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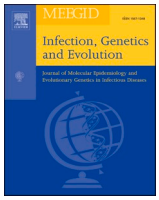

Review

Longitudinal study of the bovine cervico-vaginal bacterial microbiota throughout pregnancy using 16S ribosomal RNA gene sequences☆

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ABSTRACT

The microbiota composition of the bovine female reproductive tract influences reproductive efficiency, susceptibility to genital pathogens, and the health of newborn calves. However, knowledge about cervico-vaginal microbiota during gestation is scarce. Therefore, the present study aimed to analyze the taxonomic profile of the cervico-vaginal bovine microbiota throughout pregnancy and after calving using high-throughput sequencing of a fragment of the 16S ribosomal RNA gene.

Healthy nulliparous Holstein heifers ($n = 13$) with similar age and body conditional score were selected to collect samples from the cervico-vaginal area with a sterile swab at 5 timepoints. We sequenced the V1-V2 region of the 16S ribosomal RNA gene and analyzed data using the DADA2, phyloseq and vegan R Studio packages.

No differences were observed in alpha and beta diversity across sampling points, accounting for the stability of the microbiota throughout pregnancy. The most abundant phyla are Firmicutes, Bacteroidota, Proteobacteria and Actinobacteria, and are present as the main taxa in all five sampling points. Also, several of the least abundant taxa can be observed to change with time.

Our comprehensive study of the cervico-vaginal bacterial microbiota during the gestation period contributes to the knowledge of microbiota dynamics on the bovine reproductive tract during and after pregnancy and can serve as a baseline for future research and the development of potential therapeutic interventions.

1. Introduction

A beef and dairy cattle system's efficiency depends on the production of one calf a year per cow (Diskin et al., 2016). This goal is rarely achieved (Ault-Seay et al., 2023), partly because of reproductive failures, whose etiology is multifactorial. The main factors that influence bovine reproductive outcome are nutrition and management, infectious diseases (Moore et al., 2021), hormonal dysfunctions (Saraswat and Purohit, 2016) and genetic traits (Diskin et al., 2016). Therefore, establishing the cause of reproductive failure or impairment in cows is challenging.

One factor that has gained attention lately is the microbiota composition of the bovine female reproductive tract. In humans, multiple studies showed that the reproductive tract microbiota and its variations have a great influence on reproductive health, being associated with cases of infertility (Mor et al., 2015) and with infant health (Jašarevi et al., 2017; Prince et al., 2015). In bovines as well, reproductive efficiency, susceptibility to genital pathogens, and the health of newborn calves are all influenced by the microbiome (Ault-Seay et al., 2023). Alterations of the composition of the microbiota in the genital tract of the bovine female can have an impact in pregnancy outcome, as has been shown in several studies (Ault-Seay et al., 2022; Ault et al., 2019a, 2019b; Cassas et al., 2024; Deng et al., 2019; Laguardia-Nascimento et al., 2015; Messman et al., 2019). The microbiota variation among different regions of the reproductive tract may be influenced by the rearing, nutritional and reproductive management (Adnane and

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Chapwanya, 2022; Luecke et al., 2022). Inflammatory diseases may also disturb the composition of the microbiota in the genital tract of the bovine female and impact the pregnancy outcome (Ault-Seay et al., 2022; Ault et al., 2019a, 2019b; Cassas et al., 2024; Deng et al., 2019; Laguardia-Nascimento et al., 2015; Messman et al., 2019).

Recently, in vivo studies of the bovine genital microbiota (Bicalho et al., 2017a, 2017b, 2017c; Laguardia-Nascimento et al., 2015; Machado et al., 2012; Swartz et al., 2014) have shown that changes in the microbiota composition of the bovine female reproductive tract is associated with uterine diseases and reproductive failures. However, there is still not enough information to evaluate the main factors that influence the composition of the reproductive tract microbiota, or its correlation with the reproductive cycle (Adnane et al., 2024; Laguardia-Nascimento et al., 2015; Quereda et al., 2020; Swartz et al., 2014).

Unlike vaginal microbiota of humans and other primates, the ruminant genital bacterial communities are highly diverse (Swartz et al., 2014). *Lactobacillus*, which is the dominant genus in human vaginal microbiota, is not very abundant in bovines (Ault et al., 2019a; Clemmons et al., 2017; Laguardia-Nascimento et al., 2015; Quereda et al., 2020; Swartz et al., 2014). The most abundant phyla are Firmicutes, Bacteroidetes, and Proteobacteria (Appiah et al., 2020; Laguardia-Nascimento et al., 2015; Nesengani et al., 2017). Some additional phyla, for example Fusobacteria, were found in high proportion in a few studies (Messman et al., 2019; Quereda et al., 2020; Swartz et al., 2014).

