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Characterization of the equine placental microbial population in healthy pregnancies

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ABSTRACT

In spite of controversy, recent studies present evidence that a microbiome is present in the human placenta. However, there is limited information about a potential equine placental microbiome. In the present study, we characterized the microbial population in the equine placenta (chorioallantois) of healthy prepartum (280 days of gestation, n = 6) and postpartum (immediately after foaling, 351 days of gestation, n = 11) mares, using 16S rDNA sequencing (rDNA-seq). In both groups, the majority of bacteria belonged to the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidota. The five most abundant genera were *Bradyrhizobium*, an unclassified *Pseudonocardiaceae*, *Acinetobacter*, *Pantoea*, and an unclassified *Microbacteriaceae*. Alpha diversity ($p < 0.05$) and beta diversity ($p < 0.01$) were significantly different between pre- and postpartum samples. Additionally, the abundance of 7 phyla and 55 genera was significantly different between pre- and postpartum samples. These differences suggest an effect of the caudal reproductive tract microbiome on the postpartum placental microbial DNA composition, since the passage of the placenta through the cervix and vagina during normal parturition had a significant influence on the composition of the bacteria found in the placenta when using 16S rDNA-seq. These data support the hypothesis that bacterial DNA is present in healthy equine placentas and opens the possibility for further exploration of the impact of the placental microbiome on fetal development and pregnancy outcome.

1. Introduction

Mammals have co-evolved with bacteria colonizing niche body sites and forming distinct microbiomes [1–3]. The microbiome is important for the normal function and homeostasis of the colonized organs and tissues [1–4]. However, some organs, like the pregnant uterus and placenta, have been claimed to be sterile as part of the so called "sterile womb paradigm" [5–9]. The consequence of a fetus developing in a sterile uterus is that the development of the fetal microbiome starts during and after parturition [5–7,10,11]. This paradigm is based on evidence generated by traditional culture-based and microscopy techniques in the past decades [4–9]. However, these methods have a low sensitivity as the majority of microorganisms cannot be grown under standard, aerobic laboratory conditions [5,12]. This limitation could lead to failure of these traditional methods to detect bacteria in low biomass samples, such as the healthy placenta [5,12]. With the use of 16S rDNA sequencing and whole-genome shotgun sequencing, researchers have been able to identify microbial DNA in the uterus [13–17] and postpartum placenta in different mammals [1,18–21].

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These findings led to the 'in utero colonization' theory [5]. This theory postulates that there is an endometrial and placental microbiome that contributes to the colonization of fetal organs before birth [5,6,8,11]. Some studies have found similar bacterial communities in meconium and the placenta or amnion fluid, indicating possible colonization of fetal tissues in utero [18,22,23]. Recently, researchers have started to relate the endometrial and placental microbiome to pregnancy pathologies [1,9,15,24–45].

The microbiome is considered crucial for the maintenance of homeostasis within cells, tissues, and organs. A balanced crosstalk between host and microbes ensures the necessary homeostasis, while dynamic changes occur in both interacting organisms. In the horse, a limited number of studies describe a microbiome in the pregnant and non-pregnant reproductive tract [33,46–49]. However, the microbial population of the equine placenta prior to parturition has not yet been investigated. The placenta enables prolonged intrauterine gestation in eutherian mammals [50] and has a lifelong effect on the well-being and health of the dam and her offspring [51–53]. Confirming and characterizing the microbial population of the placenta is a crucial step to understand the role of the microbiome in the normal function of the placenta and normal development of the fetus. Identifying the bacterial population in the equine placenta may also be an important step to further understand the underlying etiology of equine placentitis, an important disease of late gestation in the horse [54–57].

One of the accepted methods for diagnosis of placentitis is the identification of abortigenic pathogens in the placenta after parturition or abortion using bacterial culture or PCR [57]. However, the influence of the flora in the caudal reproductive tract on the composition of microbial isolates has not been investigated. This limitation of microbial identification in the expelled placenta could lead to a false positive diagnosis when investigating placentitis. In a recent epidemiological study, 13.2% of healthy postpartum placentas were positive for one of the presumed causes of equine mucoid placentitis [58], suggesting there are alternative theories that explain the transition between healthy and disease. Therefore, it is important to understand the effect of parturition, including the effect of the microbiome in the caudal reproductive tract, on the microbial population of the postpartum placentae. In the present study, we described the equine placental microbial population during healthy pregnancies in pre- and postpartum chorioallantoic samples. We hypothesized that microbial DNA is present in the equine placenta and that the postpartum placenta has a distinct microbial diversity due to the passage through the cervix and vagina during parturition.

2. Material and methods

2.1. Ethics

All animal protocols were approved by and performed in accordance with the Institutional Animal Care and Use Committee of the University of Kentucky (protocol number 2014-1341 and 2019-3669).

