Animal Microbiome

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Effects of dietary restriction and one-carbon metabolite supplementation during the first 63 days of gestation on the maternal gut, vaginal, and blood microbiota in cattle

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Abstract

Background Maternal diet quality and quantity have significant impacts on both maternal and fetal health and development. The composition and function of the maternal gut microbiome is also significantly influenced by diet; however, little is known about the impact of gestational nutrient restriction on the bovine maternal microbiome during early gestation, which is a critical stage for maternal microbiome-mediated fetal programming to take place. The objective of the present study was to evaluate the impacts of diet restriction and one-carbon metabolite (OCM) supplementation during early gestation on maternal ruminal, vaginal, and blood microbiota in cattle. Thirty-three beef heifers (approx. 14 months old) were used in a 2 × 2 factorial experiment with main factors of target gain (control [CON]; targeted 0.45 kg/d gain vs restricted [RES]; targeted −0.23 kg/d gain), and OCM supplementation (+ OCM vs − OCM; n = 8/treatment; except n = 9 for RES–OCM). Heifers were individually fed, starting treatment at breeding (d 0) and concluding at d 63 of gestation. Ruminal fluid and vaginal swabs were collected on d − 2, d 35, and d 63 (at necropsy) and whole blood was collected on d 63 (necropsy). Bacterial microbiota was assessed using 16S rRNA gene (V3–V4) sequencing.

Results Overall ruminal microbiota structure was affected by gain, OCM, time, and their interactions. The RES heifers had greater microbial richness (observed ASVs) but neither Shannon nor Inverse Simpson diversity was significantly influenced by gain or OCM supplementation; however, on d 63, 34 bacterial genera showed differential abundance in the ruminal fluid, with 25 genera enriched in RES heifers as compared to CON heifers. In addition, the overall interaction network structure of the ruminal microbiota changed due to diet restriction. The vaginal microbiota community structure was influenced by gain and time. Overall microbial richness and diversity of the vaginal microbiota steadily increased as pregnancy progressed. The vaginal ecological network structure was distinctive between RES and CON heifers with genera-genera interactions being intensified in RES heifers. A relatively diverse bacterial community was detected in blood samples, and the composition of the blood microbiota differed from that of ruminal and vaginal microbiota.

Conclusion Restricted dietary intake during early gestation induced significant alterations in the ruminal microbiota which also extended to the vaginal microbiota. The composition of these two microbial communities was largely

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unaffected by OCM supplementation. Blood associated microbiota was largely distinctive from the ruminal and vaginal microbiota.

Keywords Dietary restriction, Maternal microbiota, Beef cattle, One carbon metabolite supplementation, Rumen, Vagina, Blood

Introduction

Recent work on developmental programming in livestock species has begun to unveil that just as in humans, stimuli and aberrations during gestation can lead to lifelong alterations that can impact the productivity and efficiency of food-producing animals, including cattle [87]. Increasing evidence indicates that the homeostatic mechanisms which regulate the utilization of nutrients for growth and development in animals can be programmed in utero and that maternal nutrition may be a central stimulus for programming events [17]. Irregularities in maternal nutrition, especially malnutrition, during pregnancy are not uncommon in cattle production systems, especially in extensive grazing systems, where the quality and quantity of feed resources are influenced by seasonal variations [17, 20, 109]. Changes in nutrient supply not only impact the dam, but these changes can extend to the developing fetus and may affect organogenesis, DNA synthesis, cellular differentiation, and DNA methylation [42, 68]. As a result, the growth trajectories and physiology and metabolism of the resulting offspring are modified [42].

While maternal nutrition and its impacts on fetal programming are increasingly recognized [8, 86, 98, 108], the impact of maternal nutrition-mediated gut microbiome alterations during pregnancy on fetal programming and offspring health and disease remains elusive [71]. Maternal gut microbiota has the great potential to influence fetal programming and feto-maternal microbial crosstalk [4, 46, 48], both of which may have extended influences on resulting offspring that modify their future health and production potential. In addition, the gut microbiome is critical in maintaining dam health and energy supply [91]. Alterations of the gut microbiota composition are likely to lead to changes in microbial metabolite production. Microbial metabolites are used as signaling molecules to shape the host immune system and modulate the host's metabolic pathways [51, 75]. Maternal metabolites can also be transferred to the developing fetus, where they also act as signaling molecules that regulate proper neurodevelopment [102] as well as program offspring energy homeostasis [51].