Some taxa in the microbiota of cattle genital tract are suggested to be important as biomarkers of reproductive outcome (Ault et al., 2019b; Deng et al., 2019). For instance, the presence of *Histophilus*, Clostridiaceae, and *Campylobacter* in the vagina has been identified as indicators of pregnancy failure (Deng et al., 2019). Studies comparing pregnant and non-pregnant animals also show that pregnant heifers and cows have a less diverse microbiota than non-pregnant ones (Ault-Seay et al., 2023; Laguardia-Nascimento et al., 2015). Additionally, multiple taxa abundances change throughout the estrous cycle (Ault et al., 2019a). However, studies of these changes during pregnancy are very scarce (Srinivasan et al., 2021). Therefore, knowledge about microbiota composition throughout the pregnancy is of paramount importance to prevention and treatment of reproductive problems.

Most of the abovementioned studies have reported on the uterus environment, while few studies (Appiah et al., 2020) have described the microbiota composition of the cervico-vaginal site. While the uterus has a unique microbiome during pregnancy (Karstrup et al., 2017), the cervix is responsible for controlling the flow between the external vagina and endometrial environments (Galvão et al., 2019), acting as a biological and physical barrier against microorganisms that can migrate to the endometrial site from the lower genital tract (Sheldon and Dobson, 2004). A recent study has shown that the nature of cervico-vaginal microbiota may be useful in determining uterine health status (Chen et al., 2017).

Our hypothesis is that the cervico-vaginal microbiota in pregnant heifers contain taxa that can be considered as biomarkers of a healthy reproductive tract, and our study will allow us to identify them. Thus, the present study aimed to analyze the taxonomic profile of the cervico-vaginal bovine microbiota throughout pregnancy and after calving using high-throughput sequencing of a fragment of the 16S ribosomal RNA gene.

2. Materials and methods

2.1. Animals

Healthy nulliparous Holstein heifers ($n = 13$) with similar age (14–16 months old) and body conditional score (3–3.5, scale 1 to 5, Edmonson et al., 1989) were selected for a longitudinal sampling during gestation and postpartum period. All animals were located at the experimental dairy unit of the "La Estanzuela" station of the INIA (Uruguay, 34°20′14"S, 57°41′32"W), raised and kept in a pasture-based system as previously described (Stirling et al., 2021). All cows were in outdoor paddocks around 3 weeks before the expected calving date, where cows were fed a partial mixed ration once a day (López Radcenco et al., 2021).

2.2. Sample collection and study design

Swab samples were taken from the exterior portion of cervix entrance. A double-guarded equine uterine culture swab (Minitube Ref. 17214/2950) guided by a vaginoscopy was used to collect the samples. In brief, the vulva region and perineum were washed using a disinfectant composed by iodophor and nonilfenoxi polietoxi etanol and dried with paper towels. The vaginoscopy was disinfected and cleaned among animals before introducing it into the vagina for sample collection. After identification of the cervix, the swab was exposed to touch the cervix and rotated 5 times to each side. The head of the swab was aseptically cut and placed into a dry sterile 1.5 mL minicentrifuge tube, transported to the laboratory on ice, and stored at −80 °C until DNA extractions.

A total of 5 samples per cow were collected according to the following time points: day of artificial insemination (AI, estrous phase, sampling point 1), days 30–46 (pregnancy diagnosis by ultrasonography, sampling point 2), 90–180 (sampling point 3), 180–270 of gestation (sampling point 4), and 48–78 days after calving (sampling point 5). On the day of AI, the swab collection was performed prior to insemination.

2.3. DNA Extraction and 16S rRNA gene amplicon sequencing

Genomic DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) with swabs protocol. On the first step of the protocol, 20 μL proteinase K and 200 μL of AL buffer were directly added to the swab and incubated at 56 °C for 1 h. Protocol was then continued according to the manufacturer's instructions. DNA was eluted in 50 μL of deioinized H_2O, quantified and stored at −20 °C.

The V1-V2 region of the 16S ribosomal RNA gene was amplified by PCR using specific primers 27F and 338R (Walker et al., 2020) with Illumina adapters (Illumina, 2013). The PCR mix contained 20–40 ng of genomic DNA as a template, 0.5 U of a high-fidelity DNA polymerase (Q5 Hot Start High-Fidelity DNA Polymerase, NEB), 1× reaction buffer, 200 μM dNTPs, 0.32 mg/mL bovine serum albumin (BSA) and 0.4 μM of each primer in a final volume of 25 μL. Amplification conditions were: 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 57 °C for 1 min, and 72 °C for 1 min 30 s, and finally by 72 °C for 10 min. The PCR reactions were checked by electrophoresis in 0.8% agarose gels stained with ethidium bromide (Invitrogen Corp., Carlsbad, CA) and observed under ultraviolet light. Blank and mock community (ZymoBIOMICS Microbial Community DNA Standard, Zymo Research Corp.) controls were included in every PCR reaction. Amplicons were purified with AMPure XP kit (Beckman Coulter, USA) and quantified.

