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Highlights

- Characterization of the equine endometrial microbiome in anestrus using 16S rRNA.
- Mare endometrial microbiome in anestrus has high diversity, richness and abundance.
- Significant differences between endometrial microbiome of anestrus and estrus mares.

Original Research

Metagenomic characterization of the equine endometrial microbiome during anestrus

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Abstract

The equine uterus is highly interrogated during estrus prior to breeding and establishing pregnancy. Many studies in mares have been performed during estrus under the influence of high estrogen concentrations, including the equine estrual microbiome. To date, it is unknown how the uterine microbiome of the mare is influenced by cyclicity; while, the equine vaginal microbiome is stable throughout the estrous cycle. We hypothesized that differences would exist between the equine endometrial microbiome of mares in estrus and anestrus. The aim of this study was two-fold: to characterize the resident endometrial microbiome of healthy mares during anestrus and to compare this with estrus. Double-guarded endometrial swabs were taken from healthy mares during estrus (*n*=16) and in the following non-breeding season during anestrus (*n*=8). Microbial population was identified using 16S rRNA sequencing. Our results suggest that the equine uterine microbiome in estrus has a low diversity and low richness, while during anestrus, a higher diversity and higher richness were seen compared to estrus. Despite this difference, both the estrus and anestrus endometrial microbiome were dominated by Proteobacteria, Firmicutes, and Bacteroidota. The composition of the microbial community between anestrus and estrus was significantly different. This may be explained by the difference in the composition of the endometrial immune milieu based on the stage of the cycle. Further research investigating the function of the equine endometrial microbiome and dynamics changes within the uterine environment is required.

Keywords: Endometrium; Equine; Mare; Metagenomics; Microbiome

1. Introduction

It has been widely accepted that the vagina of women is colonized with commensal bacteria [1]. Following the Human Microbiome Project launch in 2007, sequencing techniques revealed that body sites previously believed to be sterile, including the reproductive tract, actually harbour their own microbiome [2,3]. Much work has been done in the field of the vaginal microbiome, and it has been well established that this microbiome changes with age and hormone exposure, specifically estrogens [4]. Also, it has been found that the endometrial microbiome is impacted by hormonal changes throughout the menstrual cycle allowing for significant changes in bacterial abundance [5]. Contrary to women, mares are seasonal, long-day breeders. They have a period of anestrus during winter, where no cyclic levels of estrogen nor progesterone, are present. During the longer days mares have an estrous cycle consisting of a follicular phase, when estrogen is the dominating hormone, and a luteal phase, with progesterone being the predominant hormone. Equine reproductive tract microbiome research has mostly focussed on the uterine, vaginal, and placental microbiome [6–10]. A core uterine microbiome, consisting of *Lactobacillus, Escherichia/Shigella, Streptococcus, Blautia, Staphylococcus, Klebsiella, Acinetobacter and Peptoanaerobacter* has been observed in random cycling mares during the physiologic breeding season. Holyoak et al. [6] found that the microbial diversity, richness and evenness were largely dependent on the geographical location of the mare [6]. Furthermore, it has been shown that the equine endometrium in estrus has a resident microbiome, and that the sampling technique does not affect the beta diversity [7]. One study performed on Arabian mares indicated that, in contrast to women, the equine vaginal microbiome is stable throughout the estrous cycle [10]. Currently, there is no data available on the equine endometrial microbiome in anestrus versus estrus stage of the estrous cycle, to the best of the authors' knowledge.

The objectives of this metagenetic study were two-fold: first, we aimed to characterize the resident endometrial microbiome of healthy mares during anestrus, when no significant levels of sex hormones are present, and secondly, compare the resident equine endometrial microbiome in anestrus to estrus, when higher levels of estrogen are present. We hypothesized that a dissimilarity is present between the resident equine endometrial microbiome in anestrus and estrus.

2. Materials and methods

2.1. Ethical approval

The study protocol was approved by the Ethics Committee of Louisiana State University Institutional Animal Care and Use Committee (Approval number 17-046; Approval date, 20 August 2017).

2.2. Animals

The project was carried out at the School of Veterinary Medicine and the Reproductive Biology Center, Louisiana State University (LSU), Baton Rouge, Louisiana, USA. All horses included in the study were owned by LSU and all procedures were approved by LSU Institutional Animal Care and Use Committee. The estrus samples used for this study were taken during a separate project where different sample methods were compared [7]. A total of 16 mixed breed mares with a median age of 13 years (9-18 years) (range) were included in the first part of the study (collection of estrus samples) during the physiological breeding season (a 14-day period in July) and from the group sampled in estrus, eight mares with a median age of 14 years (10-19 years) (range) were enrolled in the following non-breeding season (January-March

2018) for collection of anestrus samples. Prior to and during the project the mares were housed on pasture.