2.2. Experimental design

Samples were collected from 6 prepartum and 11 postpartum placentas from healthy mares (4–22 years age). For the prepartum samples, mares were euthanized at 280 days of gestation with an overdose of pentobarbital, following the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals [59]. After euthanasia, the utero-placental unit was removed, opened on a separate table and tray, and the chorioallantois was detached from the endometrium using a sterile scalpel and forceps. Placental samples were preserved in a sterile container with RNA*later* (AM7021; Invitrogen, USA), stored overnight at 4 °C, and then frozen at −80 °C until extraction [60,61]. For the postpartum samples, the gestation length was on average 351 ± 7.2 days (median = 351 days, range = 340–363 days). The chorioallantoic tissue was collected immediately after foaling using a sterile biopsy punch. The placental samples were preserved in RNA*later*, stored overnight at 4 °C, and then frozen at −80 °C until extraction in a sterile container [58]. A sample of each placenta was submitted to the University of Kentucky Veterinary Diagnostic Lab (UKVDL) for histopathology evaluation to confirm the absence of disease. Placental membranes were assessed histologically and were cultured for bacteria and fungi pathogens. All samples were confirmed to be normal with no pathogenic bacterial or fungal growth.

Additionally, four blank samples from the PBS (Gibco 10010-023, ThermoFisher Scientific, USA), used to wash the samples prior to DNA extraction, were used as the control (blank) for contaminants in the reagents and equipment [62,63].

2.3. 16S rDNA extraction and sequencing

DNA was extracted using DNeasy PowerSoil Pro Kit (Qiagen #47014, USA), as per manufacter's instructions. Briefly, 200 mg of placental tissue was cut into small pieces using sterile scalpel blades and placed in a 1.5 ml microcentrifuge tube. The samples were washed twice with PBS (500 μl) to remove RNA*later*. The tubes were centrifuged at 15,000*g* for 5 min. All centrifugation steps were performed at room temperature (15–25 °C), unless otherwise specified. The washed tissue was added to a ZR BashingBead (PowerSoil Pro Kit). CD1 solution (800 μl) was added, vortexed briefly, and incubated at 65 °C for 10 min. Next, the samples were homogenized using a Bead Ruptor (speed = Med 5 m/s, time = 30 s, cycles = 4, dwell = 1 min). Then, the tubes were centrifuged at 15,000 g for 1 min and the supernatant was transferred to a clean microcentrifugation tube. CD2 solution (200 μl) was added and the tubes were vortexed for 5 s. After an incubation at room temperature for 3–5 min the tubes were centrifuged at 15,000 g for 1 min. The supernatant (700–800 μl) was transferred to a new tube and CD3 solution (600 μl) was added. This mixture was vortexed for 5 s and then 675 μl was transferred to a MB spin column and centrifuged at 15,000 g for 1 min. This step was repeated and then the MB spin column was placed into a clean 2 ml collection tube. EA solution (500 μl) was added and the column was centrifuged at 15,000 g for 1 min. The flow-through was discarded and C5 solution (500 μl) was added. After centrifugation at 15,000 g for 1 min, the spin column was placed in a new 2 ml collection tube and spun at 16,000 g for 2 min to dry the column. The MB spin column was placed in an elution tube, C6 solution (60 μl) was added, and incubated at room temperature for 3 min. Finally, the tubes were centrifuged at 15,000 g for 1 min.

The V3-V4 domain was amplified using primer 341F (**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**(spacer) *TG*CCTACGGGNGGCWGCAG) and 806R (**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**(spacer)*CC*GGACTACNVGGGTWTCTAAT) using a two-step PCR procedure. In step one of the amplification procedure, both forward and reverse primers contained an Illumina tag sequence (bold), a variable length spacer (no spacer, A, CA, or GCA for 341F; no spacer, G, TG, ATG for 806R) to increase diversity spectrum and improve the quality of the sequencing run, a linker sequence (italicized), and the 16S target sequence (underlined). Each 25 μl PCR reaction contained 1 unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems, USA), 1.5 mM $MgCl_2$, 0.2 mM final concentration dNTP mix, 0.2 μM final concentration of each primer, 3 μl KAPA 5X Enhancer 1, and 1 μl of DNA for each sample. PCR