The maternal gut microbiota is not the sole microbial population that can influence developmental outcomes of offspring, but the reproductive microbiota holds an important role as well. The vaginal microbiota during pregnancy can protect the uterus from pathogen invasion through the production of antimicrobial agents such as lactic acid, or through biofilm formation which can add additional protection alongside the cervicovaginal mucus [3]. Although the vaginal microbiota is also well understood to be a seeding source for the offspring microbiome, especially during parturition, it may also seed the uterus prior to gestation, possibly influencing the fetal microbiome in utero [5, 40, 71]. Given that the maternal gut microbiota has the potential to influence fetal development, gastrointestinal, and distal microbial ecosystems such as the vagina through microbial metabolic activity and multiple gut-organ axes [e.g. microbiome-gut-reproductive axis [107], maternal–fetal gut axis [72]]; establishing how moderate changes in maternal nutrition during gestation impacts the maternal gut and reproductive microbiome is important. In the present study, we evaluated the impact of dietary restriction and OCM supplementation during early gestation on the maternal ruminal, vaginal, and blood microbiota in beef cattle. Of note, the rationale behind the inclusion of OCM supplementation in this study was that it would mitigate the negative impacts of restricted diet during the critical window of time on fetal programming and subsequent offspring growth and efficiency by improving epigenetic modification-mediated biological functions in utero. Considering the increased reports on the presence of microbial community in blood [19, 80, 100], and hypotheses that blood could be a route of transfer for microbes from the gut to extra-intestinal microbial niches, and from the dam to the uterus and placenta [38, 41, 71], we characterized microbial DNA from whole blood obtained from heifers in this study. Therefore, our objective was to evaluate the impacts of diet restriction and one-carbon metabolite supplementation during the first 63 days of gestation on maternal ruminal, vaginal, and blood microbiota in beef cattle.

Materials and methods

All experimental procedures were approved by the North Dakota State University (NDSU; Fargo, ND, USA) Institutional Animal Care and Use Committee (protocol ID: 21,049).

Experimental design and animal husbandry

Thirty-three beef heifers (approximately 14-months old; initial body weight of 398 ± 32 kg) were randomly

assigned to a 2×2 factorial experiment in which two planes of nutrition (feed intake) and two levels of strategic OCM supplementation were evaluated (Fig. 1). Heifers were assigned to receive either a control intake diet (CON) which targeted an average daily gain (ADG) of 0.45 kg/day, or a restricted intake diet (RES), which targeted an ADG of −0.23 kg/day. Half of the heifers in both dietary groups received an OCM supplement (+OCM), which consisted of rumen-protected methionine (7.4 g/day; Smartamine M, Addiseo) and rumen-protected choline (44.4 g/day; ReaShure, Blachem) fed daily via a fine-ground corn carrier, as well as weekly intramuscular injections of folate (320 mg) and vitamin B_{12} (10 mg). The heifers that did not receive the OCM supplement (−OCM) were fed only the corn carrier and received weekly injections of saline. In addition to treatment diets, heifers were fed a basal diet (dry matter basis) consisting of 9% corn silage, 31% ground corn, 15% alfalfa, and 45% alfalfa/grass hay mix. Heifers were assigned to treatment groups at the time of breeding (d 0) and were individually fed using electronic Calan gate feeding systems (American Calan; Northwood, NH, USA) at the NDSU Animal Nutrition and Physiology Center (ANPC; Fargo, ND, USA). Diet allotments were adjusted weekly based on animal weights. Heifers underwent a 7-day Select Synch+CIDR estrus synchronization protocol as detailed previously [58, 105]. On d 0 which was 2 days after CIDR removal, heifers were artificially inseminated using female-sexed semen from a single sire. Of note, about 120 heifers originated from a single farm were synchronized and bred, but 73 heifers that became pregnant were remained in the study. Heifers were managed on their respected dietary treatments until harvest on d 63 (±2) of gestation. Vaginal and ruminal fluid samples were collected on d −2, 35, and 63, while blood samples were collected only on d 63 at the time of harvest. Of note, this study was designed up until d 63 of gestation, as this period of gestation in cattle encompasses organogenesis and peak primary myogenesis, which are sensitive to even moderate nutrient restriction at this time [17]. Heifers were sampled at d −2 in order to collect a baseline sample prior to treatment and pregnancy, and samples were again collected at d 35 after pregnancy was confirmed.