2.2.1. Inclusion criteria for estrus samples

Mares in estrus were enrolled in the study based on the following criteria. Estrus was defined as the presence of a follicle > 30 mm in diameter, endometrial edema, absence of a corpus luteum detected via transrectal ultrasound, serum progesterone concentration of < 1 ng/mL and an open cervix on digital transrectal palpation. Only mares without signs of endometritis were included. This was determined by the presence of < 1-2 neutrophils per high power field cytology brush, no histologic evidence of inflammation or infection (blinded boarded theriogenologist evaluated), no intraluminal uterine fluid present on transrectal ultrasound examination during estrus, and a negative aerobic culture of each sample obtained (endometrial biopsy, swab, low volume lavage (LVL) and cervical swab).

2.1.2. *Inclusion criteria for anestrus samples*

Anestrus was defined as a minimum of 3 consecutive weeks with a plasma progesterone level < 1 ng/mL, follicles ≤ 20 mm in diameter and the absence of corpora lutea via transrectal ultrasound and a flaccid cervix on digital transrectal palpation. No intra-uterine procedures were performed on the mares in the time between the estrus and anestrus samples.

2.3. Methods

After transrectal ultrasound examination, the mare's perineum was cleaned with 7.5% povidone-iodine scrub (Betadine Surgical scrub Veterinary, Aviro helath L.P., USA) prior to sterile collection of the samples. All samples were taken in a clean, climate controlled, closed examination room.

2.2.1. Methods for anestrus samples

During anestrus an endometrial swab sample was collected in sterile fashion, by a single operator as previously published [7].

2.2.1.1 Endometrial swab

A double guarded swab (Minitube, Verona, WI, USA) was transcervical introduced into the uterus and an endometrial sample was obtained. The obtained swab was frozen and stored at -80°C for molecular analyses as previously published [7].

2.2.1.2. Negative control

A sterile, unused swab, was submitted for genomic DNA isolation on the same days of sample collection.

2.2.2. Sample collection for estrus samples

During estrus an endometrial swab sample was collected in sterile fashion, by a single operator as previously published [7].

2.2.2.1. Negative control

A sterile, unused swab, was submitted for genomic DNA isolation on the same days of sample collection.

2.3 Metagenomic analyses.

The anestrus and estrus samples were analysed in two different batches, one containing only anestrus samples and one containing only estrus samples, and analysed at different time points. Genomic DNA was extracted from all uterine samples using Qiagen DNeasy PowerSoil

extraction kits (Qiagen, Hilden, Germany). Swabs were extracted by removing the swab tips from the applicators with sterile razor blades, and then transferring the swab material directly to bead-beating tubes. After removing the supernatant, pellets were resuspended in a small volume of bead-beating solution (from Qiagen DNeasy PowerSoil extraction kits) and transferred to bead-beating tubes. Subsequent steps followed the manufacturer's instructions. In addition to the various uterine samples, a set of blanks were processed similarly as well as a no template control (no sample material was added to the bead-beating tubes). DNA extracts were visualized by gel electrophoresis, transferred to 96-well plates, and then shipped overnight on dry ice to the Research Technology Support Facility of Michigan State University for 16S rRNA sequencing using primers 515f and 806r (V4-V5 region). Barcoding and library preparation was performed. Sequencing was done on a Miseq platform (Illumina, Inc.) with 2 x 250 bp paired-end according to the Kozich et al. [11] protocol. All samples were sequenced twice.

Samples were filtered and trimmed based on their quality scores and error rates using the dada2 pipeline [12]. Next, an amplicon sequence variant (ASV) table was made, and chimeras were removed. The 16S rRNA SILVA v138.1 database [13] was used for mapping and assigning taxonomy. Next, contaminating reads were removed from the samples using Microdecon [14] based on the negative controls (blank and no template control). Downstream analysis was performed using the Phyloseq package [15]. Alpha diversity calculation (Shannon, Chao1, and inverse Simpson), beta diversity (weighted UniFrac), and analysis of similarity (ANOSIM statistic) was performed using the *microbiome*, *amplicon*, *microeco*, and *vegan* packages [16–18]. Differentially abundant taxa were identified using DESeq2, adapted from the pipeline published by Hagey et al [19,20]. Graphs were generated using *ggplot2*, *dplyr*, *RColorBrewer*, *ggpubr*, and *lattice* packages in R. Bar and pie plots were generated using Microsoft Excel.

Sequences have been deposited in the NCBI SRA as SRP267434.

