreased fertility should be explored further.

The assessment of the microbiome present on the stallions' penises at different time points highlighted the variation in bacteria abundance during the season. The finding that the microbiome is influenced by travel and breeding activity leads to new hypotheses that can characterise these factors in further studies. The finding that the microbiome on stallions' penises is highly variable is of note, as this suggests that stallions can readily pick up and pass on new bacteria, which highlights the need for good hygiene. In the past, attention was mostly paid to known venereal pathogens in the stallion, but future studies should investigate whether this is sufficient, as sequences of pathogens that are implicated in human infertility have been found in this study.

The stallions in this study were all of acceptable fertility but including subfertile stallions in future studies could potentially lead to new insights into the role of the microbiome in stallion fertility.

The finding that no specific bacteria population was found to be uniquely present or absent in either fertile/infertile mares and/or stallions with high or low fertility is important.

Recommendations

Barren mares that fail to fall pregnant have a higher bacterial abundance in their uterus than successfully bred barren and maiden mares. Further research is recommended as it may be possible to design a prognostic test that enables prediction of fertility success for barren mares. Further research should also investigate whether transferring bacteria from mares with known good fertility to barren mares improves pregnancy rates.

Assessment of the microbiome revealed the presence of known and potentially novel equine pathogens that could not be isolated by traditional culture methods. It is recommended that this phenomenon is further investigated by increasing the sample size, as this may reveal the need for more sensitive diagnostic tests for these newly described pathogens (e.g. *Moraxella* sp., *Flavobacterium*). If they are found to be of significance in infertile mares, a more sensitive diagnostic test may be warranted (e.g. a diagnostic PCR test).

The stallion's penis harbours a dynamic microbiome that shifts during the season and seems to correlate with its environment. This highlights the importance of good hygiene to minimise the biosecurity risk associated with pathogens being passed between horses. Future studies should explore in more depth the risk of bacteria being transferred between Australian mares and stallions arriving from overseas.

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