conditions were: an initial incubation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 45 s, 50 °C for 30 s, 72 °C for 30 s and a final extension of 72 °C for 3 min. In step two, each sample was barcoded with a unique forward and reverse barcode combination using forward primers (**AATGATACGGCGACCACCGAGATCTACAC**NNNNNNNNTCGTCGGCAGCGTC) with an Illumina P5 adapter sequence (bold), a unique 8 nucleotide barcode (N), a partial matching sequence of the forward adapter used in step one (underlined), and reverse primers (**CAAGCAGAAGACGGCATACGAGAT**NNNNNNNNGTCTCGTGGGCTCGG) with an Illumina P7 adapter sequence (bold), unique 8 nucleotide barcode (N), and a partial matching sequence of the reverse adapter used in step one (underlined). The PCR reaction in step two contained 1 unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems, USA), 1.5 mM $MgCl_2$, 0.2 mM final concentration dNTP mix, 0.2 μM final concentration of each uniquely barcoded primer and 1 μl of the product from the PCR reaction in step one diluted at a 10:1 ratio in water. PCR conditions were: an initial incubation at 95 °C for 3 min, followed by 9 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension of 72 °C for 3 min. The final product was quantified on a Qubit instrument using the Qubit High Sensitivity dsDNA kit (Invitrogen) and individual amplicons were pooled in equal concentrations. The pooled library was cleaned utilizing Ampure XP beads (Beckman Coulter, USA). Quality analysis was performed using the Nanodrop (ThermoFisher Scientific, USA). For the placenta samples, the 260/280 ratio was ≥1.8 and the 260/230 ratio was ≥2.07. For the blank samples, the 260/280 ratio was >1.8 and the 260/230 ratio ranged between 0.37 and 2.29. The library was quantified via qPCR followed by 300-bp paired-end sequencing using an Illumina MiSeq instrument (Illumina, USA) in the Genome Center DNA Technologies Core, University of California, Davis. DNA extractions and library preparation were performed by the UC Davis Host Microbe Systems Biology Core Facility. Sequences are available in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA939134.

2.4. Downstream analyses

Denoising, filtering, and trimming were performed in Mothur (version 1.48.0) [64]. The 16S rDNA SILVA v138.1 database [65] was used for mapping and assigning taxonomy. First, the contigs were obtained using the "make.contigs" command. Next, sequences were filtered using the "screen.seqs" command with maximum ambigous sequences set to 0 and maximum number of polymers set to 8. From the SILVA v138.1 database a custom database was built for the V3-V4 region using the "pcr.seqs" command. Unaligned sequences were removed using the "screen.seqs" and the "filter.seqs" commands. Sequences were further denoised using the "pre.cluster" command with the difference argument set to 2. Chimeras were removed using the "chimera.vsearch" command. Then, an ASV table was generated using the "make.shared" command and imported into R. For decontamination, contaminating reads identified in the negative control samples (PBS, blank; n = 4) were removed from the samples using Microdecon [66]. After decontamination, low abundance taxa with three or less counts in all samples were removed. The remaining taxa were split into taxonomy levels and relative abundances were calculated using the Phyloseq package [67]. Alpha diversity calculation (Shannon, Chao1, and Fisher) and beta diversity calculation (weighted UniFrac and Bray-Curtis) was performed using the *microbiome*, *amplicon*, and *vegan* packages. Alpha diversity was compared using the Tukey HSD test and beta diversity was compared using ANOVA and Analysis of Similarity (ANOSIM). Using ANCOM-BC [68], with p_adj_method set to Benjamini-Hochberg (BH), differently abundant taxa were identified. Graphs were generated using *ggplot2*, *dplyr*, *RColorBrewer*, *ggpubr*, and *lattice* packages. Bar and pie plots were generated using Microsoft Excel (Microsoft Corporation, USA). Statistical differences were declared at $p < 0.05$ throughout all analyses, unless stated otherwise.

3. Results

After applying the Mothur pipeline [64,69–76], we could identify 418,525 sequences and from these sequences, we identified 51,300 unique amplicon sequence variants (ASVs) in the samples and blanks combined. After the decontamination step, 2889 ASVs were left in the placental samples.

3.1. Prepartum

In healthy prepartum placentas, the majority of the taxa belonged to the phyla Firmicutes, Actinobacteriota, and Proteobacteria with an abundance of 29%, 29%, and 28%, respectively (Fig. 1A). The two lower abundant taxa were Bacteroidota and Acidobacteriota (Fig. 1A). When comparing individual samples, the microbial composition in five samples showed a similar pattern at the phylum level, while one sample (CA3) had a 100% abundancy of the phylum Actinobacteriota (Fig. 1B). The reason for the divergent composition in sample CA3 is unclear and could represent a natural variation of the microbiome in this apparently healthy pregnancy, or could be due to an undetected pathology, contamination of the sample or the misclassification of unknown genera.

At the genus level, the top three bacteria in the healthy prepartum samples were *Bradyrhizobium*, an unclassified *Pseudonocardiaceae*, and *Mycobacterium* (Fig. 2A). When comparing individual samples to each other, they showed diversity in the microbial composition, except for two samples. One had a high abundancy of unclassified *Pseudonocardiaceae* (68.4%, CA3) and one sample had a high abundancy of *Bradyrhizobium* (69.7%, CA5) (Fig. 2B).

3.2. Postpartum

Similar to the prepartum samples, Proteobacteria, Firmicutes, and Actinobacteriota were the top three phyla in postpartum samples, with an abundance of 33%, 23% and 21%, respectively (Fig. 3A). Bacteroidota was the fourth most abundant microbial phylum with an abundancy of 14% (Fig. 3A). A similar microbial composition was found among the individual samples (Fig. 3B).