3. Results

3.1. Sequencing results

During anestrus a total of eight individual samples were obtained and sequenced once. A total of 2,173 ASV's were found after quality filtering and mapping. After applying Microdecon, 2,027 ASV's were left for further analysis. During estrus a total of 16 individual samples were obtained. One sample was not run as the sample was lost. In total, 15 individual samples were sequenced twice. A total of 957 ASV's were found after quality filtering and mapping. After applying Microdecon, 811 ASV's were left for further analysis.

3.2. Alpha diversity

The microbial communities within the anestrus and estrus groups were assessed using alpha diversity and compared using Tukey HSD. A significant difference in alpha diversity between the anestrus and estrus groups was found for the Chao1, Inverse Simpson and Fisher index (P<0.05). However, the Shannon index did not show a significant difference between the anestrus and estrus groups (P=0.076) (Fig. 1).

3.3 Relative abundance at phyla and genus level

The relative abundance of the most abundant bacteria followed a similar pattern in both sample groups at phylum level (Fig. 2, Fig. 3). At phyla level, Proteobacteria, Firmicutes and Bacteroidota were the most abundant phyla with a combined total relative abundance of 83% and 82% respectively for the anestrus and estrus groups. At genus level the most abundant bacteria in the anestrus group were *Rikenellaceae RC9 gut group* and *Peptoanaerobacter*, with only a

combined total relative abundance of 14%. This contrasts with the estrus group, in which the most abundant bacteria were *Klebsiella, Aeromonas, Mycoplasma* and *Citrobacter* with a combined total relative abundance of 54% (Fig. 4). Further individual differences in the relative abundances of microbes could be seen in both sample groups at both phylum and genus level (Fig 3, Fig. 5). The ANOSIM statistic was 0.88 with P=0.001, showing that the microbial communities in anestrus an estrus were significantly dissimilar from each other.

Out of 29 individual genera analyzed, it was found that 19 showed a significant increase during anestrus as compared to estrus (Fig. 6). On the other hand, 10 individual genera showed a decrease during anestrus as compared to estrus (Fig. 6).

3.4 Analysis of similarity (ANOSIM)

The ANOSIM statistic between all groups was r=0.88 (P=0.001) (Table 1). The ANOSIM statistic ranges between 0 and 1, and the closer this statistic is to 1, the more dissimilarity is present between the groups.

3.5 Beta diversity

The ANOSIM tests if there is a significant difference between the two groups, and the weighted UniFrac accounts for the abundance of observed organisms and incorporates the phylogenetic distance between microbes in the distance calculation. The composition of the microbial community between the two sample groups was significantly different (P<0.05, ADONIS on weighted Unifrac (Fig. 7)). The ANOSIM tests if there is a significant difference between the two groups, and the weighted UniFrac accounts for the abundance of observed organisms and incorporates the phylogenetic distance between microbes in the distance calculation. There was no overlap visible in the PCoA plot of weighted UniFrac PCoA plot, meaning that the two groups did not share many microbes.

4. Discussion

We demonstrated that the resident endometrial microbiome of healthy mares during anestrus is dominated by Proteobacteria, Firmicutes and Bacteroidota, with a combined total relative abundance of 83%. This is similar to what has been found during estrus (82%) and what was found by Holyoak et al. in random cycling mares during the physiologic breeding season [6,7]. In our study, we focused on the comparison between estrus, in the cycling season, and anestrus (non-cycling season) rather than diestrus. At genus level, the most abundant bacteria in the anestrus group were *Rikenellaceae RC9 gut group* and *Peptoanaerobacter*, with only a combined total relative abundance percentage of 14%. This contrasts with the estrus group, in which the most abundant bacteria were *Klebsiella, Aeromonas, Mycoplasma* and *Citrobacter* with a combined total relative abundance percentage of 54%. In agreement with our hypothesis, the ANOSIM statistic was 0.88, indicating that the microbial composition is highly dissimilar between the anestrus and estrus groups. This high dissimilarity was present despite that not all mares sampled during estrus (n=16) were available for follow up during anestrus (n=8), a limitation of the current study.

During equine anestrus, when both estrogens and progesterone are low, the endometrial microbiome had a high diversity and high richness. This is similar to what is seen during early puberty and in the post-menopausal vaginal microbiome of women [21,22]. However, this is contrary to the bitch, where no changes in the endometrial microbiome were found during the different stage of the estrous cycle [23]. In our study, the difference between anestrus and estrus groups was also observed in the alpha diversity, a significant difference was present for Fisher, Inverse Simpson and Chao1 indexes, however, no difference was observed in the Shannon index

between anestrus and estrus. This could be explained if the high diversity, richness, and abundance observed in anestrus was caused by rare species or singletons and the observed high relative abundance, since these are not taken into account in the Shannon index.