Libraries were prepared by indexing 25–100 ng of PCR product with the Nextera XT Index Kit v2 (Illumina Inc., San Diego, CA) using the same PCR conditions as the previous one but without the addition of BSA. Cycling conditions were: 68 °C for 3 min, 98 °C for 3 min, followed by 8 to 12 cycles of 98 °C for 45 s, 62 °C for 30 s, and 68 °C for 2 min, and finally by 68 °C for 1 min. Then, the libraries were quantified, normalized, pooled, denatured, and sequenced in an Illumina MiniSeq System using a MiniSeq Mid Output Reagent Cartridge (300 cycles). The samples were randomly grouped at the PCR stage and again at the sequencing stage. The mock community libraries were included, ensuring at least one was present in every sequencing run as a control.

All DNA quantifications were made in a Qubit 3.0 fluorometer (Thermo Scientific) using DNA High-Sensitivity kit (Invitrogen, USA).

2.4. Sequence processing and statistical analyses

All sequence processing and statistical analyses were performed in R

4.3.2 (R Core Team, 2023). The sequencing reads were filtered and trimmed, chimeras were removed and ASVs (Amplicon Sequence Variants) were detected with the DADA2 package v.1.30.0 (Callahan et al., 2016). Taxonomy was assigned using the Silva database v.138 (Quast et al., 2013). ASVs classified as Eukaryota were removed from the dataset. Rarefaction curves were made using MicrobiotaProcess package v.1.14.0 (Xu et al., 2023). Then, data were rarefied to 9000 reads per sample, ensuring alpha diversity reached a plateau (Fig. S1). Lack of sequencing bias was ensured by comparing bacterial mock community DNA controls composition obtained in this work with its manufacturers' declared compositions.

Statistical analyses were performed with the phyloseq v.1.46.0 (McMurdie and Holmes, 2013) and vegan v. 2.6–4 (Oksanen et al., 2022) packages.

Alpha diversity was analyzed using observed richness (the number of ASVs per sample), Shannon's diversity and InvSimpson richness indexes. Differences in alpha diversity of the five sampling points were tested using Kruskal Wallis non-parametric test. To assess changes in community structure during pregnancy (β-diversity), a Principal Coordinates Analysis (PCoA) of Bray–Curtis distances was performed. Clustering between samples from different time points was evaluated using a PERMANOVA analysis with "adonis" function in the vegan package. The dispersion of time point groupings was analyzed using "betadisper" function in vegan and evaluated with ANOVA. Venn diagrams were generated to show the overlapping of taxa among sampling points, without considering their abundance. Relative abundance of most abundant taxa was graphically explored, and differential relative abundances of taxa between sampling points were analyzed using ANCOM-BC v. 2.4.0 (Lin and Peddada, 2020).

For all analyses, significance cutoff was set at $p \leq 0.05$. Graphs were created using R packages ggplot2 v.3.5.0 (Wickham, 2016), vegan and VennDiagram v.1.7.3 (Chen, 2022).

3. Results

3.1. Sequence information

Five samples from each animal ($n = 13$) were successfully sequenced, giving between 40,451 and 1,048,962 raw reads (mean 119,175). After trimming, denoising, chimera removal and exclusion of eukaryotic sequences, the minimum number of reads per sample was 9079.

Analysis of mock community sequences ($n = 23$, at least one for every sequencing run) was conducted separately. The lack of sequencing bias was ensured by comparing the observed composition with the theoretical compositions provided by the manufacturers (data not shown).

A total of 14,028 different ASVs were detected among all 65 samples. After rarefaction, 13,012 ASVs remained.

3.2. Diversity analysis

The diversity of bacterial communities and comparison among sampling points were explored through alpha and beta diversity analyses.

For all alpha diversity metrics, there were no differences ($p > 0.05$) observed between the five sampling points nor between individuals using the Kruskal-Wallis test (Fig. 1A-C).

A Principal Coordinates Analysis (PCoA) revealed no distinct clustering of samples by sampling point, with minimal variation explained by the first ordination axes (Fig. 2A, B). The adonis test indicated a weak but significant effect of sampling point on community structures (pseudo-F = 1.3, R2 = 0.08, $p = 0.003$). However, betadisper analysis demonstrated significant heterogeneity in data dispersion ($p = 0.035$; Fig. 2C), suggesting that these differences may be attributed to that. Also, very little of the variability (8.5%) can be explained by the sampling point variable.

3.3. Taxonomic composition exploration

Among the 13,012 ASVs detected in the rarefied dataset, two belong to the kingdom Archaea, but no further classification was achieved. Abundance of these ASVs was very low (0.01%) and they were present only in sampling points 2 and 3 (Table S1). The remaining ASVs belonged to kingdom Bacteria and were classified in 31 phyla, 404 families and 979 genera.