At the genus level, the top three bacteria in healthy postpartum samples were *Acinetobacter*, Pantoea, and an unclassified *Microbacteriaceae* (Fig. 4A). The microbial compositions of individual samples were diverse (Fig. 4B).

3.3. Alpha diversity

To estimate alpha diversity, we calculated the Shannon diversity index, chao1, and Fisher index. Alpha diversity was calculated and compared using the Tukey HSD test. We found a significant higher alpha diversity in postpartum samples compared to prepartum samples for all indexes ($p < 0.05$; Fig. 5). Shannon is an estimator which evaluates the relative abundance of different OTUs, while Chao1 takes rare species into account, and Fisher is calculated based on number of taxa and number of individuals [77]. The higher these estimators, the more diverse and/or rich the sample is. These results indicate a significant higher richness and diversity in postpartum samples, with postpartum sample also having more rare species than prepartum samples.

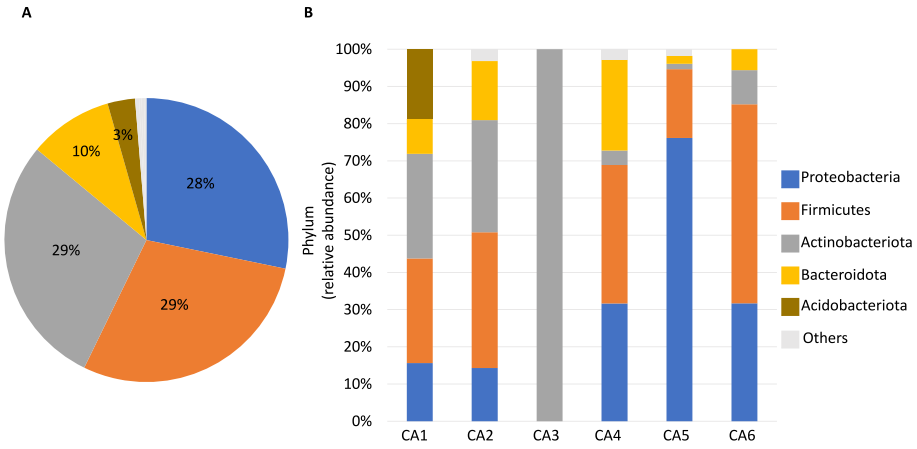

Fig. 1. Relative abundance in healthy prepartum samples (n = 6) at the phylum level. A: pie chart of the composition of the microbiome. B: relative abundance in individual samples (CA1-CA6). CA = chorioallantois.

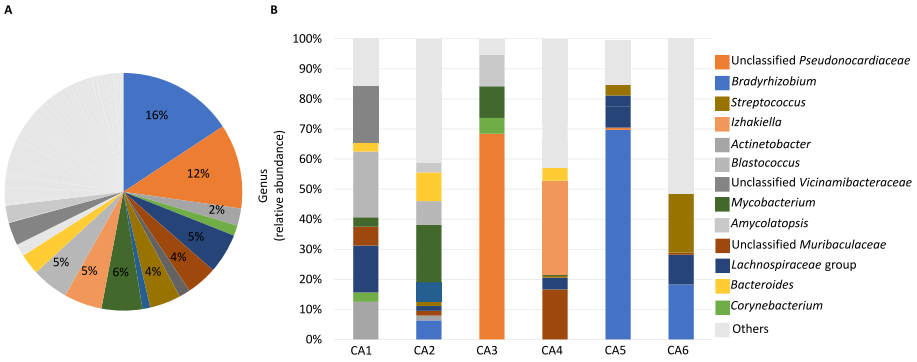

Fig. 2. Relative abundance in healthy prepartum samples (n = 6) at the genus level. A: pie chart of the composition of the microbiome. B: relative abundance in individual samples (CA1-CA6). CA = chorioallantois.

3.4. Beta diversity

To estimate beta diversity, the Bray-Curtis distance was calculated and compared using ANOVA and Analysis of Similarity (ANOSIM). A significantly different beta diversity between the two groups was observed ($p < 0.01$; Fig. 6). The ANOSIM statistic for the Bray-Curtis distance was 0.75 ($p < 0.01$; Fig. 6), with the closer this statistic is to 1, the more different the two groups are.

3.5. Differentially abundant taxa

A total of seven differentially abundant phyla between post- and prepartum samples were identified ($p > 0.05$): five phyla with a higher abundancy and two with a lower abundancy in postpartum samples compared to prepartum samples (Fig. 7). We observed 55 genera that were significantly more abundant in postpartum samples as compared to prepartum samples (Supplementary Table 1, $p < 0.05$).