Focusing on the genus level specifically, 29 individual genera were identified that were significantly more or less abundant in anestrus compared to estrus. 19 genera were found to be in significantly higher abundance in anestrus compared to estrus, most of these belonged to the Firmicutes and Proteobacteria phyla. Based on their known role in the microbiome of other species and organ systems they could be split in five groups: 1. *Anaerovorax, Phascolarctobacterium, UCG-002, NK4A214 group, Treponema, Ruminococcus, Prevotellaceae UCG-001, Sp3-e08* and *Rikenellaceae RC9 group* are involved in short chain fatty acid (SCFA) production and degradation of structural carbohydrates [24–32]. No clear function is described for the *Family XIII AD3011 group or UCG-005*, however, they are respectively part of the *Anaerovoracaceae* (as is *Anaerocorax*) and *Ruminococcus* families. The *DgA-11 gut group* is found in the fecal microbiome of sheep and is part of the Rikanellacea family [33]. *Methanocopuscullum* is involved in long chain fatty acid degradation [34]. 2. *Candidatus soleaferrea,* and *Sutterella* are involved in inflammatory and immune responses [35,36]. 3. *Colidextribacter* is involved in impaired intestinal permeability and gut barrier dysfunction [37] 4. *Oribacterium* is found in the human oral microbiome [38]. 5. *Mobiluncus* has been associated with human bacterial vaginosis [39].

Ten individual genera, *Achromobacter, Acidipila Silvibacterium, Aeromonas, Citrobacter, Enterobacter, Hydrotalea, Klebsiella, Mycoplasma, Latilactobacillus* and *Stenotrophomonas* were significantly decreased in anestrus compared to estrus. Of these, *Aeromonas, Citrobacter, Enterobacter, Klebsiella* and *Stenotrophomonas* are known pathogens in equine reproduction

[40–49]. *Mycoplasma* has been suggested to be a commensal in the genital tract of stallions [50], however other authors were not able to identify *Mycoplasma* in the semen and vaginal swabs of Danish stallions and mares [51]. *Achromobacter*, *Acidipila Silvibacterium*, *Hydrotalea*, and *Latilactobacillus* have not been associated with the equine reproductive tract.

Interestingly, many of the genera identified to be present in increased abundance in anestrus are involved in SCFA production, fatty-acid, and glycolipid metabolism. However, the genera with a decreased abundance during anestrus are mostly known pathogenic microbes in equine reproduction [40–49]. Human studies have indicated that hormonal fluctuations modulate antimicrobial peptides in the uterine mucosa and endometrial fluid [52]. Thus suggesting that the human endometrial microbiome is cycle-dependent with possible metabolic activity in the host-microbiota crosstalk in supporting endometrial functions in the receptive phase. Something similar could be present in the mare; particularly as the main difference between the two groups investigated in our study, anestrus and estrus, is the hormonal milieu. As mentioned previously, during anestrus no significant levels of estrogen or progesterone are present, while during estrus the endometrium is under the influence of estradiol and has previously been exposed to progesterone during the luteal phase. Using staining techniques on endometrial biopsies, it has been shown that glycogen is most abundant in equine endometrial glands during the physiological breeding season and that luminal epithelial cells predominantly contain carboxylated acid mucins during the physiological breeding season, spring and fall transition periods [53]. More recently, an increase of uterocalin and glycogen staining intensity of equine endometrial biopsy samples was found with increasing peripheral blood progesterone concentrations and an increase in uteroglobulin and uteroferrin was associated with a decrease in circulating progesterone concentrations [54]. This is in agreement with earlier studies from Zavy

et al. [55], who showed that acid phosphatase activity peaked in uterine luminal fluid on day 12 of the estrous cycle and subsequently decreased. Despite these studies investigating the presence of mucins and proteins in endometrial biopsy samples and the early work performed on uterine luminal proteins, to the authors' knowledge, there is no recent data available on the specific composition of the equine endometrial mucus during different stages of the estrous cycle. Therefore, we can only postulate that the rich and high diverse microbiome found in anestrus and the low diverse microbiome in estrus is related to the composition of the equine endometrial mucus and furthermore that if the differences found between the anestrus and estrus equine endometrial microbiome could be driven by sex steroid hormonal mechanisms.

It has been reported that in the mare the switch from estradiol to progesterone during the transition from estrus to diestrus affects endometrial gene expression to support embryo survival [56] and that a longer preceding estrus increases the likelihood of subsequent pregnancy [56,57]. However, it remains unclear what genes or pathways contribute to the positive effect of estrogen exposure on pregnancy success in the mare [58]. We could hypothesize that the duration of estrogen exposure influences the uterine milieu and subsequently the endometrial microbiome, potentially allowing for an increase in the abundance of different microbes, ultimately affecting fertility. Further investigations into the influence of sex steroid hormones on the endometrial environment and microbiome are needed and ongoing in the field of equine reproduction.