Venn diagrams were generated to show the overlap of taxa among sampling points. At the phylum level, most of the taxa (21) are shared between all five sampling points, with 8 phyla shared between 2, 3 or 4 sampling points, and 3 phyla exclusively found in one sampling point (Fig. 3A). Similar patterns are observed at the family (Fig. 3B) and genus (Fig. 3C) levels.

3.4. Temporal dynamics: comparative relative abundance of taxa across sampling points

The relative abundance of taxa in heifers throughout pregnancy is illustrated in Fig. 4A-C. Firmicutes (45–59%), Bacteroidota (10–16%), Proteobacteria (11–29%) and Actinobacteria (7–13%) were consistently the most abundant phyla across all five sampling points, collectively comprising over 90% of the total microbiota in each sampling point (Table S1). No significant differential relative abundances of these four taxa between sampling points were detected (ANCOM-BC q value >0.05; Table S2). Additionally, each sampling point exhibited between 5 and 6 phyla with relative abundance exceeding 1% (Table S1).

At the family level, the twelve most abundant taxa collectively account for more than 50% of the total variability at each sampling point. Five taxa consistently rank among the twelve most abundant across all five sampling points: Oscillospiraceae (6–9%) and Lachnospiraceae (4–6%) families, orders Bacteroidales (3–6%) and Oscillospirales (2–3%), and class Clostridia (3–4%; Table S1). ANCOM-BC analysis did not reveal significant differences for these taxa (q value >0.05; Table S2; Fig. 5). Additionally, families such as Pseudomonadaceae, Mycoplasmataceae, Rikenellaceae, UCG-010, Peptostreptococcaceae, Comamonadaceae, and Bifidobacteriaceae were consistently among the most abundant on three or four sampling points. Each sampling point contained between 23 and 28 taxa with relative abundances greater than 1% (Table S1).

At the genus level, the twelve most abundant taxa collectively

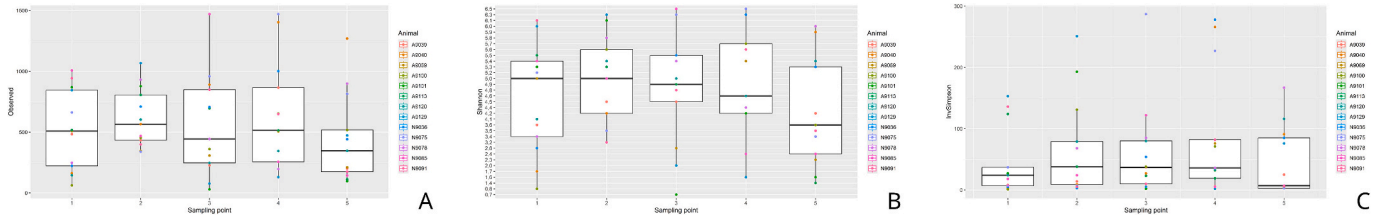

Fig. 1. Alpha diversity boxplot of the cervico-vaginal bacterial microbiota in heifers with different measures. A. Observed number of ASVs. B. Shannon diversity index. C. InvSimpson index. Each box represents a sampling point. Dots represent values for individual samples, colored by the animal.

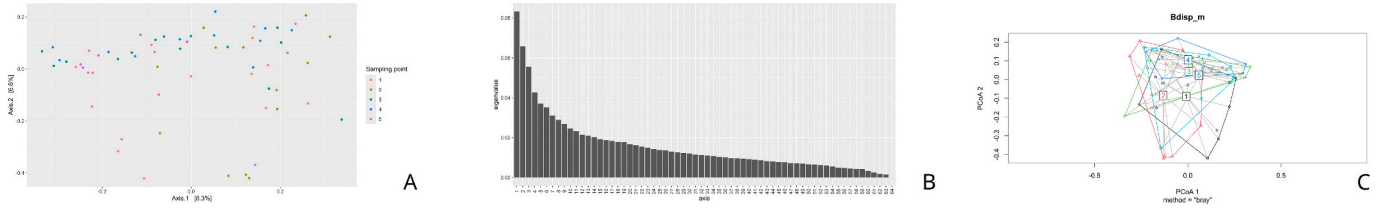

Fig. 2. Beta diversity analysis. A. PCoA plot of Bray–Curtis distances between samples. Each dot corresponds to a sample, colored according to the sampling point. B. Eigenvalues of the PCoA plot axes. C. Betadisper test graphic visualization. Each point is a sample. The colors and shapes of the symbols represent a sampling point. Each sample is connected to the centroid of its group by a line.