4. Discussion

Recently, the human placental microbiome has been extensively researched during healthy and compromised pregnancies [1,18–20,39,41–44,75,78–80], but few publications are available in

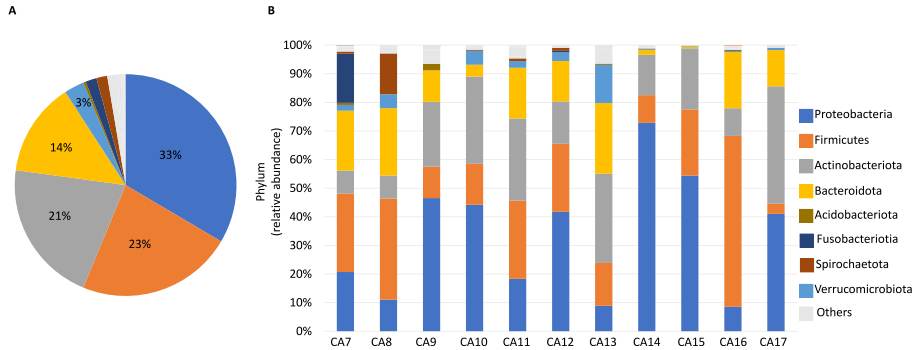

Fig. 3. Relative abundance in healthy postpartum samples (n = 11) at the phylum level. A: pie chart of the composition of the microbiome. B: relative abundance in individual samples (CA1-CA6). CA = chorioallantois.

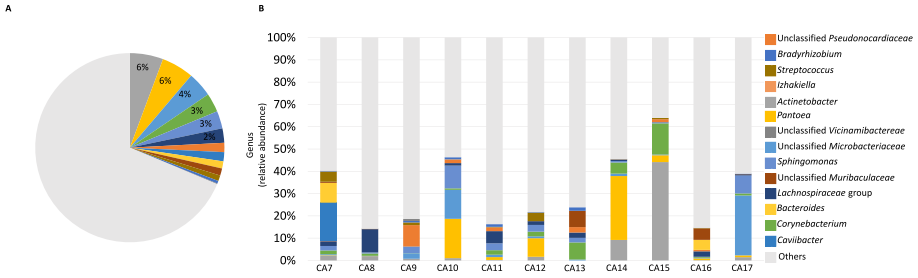

Fig. 4. Relative abundance in healthy postpartum samples (n = 11) at the genus level. A: pie chart of the composition of the microbiome. B: relative abundance in individual samples (CA1-CA6). CA = chorioallantois.

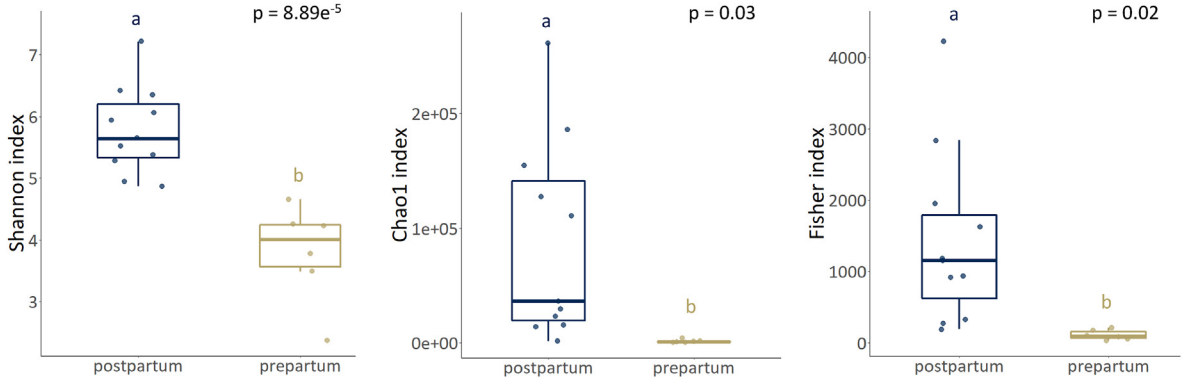

Fig. 5. alpha diversity (Shannon, Chao1, and Fisher index) in healthy pre-and postpartum samples. Alpha diversity was significantly different ($p < 0.05$) between the two groups for any index.

other mammals such as mouse, cattle, primates, and sheep [13,40,45,81–83]. In human placenta, Proteobacteria was identified as the dominant phylum in samples collected by C-section and vaginal delivery (Table 1) [1,18,39,41,81,84]. Other abundant phyla identified in healthy human placenta collected by C-section or after vaginal delivery were Firmicutes, Cyanobacteria, Spirochaetes, Tenericutes, Bacteroidetes, Fusobacteria, Actinobacteria, Acidobacteria, and Thermus [1,12,18,39,41,43,81,84,85]. In cattle, the most prevalent phyla identified in the placenta were Firmicutes, Bacteroidetes, and Proteobacteria in post-mortem samples collected in slaughterhouses [13] or after normal vaginal delivery [83] (Table 1).