5. Conclusions

In conclusion, as determined using by 16S rRNA sequencing, the equine uterus does harbor a resident microbiome during anestrus. This endometrial microbiome had a high diversity, high

richness, and high abundance and is significantly different from the equine endometrial microbiome in estrus.

A significant difference in alpha diversity was present for Fisher, Inverse Simpson and Chao1 indexes, however, no difference was observed in the Shannon index between anestrus and estrus. This could be explained if the high diversity and richness observed in anestrus was caused by rare species or singletons, since these are not taken in into account in the Shannon index. These findings may have implications for mares with a hormonally hastened transition from anestrus to estrus. Timing of estrogen exposure and the uterine microbiome prior to breeding may impact mare fertility and pregnancy outcomes.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Author Contributions

Conceptualization, J.L. Sones; Methodology, J.L. Sones, G. King, M. van Heule; Formal Analysis M. van Heule and B.A. Heil; Investigation, B.A. Heil, J.L. Sones, S.K. Thompson, T.A. Kearns, K.F. Beckers, E.L. Oberhaus.; Writing – Original Draft Preparation, B.A. Heil and M. van Heule.; Writing – Review & Editing, B.A. Heil, J.L. Sones, P. Dini.; Visualization, M. van Heule; Supervision, J.L. Sones.; Project Administration, J.L. Sones.; Funding Acquisition, J.L. Sones, P. Deals.

Data Availability Statement

Sequences have been deposited in the NCBI SRA as SRP267434.

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Table 1. Analysis of similarity between anestrus and estrus groups and the *P*-value.