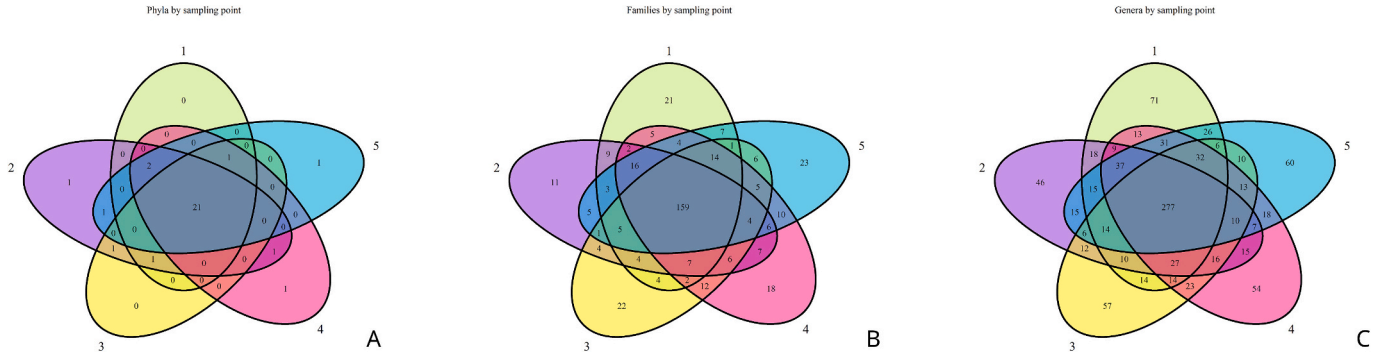

Fig. 3. Venn Diagrams showing the number of shared and unique taxa between the sampling points. A. Phyla (n = 31). B. Families (n = 404). C. Genera (n = 980).

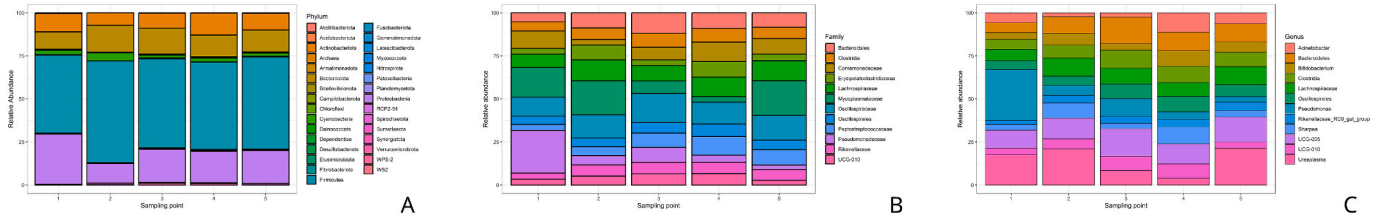

Fig. 4. Mean relative abundance of identified taxa in the 13 samples from each sampling point. A. All 31 phyla B. The top 12 families C. The top 12 genera.

represent more than 40% of the total variability at each sampling point. Five taxa consistently rank among the twelve most abundant across all five sampling points: genus UCG-005 (4–7%), family Lachnospiraceae (3–5%), orders Bacteroidales (3–6%) and Oscillospirales (2–3%) and class Clostridia (3–4%; Table S1). ANCOM-BC analysis did not show significant differences for these taxa (q value >0.05; Table S2). Additionally, genera such as *Pseudomonas*, *Ureaplasma*, *Bifidobacterium*, UCG-010, *Sharpea*, and Rikenellaceae_RC9_gut_group were consistently among the top twelve most abundant in three or four sampling points (Table S1).

ANCOM-BC results indicate a temporal pattern effect on the relative abundance of taxa. Several taxa show significant changes in their relative abundance throughout pregnancy. Eight phyla, 72 families, and 190 genera exhibit significant changes in relative abundance in some sampling points (Table S2).

Phyla with the most notable changes include: Gemmatimonadota, which diminished from sampling point 1 to 2 (lfc = −1.15) and raises its abundance from the sampling point 2 to 3 (lfc = 1.79); Acidobacteriota, which diminished strikingly from sampling point 1 to 2 (lfc = −2.09); Sumerlaeota, which raises its abundance from the sampling point 2 to 3 (lfc = 2.27) and Armatimonadota, which diminished from sampling point 1 to 2 (lfc = −1.92) and raises from the sampling point 3 to 4 (lfc = 1.07) (Table S2).

The families with the most relevant changes are: from sampling point 1 to 2: Fimbriimonadaceae (lfc = −1.55), Hyphomicrobiaceae (lfc = −1.31), Gemmatimonadaceae (lfc = −1.26), Succinivibrionaceae (lfc = 2.60), Sandaracinaceae (lfc = 1.25) and Azospirillaceae (lfc = 1.13); from sampling point 2 to 3: Succinivibrionaceae (lfc = −3.11), Fusobacteriaceae (lfc = −1.20), Leuconostocaceae (lfc = −1.16), Sumerlaeaceae (lfc = 1.81), p-2534-18B5_gut_group (lfc = 1.13) and Gemmatimonadaceae (lfc = 1.08); from sampling point 3 to 4: Thermaceae (lfc = −1.04), Vibrionaceae (lfc = −0.92), M2PB4-65_termite_group (lfc = −0.82), Hyphomicrobiaceae (lfc = 1.25), Sanguibacteraceae (lfc = 1.05) and Chthonomonadaceae (lfc = 1.01); from sampling point 4 to 5: Sanguibacteraceae (lfc = −0.99), Mycobacteriaceae (lfc = −0.95), Iamiaceae (lfc = −0.88), Fusobacteriaceae (lfc = 2.07), Rhizobiales_Incertae_Sedis (lfc = 1.18) and Reyranellaceae (lfc = 1.08) (Table S2).