In the present study, we described the presence of microbial DNA in the healthy pre- and postpartum equine placenta. The majority of the identified microbes belong to the phylum Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidota, which account for more than 75% of the phyla present in the pre- and postpartum samples. Previous studies also identified these four phyla as the dominant microbes in the equine placenta collected after normal vaginal delivery and in the non-pregnant equine endometrium (Table 1) [12,49,86–90]. The top three genera in the prepartum samples were *Bradyrhizobium*, an unclassified *Pseudonocardiaceae*, and *Mycobacterium* and in the postpartum samples were *Acinetobacter, Pantoea*, and an unclassified *Microbacteriaceae*. Bacteria previously identified in the postpartum equine placenta and equine endometrium [12,49,86,87,89] were also identified in the postpartum equine placentas in the present study (Table 2), except for *Gemella*, *Moraxella, Klebsiella*, *Peptoanaerobacter,* and *Lonsdalea* spp. In the bovine uterus, *Trueperella*, *Acinetobacter*, *Fusobacteria*, *Proteus*, *Prevotella*, *Corynebacterium*, *Staphylococcus*, *Microbacterium*, *Butyrivibrio*, *Ralstonia* and *Peptostreptococcus* spp. were previously described [13,90]. *Acinetobacter*, *Proteus*, *Prevotella*, *Corynebacterium*, *Staphylococcus*, and *Peptostreptococcus* spp. were also present in the equine placentas. Similarly, several genera that have been identified in the human placenta were also identified in our equine placental samples (Table 2). Overall, there was a notable overlap among the genera found in the placenta of different mammals, suggesting the presence of a micro-environment that allows the presence of these bacteria. Bacterial function can be different depending on the environment and substrate availability. A better insight in the function of these bacteria in the placenta could expand our understanding of the effect of the placental microbiome on the outcome of pregnancy and the impact on the dam and her offspring.

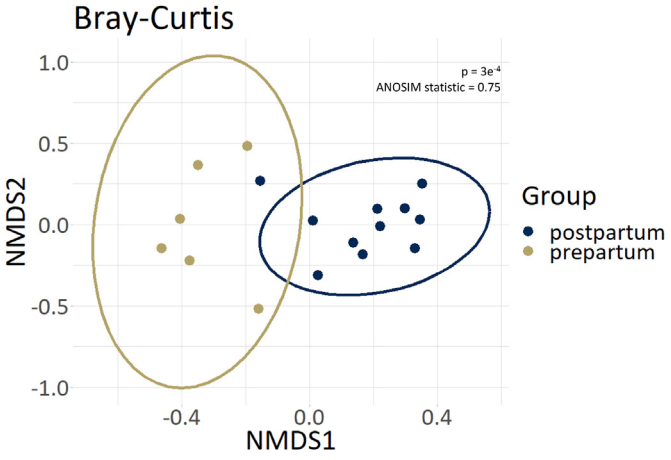

Fig. 6. Non-metric multidimensional scaling (NMDS) of beta diversity (Bray-Curtis distance) in healthy pre-and postpartum samples. Beta diversity was significantly different ($p < 0.01$) between the two groups. The ANOSIM statistic is 0.75 for the Bray-Curtis distance ($p < 0.01$).

A

phylum	LFC	Standard error	Adj. p (FDR)	Diff. abundant
Proteobacteria	2.95	0.75	0.00	True
Actinobacteriota	3.02	0.37	0.00	True
Firmicutes	2.46	0.65	0.00	True
Bacteroidota	2.82	0.48	0.00	True
Gemmatimonadota	-0.94	0.34	0.02	True
Verrucomicrobiota	2.81	0.39	0.00	True
Armatimonadota	-0.83	0.27	0.01	True

B

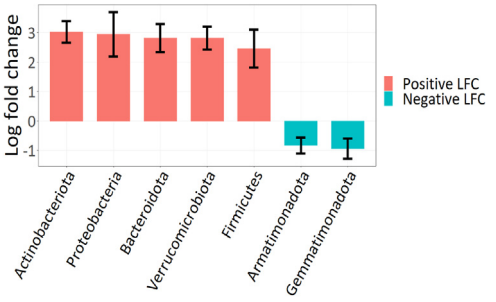

Fig. 7. A: Differentially abundant taxa in healthy postpartum samples compared to healthy prepartum samples. LFC: log fold change, negative fold change indicates lower abundance in postpartum samples. FDR: false discovery rate, using the Benjamini-Hochberg method (BH). B: box plot of the log fold change in postpartum samples compared to prepartum samples.

Of note, similar to previous reports [4,63,85,91,92], some of the above-mentioned genera (*Acinetobacter*, *Bradyrhizobium*, *Pseudomonas*, and *Coryenbacterium,* among others) were also found in our blank samples and consequently could be considered as contaminants. However, even after *in silico* decontamination, these genera were still present in the ASV table. This finding highlights the importance of the decontamination step during data analysis. Additionally, these genera were previously identified in the endometrium and/or placenta using 16S and/or WGS in different mammals [1,13,18–20,39,49,78,79,84,85,90], suggesting that these genera are indeed present in placental samples with a higher abundance than those in the blank samples. It is also important to consider that due to the high sequence similarity of the 16S gene, the 16S rDNA-sequencing method has a limited power in detecting bacteria at the genus level. In future studies, the presence of the identified genera needs to be confirmed with higher resolution sequencing techniques such as metagenomic and metatranscriptomic techniques.