Group 1	Group 2	p-Value
Anestrus	Estrus	0.001

Figures

Please print all figures in color

Figure legends

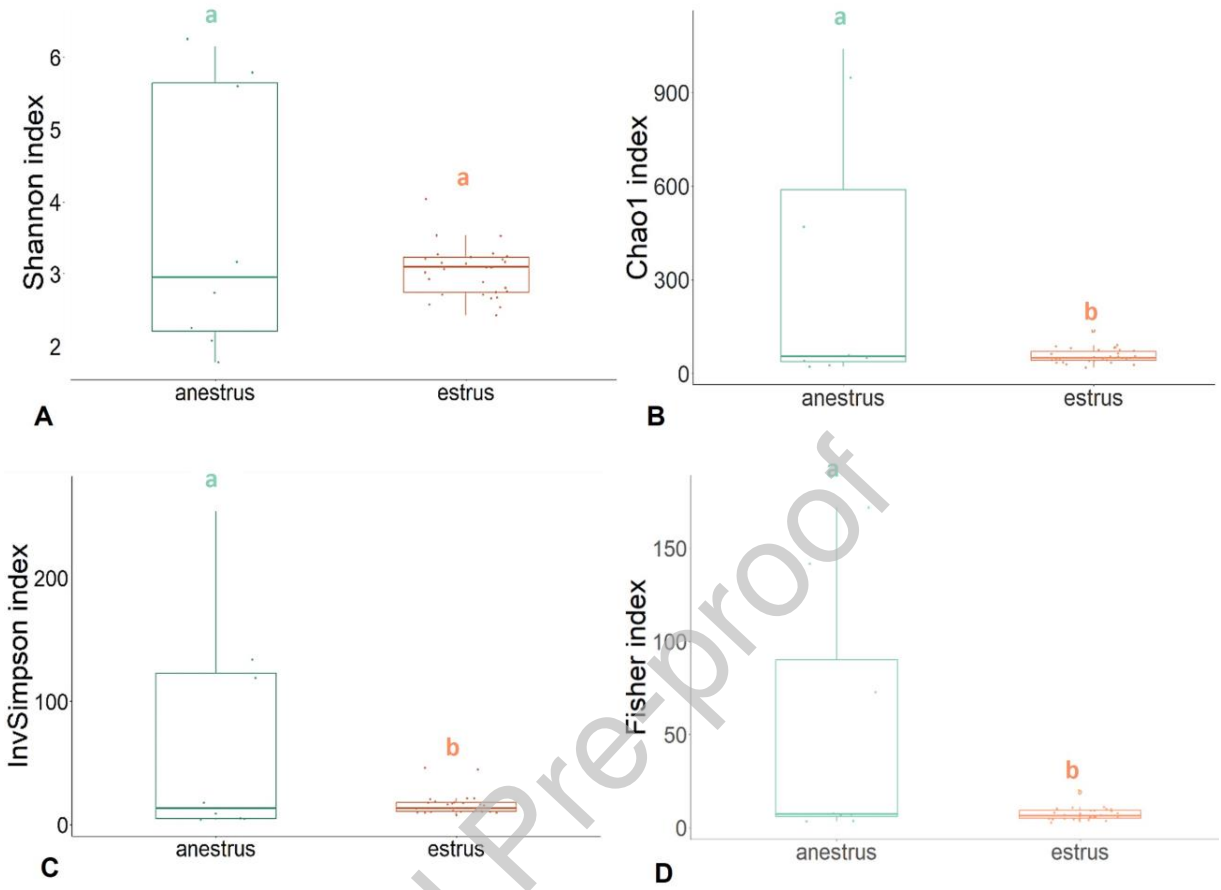

Fig. 1. Alpha diversity index in the anestrus and estrus groups. A) Shannon index, B) Chao1 index, C) Inverse Simpson index, D) Fisher index. A significant difference in alpha diversity between the anestrus and estrus groups was found for the Chao1, Inverse Simpson and Fisher index (P<0.05). The Shannon index did not show a significant difference between anestrus and estrus groups (P=0.076).

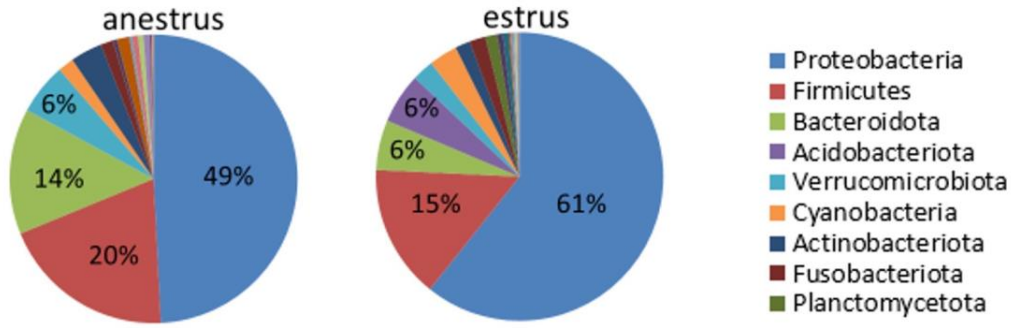

Fig. 2. Relative abundance on phylum level in anestrus and estrus groups. Proteobacteria, Firmicutes and Bacteroidota were the three most abundant phyla in both groups with a combined total relative abundance of 83% and 82% respectively for the anestrus and estrus groups.

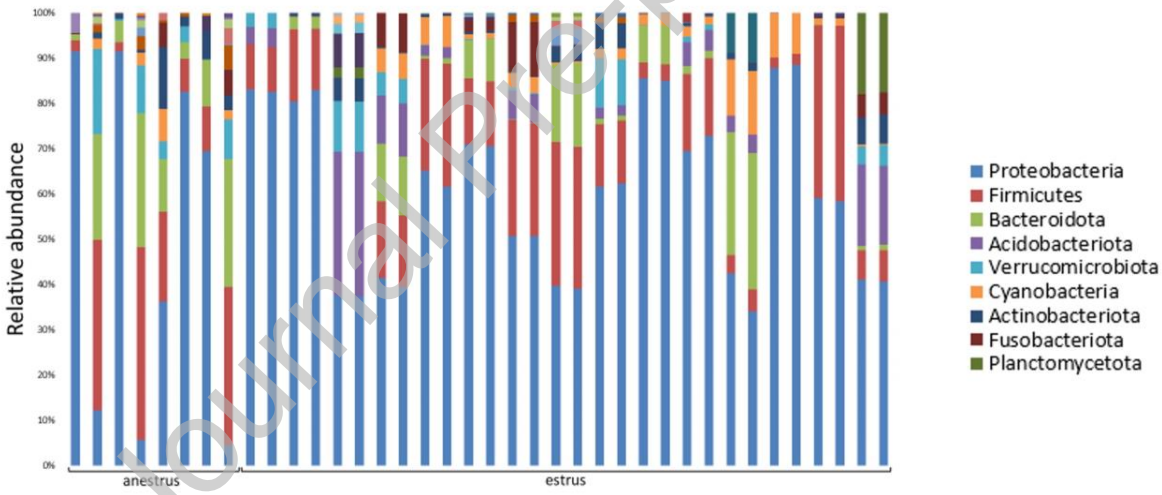

Fig. 3. Relative abundance on phylum level in anestrus and estrus groups. Proteobacteria, Firmicutes and Bacteroidota were found to be the three most abundant phyla in both groups. There are individual differences between samples in both sample groups.