4. Discussion

In bovines, humans and other mammals, the paramount importance of a healthy microbiota to warrant a good outcome of pregnancy has been described (Barba et al., 2020; Cassas et al., 2024; Garcia-Garcia et al., 2022; Kiefer et al., 2021; Li et al., 2017; Mahalingam et al., 2019; Messman and Lemley, 2023; Miller et al., 2017; Moreno and Simon, 2019; Rhoades et al., 2021; Wang et al., 2022). However, information about the bovine reproductive tract microbiota profile throughout the pregnancy is scarce. To our knowledge, only one study (Deng et al., 2019) has described the lower reproductive tract (vagina) microbiota profile throughout gestation. Therefore, our study describes for the first time the microbiota profile of the cervico-vaginal site from the day of

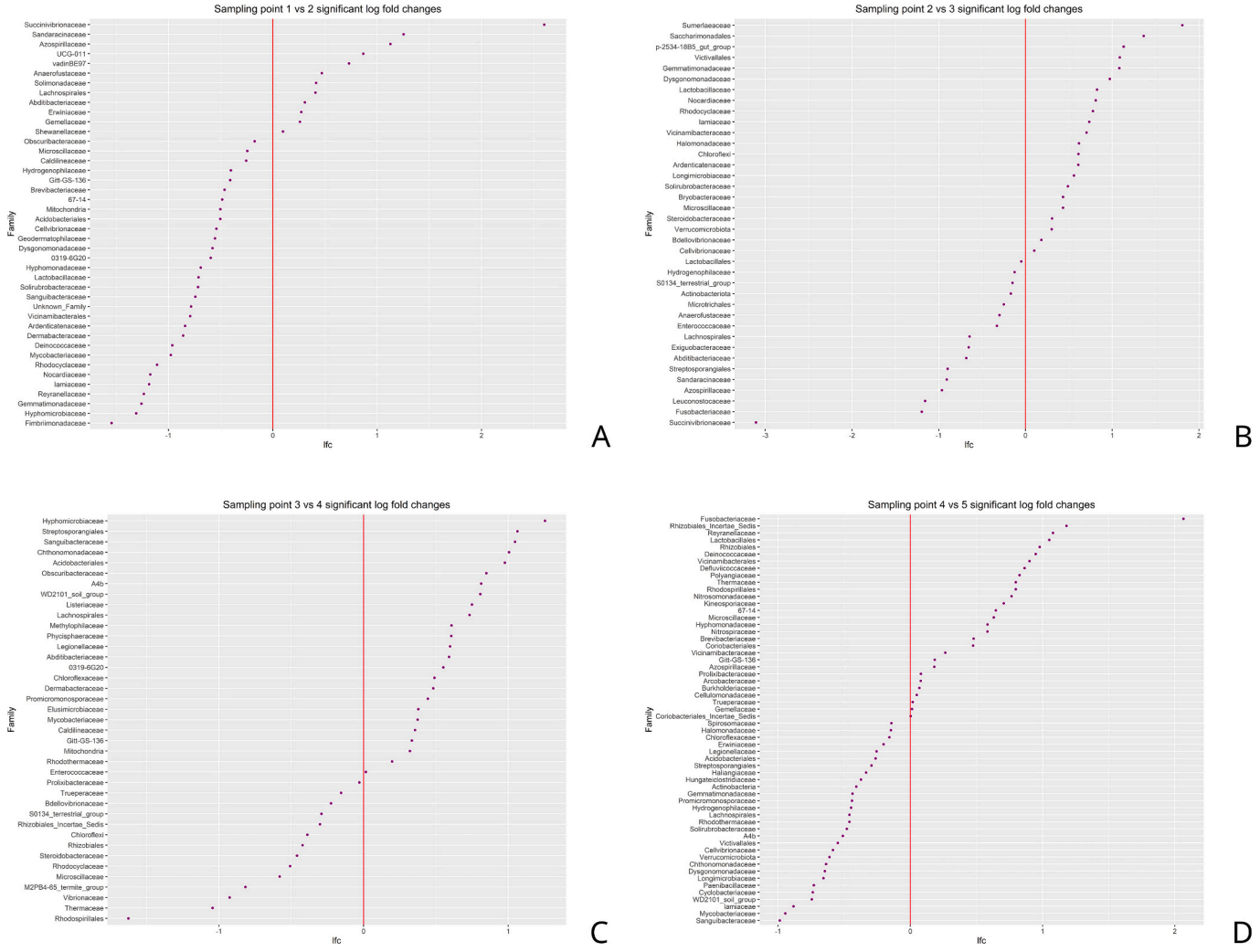

Fig. 5. ANCOM-BC significant results. Significant differences in relative abundance (q value >0.05) at family level are shown. A. Sampling point 1 to 2. B. Sampling point 2 to 3. C. Sampling point 3 to 4. D. Sampling point 4 to 5. lfc: log fold change. lfc negative values indicate the relative abundance decreases from one sampling point to the next, positive values indicate an increase.