Although we identified several bacteria in equine healthy placentas and several other studies have reported the presence of microbes in the healthy placentae of other species, the existence of a placental microbiome remains a controversial topic [8,12,26,93,94]. This controversy is mainly due to limitations associated with microbial growth and the detection methods [63,95]. The disagreement around the presence of a placental microbiome revolves around the question whether the microbial DNA found in the placenta is originating from viable microbes that are part of a microbiome, or from microbial DNA that reached the placenta through the maternal blood circulation or contaminating microbial DNA present in the environment such as the extraction kits (so called 'kitome') [7,8,21,91,94–97]. Most previous studies and the current study were not aimed to distinguish between live or dead microbes. Thus, the current results demonstrate at the minimum the presence of microbial DNA in this body niche, rather than an active microbiome [98]. One of the main limitations of 16S rDNA-seq and a metagenomic method is the difficulty to distinguish between microbial DNA that is part of an active microbiome

Table 1
Overview of phyla present in equine, human and bovine placenta and endometrium.

Species	Equine						Human			Bovine		
Tissue	Prepartum placenta	Postpartum placenta	Postpartum placenta	Postpartum placenta	Non-pregnant endometrium	Non-pregnant endometrium	Term (VD and CS) placenta	Term and preterm (VD and CS) placenta	Term (CS) placenta	Abortion (at 30 –60 days) placenta	Uterus and placenta (virgin and pregnant resp)	Postpartum placenta
Reference	Current study	Current study	Xia et al., 2017 [86] (abstract)	Sones and Heil, 2018 [87] (abstract)	Sathe et al., 2017 [88] (abstract)	Holyoak et al., 2018 [89] (abstract)	Holyoak et al., 2022 [49]	Parnell et al., 2017 [39]	Aagaard et al., 2014 [1]	Collado et al., 2016 [18]	Zhu et al., 2018 [84]	Moore et al., 2017 [13] Hummel et al., 2021 [83]
Firmicutes	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓
Proteobacteria	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Bacteroidetes	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓
Actinobacteria	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓
Acidobacteria	✓	✓					✓					
Fusobacteria	✓	✓						✓				✓

VD = vaginal delivery; CS = caesarean section.

Table 2
Overview of genera present in equine placental samples and in the literature.

	Equine						Human						
Tissue	Prepartum placenta	Postpartum placenta	Postpartum placenta	Postpartum placenta	Non-pregnant endometrium	Non-pregnant endometrium	Term and preterm (VD and CS) placenta	Term placenta (CS)	Term (CS) placenta	Abortion (at 30–60 days) placenta	Term and preterm placenta (VD)	Term placenta (VD)	Term and preterm placenta (VD and CS)
Reference	Current study	Current study	Xia et al., 2017 [86] (abstract)	Sones and Heil, 2018 [87] (abstract)	Holyoak et al., 2018 [89] (abstract)	Holyoak et al., 2022 [49]	Aagaard et al., 2014 [1]	Benny et al., 2021 [41]	Collado et al., 2016 [18]	Zhu et al., 2018 [84]	Prince et al., 2016 [20]	Zheng et al., 2015 [78]	Doyle et al., 2014 [79]
Bacillus		✓	✓	✓		✓					✓	✓	
Mycoplasma		✓	✓	✓			✓						✓
Clostridium		✓	✓	✓		✓			✓		✓	✓	
Pseudomonas	✓	✓			✓	✓						✓	
Porphyromonas		✓			✓	✓							
Streptococcus	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓	✓
Lactobacillus	✓	✓				✓		✓	✓		✓	✓	✓
Prevotella		✓				✓	✓					✓	✓
Escherichia/ Shigella		✓				✓	✓		✓	✓	✓	✓	
Blautia	✓	✓				✓							
Staphylococcus		✓				✓	✓		✓	✓		✓	✓
Acinetobacter	✓	✓				✓	✓			✓	✓	✓	✓
Ureaplasma		✓					✓				✓		✓
Paenibacillus		✓					✓						
Haemophilus	✓	✓				✓	✓	✓					
Enterococcus	✓	✓				✓		✓		✓		✓	
Chryseobacterium	✓	✓						✓				✓	
Lysinibacillus	✓	✓						✓				✓	
Citrobacter		✓								✓			
Aeromonas		✓								✓			
Sphingomonas		✓								✓			
Micrococcus		✓								✓			
Peptostreptococcus		✓				✓							
Bradyrhizobium	✓	✓									✓		
Blastococcus	✓	✓											
Corynebacterium	✓	✓				✓						✓	✓
Actinomyces	✓					✓							
Ruminococcus	✓	✓				✓					✓	✓	
Chryseobacterium	✓	✓											
Paracoccus	✓	✓										✓	

VD = vaginal delivery; CS = caesarean section.