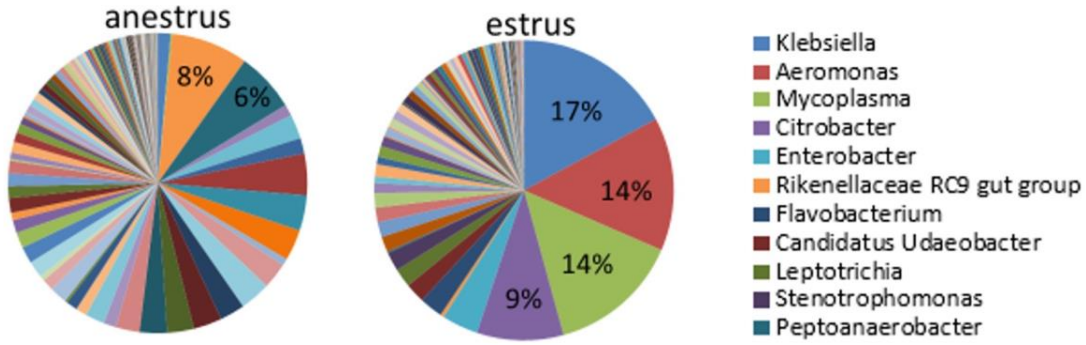

Fig. 4. Relative abundance on genus level in anestrus and estrus groups. In the anestrus sample *Rikenellaceae RC9 gut group* and *Peptoanaerobacter* were the most abundant genera with a combined total relative abundance of 14%, while in the estrus group *Klebsiella*, *Aeromonas*, *Mycoplasma* and *Citrobacter* were the most abundant genera with a total relative abundance of 54%.

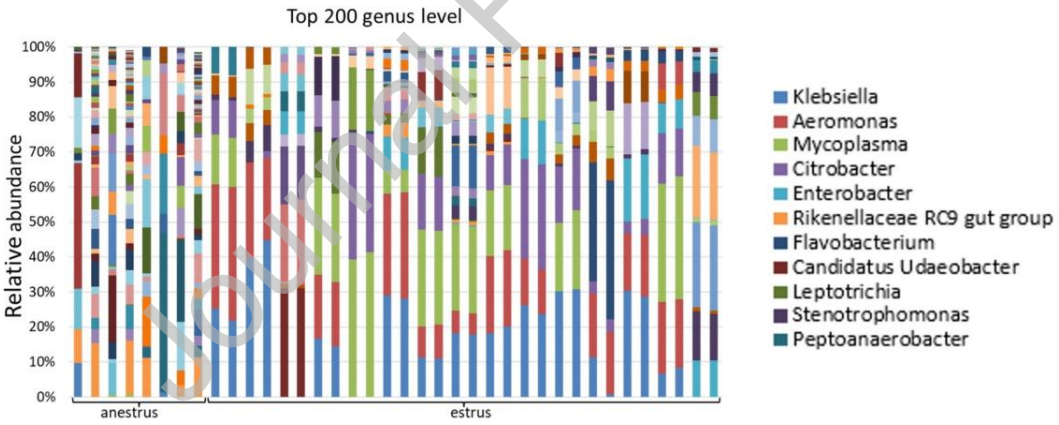

Fig. 5. Relative abundance of the top 200 genera in anestrus and estrus groups.