insemination until after parturition in pregnant heifers.

In several studies, the microbiota of the bovine reproductive tract showed variations between stages of the cycle: proestrus, estrus, metestrus, and diestrus have different populations, and a comparison of follicular and luteal phases also showed changes (Ault et al., 2019a; Quereda et al., 2020). Our results indicate that, once pregnancy is established, the cervico-vaginal microbiota remains stable during the entire gestation period and several days after calving. Most of the phyla are consistently present across all five sampling points, and the most abundant phyla—Firmicutes, Bacteroidota, Proteobacteria and Actinobacteria—were present as the main taxa in all five sampling points, accounting for the stability of the microbiota along the pregnancy and after parturition. These results are consistent with those of Laguardia-Nascimento et al. (2015) and Nesengani et al. (2017) who analyzed the vaginal microbiota in heifers on the first trimester of pregnancy. Furthermore, these taxa have been identified as the most abundant in the pregnant uterus (Karstrup et al., 2017; Moore et al., 2017). It has been stated that there is a close relationship between the vaginal and uterine microbiota, with regular interchange occurring between these two environments, although vaginal microbiota is more diverse (Clemmons et al., 2017; Garcia-Garcia et al., 2022; Sheldon et al., 2019; Wang et al., 2021). As suggested by other authors in humans (Moreno and Simon, 2019), we propose that the stability of the cervico-vaginal microbiota is crucial for maintaining a healthy environment during pregnancy.

Coinciding with the results from the literature (Deng et al., 2019; Jeon et al., 2015; Knudsen et al., 2015; Santos et al., 2011), Firmicutes was the most abundant phylum (45–59%; Table S1) in all five sampling points. The abundance of Firmicutes does not significantly vary between sampling points (Table S2), even in the post-partum stage (sampling point 5). Firmicutes has been associated with postpartum uterine disease (Carneiro et al., 2016; Moore et al., 2017), but it is also described as important part of a healthy microbiota (Machado et al., 2012; Laguardia-Nascimento et al., 2015). Our results agree with the later, suggesting that Firmicutes is important in the healthy vaginal microbiota of pregnant heifers. Our data do not associate the presence of this phylum with post-partum disease in primiparous cows.

At the family level, the taxa that were among the most abundant in all five sampling points are families Oscillospiraceae and Lachnospiraceae, order Bacteroidales and class Clostridia (Table S1). The families Oscillospiraceae and Lachnospiraceae have been detected in the vagina of healthy heifers in previous studies (Ault et al., 2019a; Moreno et al., 2022) and are also associated with healthy post-partum microbiota (Clemmons et al., 2017). Both families have recently been proposed as biomarkers of reproductive success (Moreno et al., 2022; Valderrama et al., 2023), and our data support this hypothesis. Order Bacteroidales has been found in the vaginal and fecal microbiota of pregnant heifers (Ault et al., 2019a; Deng et al., 2019), showing that is a resident

microorganism in healthy cows, consistent with several studies that have proven that gut and reproductive tract microbiota share certain taxa (Garcia-Garcia et al., 2022). Regarding class Clostridia, as lower taxonomic ranks are undetermined, we cannot propose a hypothesis about its role in the vaginal microbiota. While the family Clostridiaceae has been associated with pregnancy failure (Deng et al., 2019), the order Clostridiales has been proposed as a marker of reproductive success (Ault et al., 2019a).

We found that *Ureaplasma* genus is amongst the most abundant genera in sampling points 1 (insemination day), 2 (~30 days of gestation) and 5 (~60 days post-partum; Table S1). The role of this genus remains controversial. Some authors have found that its presence is a marker of infertility and uterine disease, acting as an opportunistic pathogen (Messman and Lemley, 2023). Nevertheless, it can be harmless in a balanced microbiota, as it appears consistently as part of the healthy microbiota in several studies (Jeon et al., 2015; Messman et al., 2019; Poole et al., 2022; Quadros et al., 2020). Our data support that *Ureaplasma* contributes to maintaining a healthy cervico-vaginal microbiota.