in the placenta and contaminating DNA [7,8,21,29,69,91,94,95,97]. Some of the disadvantages associated with DNA-based sequencing methods in low biomass samples may be avoided by using techniques such as metatranscriptomics to look at the gene expression of live bacteria in combination with metametabolomics. Generating 'multi-omics' datasets would help in elucidating the presence of a placental microbiome [99,100]. During the last few years, there has been more evidence regarding the presence of microbes in the placenta in health and disease [1,18–20,39,41–44,75,78–80]. Yu et al. (2021) were able to track labelled *Staphylococcus aureus* to the placenta with a bacterial tracking technique using fluorescent microscopy [45]. The presence of an endometrial microbiome is increasingly accepted in several mammals, including humans [13,17,24,25,30,35,38,49,70,89,101–104]. The disappearance of the endometrial microbiome after establishment of pregnancy and before the development of the placenta would require a process which has not been yet characterized. Using multi-omics to investigate the presence of a microbiome could further shed light on the endometrial and placental microbiome.

Comparing alpha and beta diversity between pre- and postpartum placentas revealed significant differences, with postpartum samples having a higher diversity. We also identified seven differentially abundant phyla and 55 differentially abundant genera between the two groups. These observations suggest possible contamination of the placenta during its passage through the birth canal. However, our findings are different from the findings of Parnell et al. (2017), who found no significant differences between vaginal and C-section delivered placenta in women [39]. This could be attributed to differences in the anatomy of the birth canal and the environment in which parturition takes place in human and horses. Undoubtedly, the foaling environment could lead to more contamination than a human hospital environment would, and this limitation needs to be considered when assessing microbial DNA found in clinical samples, especially if the placenta has been separated and collected in a nonsterile environment. In the present study, the samples were taken immediately after passage through the birth canal. Therefore, several of identified microbes are likely related to the caudal reproductive tract and vaginal flora. Barba et al. (2020) reported that *Corynebacterium*, *Akkermansia*, *Campylobacter*, *Fusobacterium*, *Porphyromonas*, *Helcococcus*, *Arcanobacterium*, and *Petoniphilus* were among the most abundant genera in the vagina of the mare [48]. These genera were only present in our postpartum samples, except for *Corynebacterium* that was present in both pre- and postpartum samples. *Corynebacterium* was the fourth most abundant bacteria in the postpartum samples and had a significantly higher abundancy in postpartum samples than prepartum samples (35% vs 8%, respectively). Additionally, Rodríguez-Lázaro et al. (2022) isolated *Acinetobacter* spp. from the vagina of mares [105]. In the present study, *Acinetobacter* is the most abundant genera found in postpartum samples. These parallels further suggest that the difference in microbial composition between pre- and postpartum samples is likely caused by vaginal contamination during parturition.

Understanding the microbial population in the healthy placenta will also allow us to better diagnose and treat conditions associated with pathogenic microbes, such as those involved in equine placentitis. For example, one of the routine methods for identifying the cause of abortion in mares is microbiologic assessment using culture methods and/or polymerase chain reaction (PCR) to detect the presumed causative agents in samples submitted for diagnosis [57]. Previous studies demonstrated the possibility of false positive detection of bacteria in the healthy equine placenta [57,58,106,107].

In conclusion, we have demonstrated the presence of bacterial DNA in the placenta of healthy equine pregnancies. The majority of bacteria belonged to the genera *Bradyrhizobium*, an unclassified *Pseudonocardiaceae*, *Acinetobacter*, *Pantoea*, and an unclassified *Microbacteriaceae*. We observed a significant difference in the microbial composition in pre- and postpartum placentae, which could be due to contamination of the samples with vaginal flora during the passage of the placenta through the birth canal. This contamination could have a significant influence on microbes found in postpartum placental tissue when using DNA based methods. This information needs to be taken into consideration in future studies and in clinical cases where the causative agent(s) of abortion need to be determined. Overall, using a DNA-based method, it remains unclear if the bacteria found in pre- and post-partum samples are metabolically active and crosstalk with the host.

CRediT authorship contribution statement

Machteld van Heule: Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **Hugo Fernando Monteiro:** Investigation, Writing – review & editing. **Ali Bazzazan:** Investigation, Writing – review & editing. **Kirsten Scoggin:** Sample collection, Methodology, Conceptualization. **Matthew Rolston:** Methodology. **Hossam El-Sheikh Ali:** Investigation, Writing – review & editing. **Bart C. Weimer:** Resources, Investigation, Conceptualization, Writing – review & editing. **Barry Ball:** Resources, Conceptualization, Funding acquisition. **Peter Daels:** Resources, Investigation, Writing – review & editing. **Pouya Dini:** Funding acquisition, Conceptualization, Investigation, Methodology, Writing – review & editing, Supervision, All of the authors have agreed to the submission of this manuscript and to be responsible for its contents and declare no conflicts of interest.

Declaration of competing interest

The authors have no conflict of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other journal.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.04.022.

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