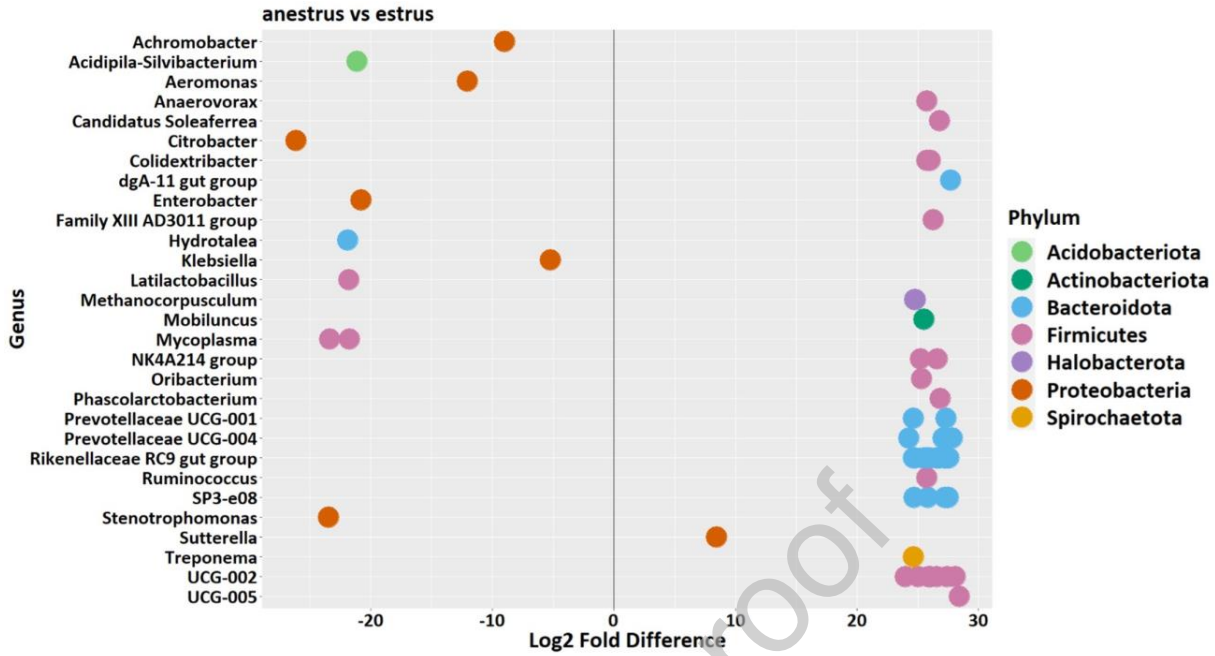

Fig. 6. Log fold changes at genus level between anestrus and estrus. Of the 29 individual genera analyzed, 19 showed a significant increase during anestrus as compared to estrus and 10 individual genera showed a decrease during anestrus as compared to estrus.

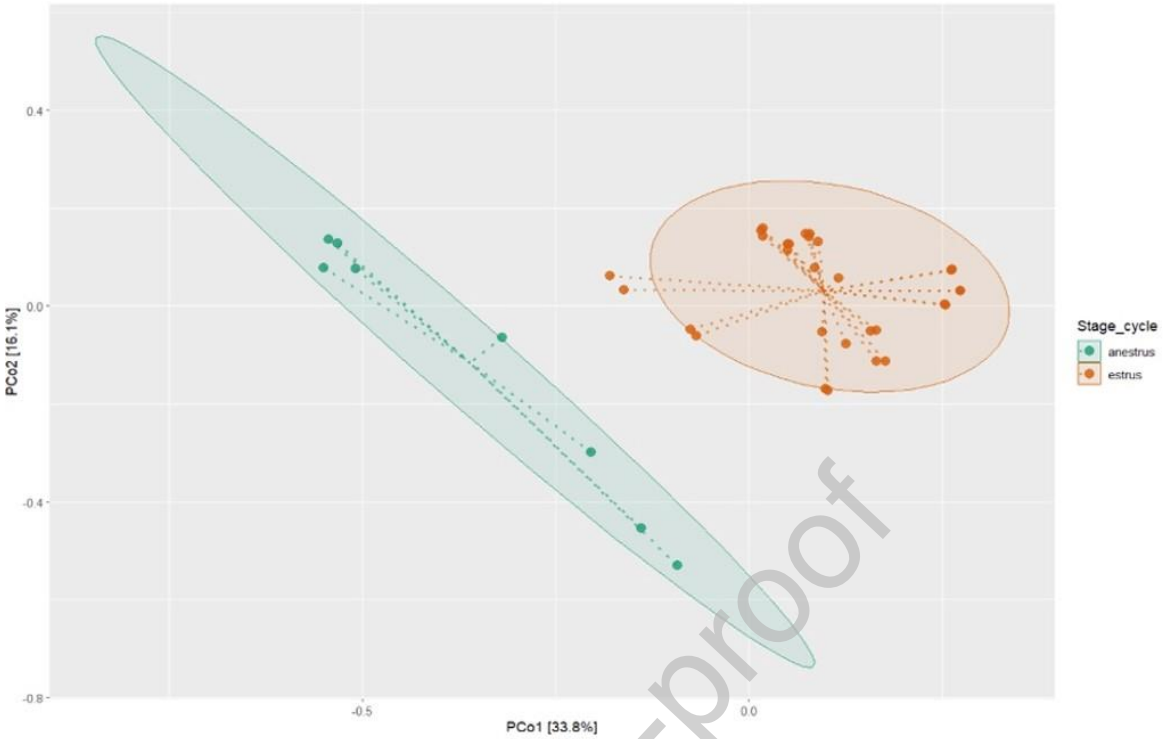

Fig. 7. PCoA plot of weighted UniFrac distance. A significant difference in beta diversity was found between anestrus and estrus groups (P<0.05)