Regarding the *Pseudomonas* genus, despite it has been described as an opportunistic pathogen that causes abortion in bovines (Anderson, 2007; Vidal et al., 2017), several studies found it in great abundance in healthy uterine and vaginal microbiota in cattle, pointing out that it is part of the resident microbiota (Becker et al., 2023; Bicalho et al., 2017b; Chen et al., 2020). Our results are then in agreement with this observation. Nevertheless, it is important to surveil their abundance at the beginning of the pregnancy to prevent its possible ulterior consequences. On the other hand, *Bifidobacterium* is present in ruminal, uterine and vaginal microbiota of healthy cattle, not being associated to any disease (Amat et al., 2021; Jeon et al., 2015; Winders et al., 2022). Moreover, *Bifidobacterium* abundance was diminished in metritic cows when compared to control in one study (Basbas et al., 2023), being then a possible biomarker of a healthy microbiota. Conversely, *Sharpea* genus is mainly detected in ruminal or fecal samples of cattle (Obregon-Gutierrez et al., 2022; Seshadri et al., 2018). Therefore, we think it is a possible contaminant from feces, which is not rare in this kind of studies (Ault-Seay et al., 2022; Clemmons et al., 2017), but more investigation is needed to determine its possible rol in cervico-vaginal microbiota.

Concerning the kingdom Archaea, it was poorly represented in our data. This result agrees with previous studies, which only found sequences classified as Archaea sporadically and with low frequency (Appiah et al., 2020; Quereda et al., 2020).

This new knowledge helps to identify which taxa are possible biomarkers of a healthy microbiota, due to their permanence over time and high abundance in the reproductive tract, indicating they are part of a resident core microbiota in healthy pregnant animals.

Additionally, several taxa can be observed to change over time. Taxa with the most striking changes are also amongst the least abundant (Tables S1 and S2), suggesting that it is important to pay attention to the less abundant bacteria to obtain a comprehensive understanding of the temporal changes in the microbiota of the pregnant bovines. To the best of our knowledge, none of these taxa have been studied in relation to the cervico-vaginal, uterine or vaginal microbiota in cows, so these data open a path to investigate their importance and possible roles in the health of the bovine genital tract.

Our findings differ from previous studies in some respects. The only previous study of the vaginal microbiota throughout pregnancy (Deng et al., 2019) showed significant changes in alpha and beta diversity over time, a pattern we did not observe. Several factors could contribute to these differences, including the 16S region analyzed, the breed of the animals, light discrepancies in sampling point timing, and the amount of sequence data analyzed. All these factors lead us to point out the need for an advance in the standardization of methodologies used to assess microbiota diversity in cattle.

Our study has some limitations that should be pointed out. These limitations are inherent to all studies based on sequencing different variable regions of the 16S ribosomal DNA gene: on one hand, each variable region presents different biases in the detection of certain taxa. Regarding the V1-V2 region analyzed here, the bibliography on this subject is quite scarce, so a deep analysis of these biases is not possible with the information we have. Another limitation inherent to the approach is the taxonomic resolution power that the short reads obtained with Illumina technology allow. Other studies have limitations regarding the depth of sequences obtained, but we consider that this is not our case. Despite these limitations, we believe that our study provides significant information about the bovine cervico-vaginal microbiota.

While further studies are necessary, our results represent a step forward in the understanding of the healthy cervico-vaginal microbiota in pregnant cows.

5. Conclusions

Our comprehensive study of the cervico-vaginal bacterial microbiota during the gestation period contributes significantly to the understanding of microbiota dynamics on the bovine reproductive tract during pregnancy and after parturition. The cervico-vaginal microbiota is stable throughout the pregnancy, although some taxa exhibit temporal changes. Differences in microbiota diversity compared to previous studies underscore the need for further studies to fully comprehend the microbiota dynamics during pregnancy in cows. This study sheds light on the composition of the healthy reproductive tract microbiota and can serve as a baseline for future research and the development of potential therapeutic interventions.

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Ethical statement

This study was carried out in strict accordance with the recommendations of the Uruguayan Animal Welfare Act of 2009 (law # 18,611) for the use of animals in experimentation, teaching, and scientific research activities. The research protocol was reviewed and approved by the Institutional Animal Care and Use Committee of National Institute of Agricultural Research of Uruguay (INIA) (protocol # INIA 2022.5).

CRediT authorship contribution statement

Lucía Calleros: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Maila Barcellos:** Writing – review & editing, Methodology, Investigation, Data curation. **Sofía Grecco:** Writing – review & editing, Methodology, Investigation, Data curation. **Juan Pablo Garzón:** Writing – review & editing, Investigation. **Joaquín Lozano:** Writing – review & editing, Investigation. **Victoria Urioste:** Writing – review & editing, Investigation. **Gustavo Gastal:** Writing – review & editing, Resources, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

Sequence data from this article have been deposited with the DBJ/EMBL/GenBank Data Libraries under Bioproject number PRJNA1122579, BioSample accession numbers SAMN41788669 to SAMN41788733.

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