Ruminal fluid, vaginal, and blood sampling

Ruminal fluid was collected on d −2 and 35 in a similar method as described previously [6, 107]. Briefly, heifers were restrained in a hydraulic cattle chute and a metal speculum was placed in the mouth so that a flexible PVC

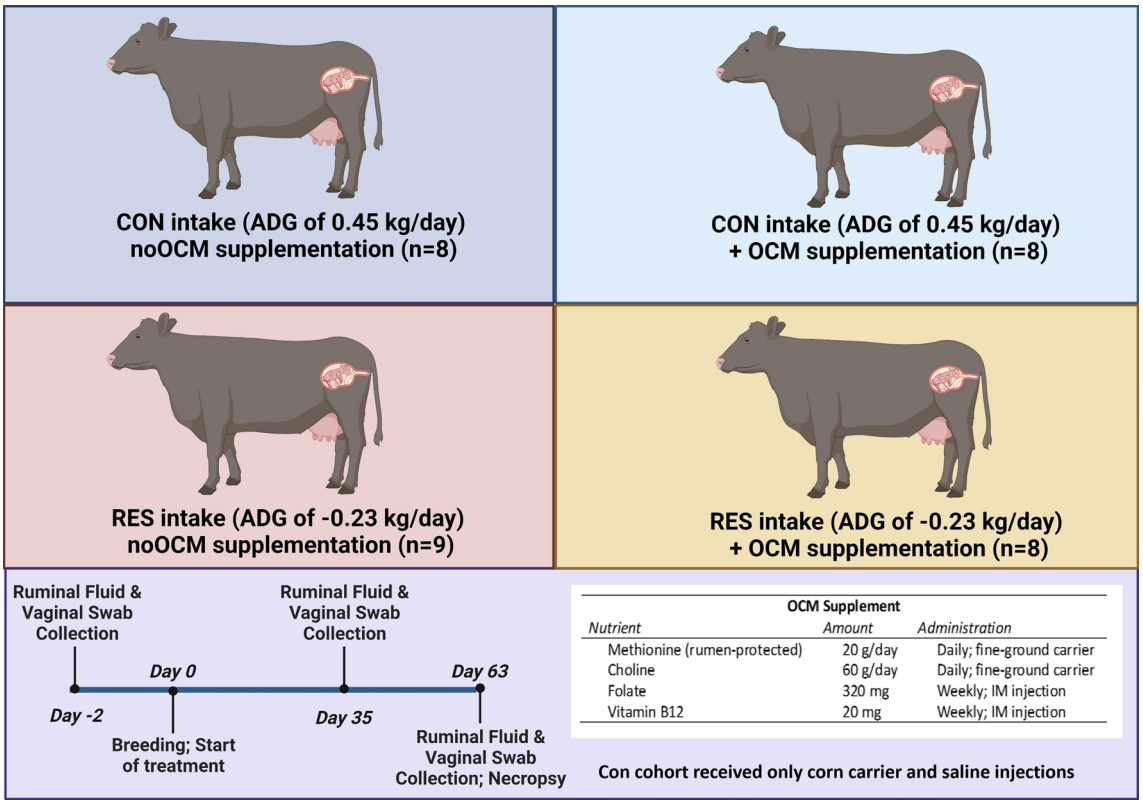

Fig. 1 Schematic overview of the experimental design including dietary gain, OCM supplmentation, and sampling timeline

stomach tube could be passed through the esophagus and into the rumen. The tube was worked through the ruminal mat and then a light vacuum was applied to collect the ruminal fluid. Ruminal fluid was collected into a clean, side-arm Erlenmeyer flask, gently swirled, and approximately 40 mL of ruminal fluid was immediately aliquoted into a sterile 50-mL falcon tube and flash frozen on dry ice. To avoid cross-contamination between animals, separate collection tubes and flasks were used for each heifer and were cleansed with a bleach solution after each use. Ruminal fluid samples were transported to the laboratory and stored at −80 °C until DNA extraction. Ruminal fluid on d 63 was collected during necropsy using a new, sterile 50 mL syringe and a 16-gauge needle to draw ruminal fluid from the rumen.

Vaginal swabs were collected on d −2 and 35 using the same techniques described previously [6]. The exterior of the vulva was sprayed with 70% ethanol and wiped clean using a paper towel. Using a gloved hand, the left and right labia were held open, while a sterile cotton tipped swab (Puritan, Guilford, ME, USA) was gently inserted into the vagina and swirled four times. The swab was carefully removed and placed in a Whirl–Pak bag and stored on ice until transferred to the laboratory, where they were then stored at −80 °C until genomic DNA extraction. On d 63, vaginal swabs were collected in the same manner, however, they were collected after the animal was euthanized. Ambient air swabs were collected during sampling to account for any environmental contamination by holding a cotton tipped swab to the air and swirling around the collection area. These control swabs were included in the DNA extraction and 16S rRNA gene sequencing steps as well.

Blood samples were collected from each heifer at the time of exsanguination using sterile 50-mL falcon tubes and free catch. Blood samples were left whole and immediately flash frozen on dry ice, transferred to the laboratory, and stored at −80 °C until genomic DNA extraction.

It is important to highlight that day 63 samples were collected immediately upon animal euthanasia. Heifers were harvested at the USDA inspected North Dakota State University Meat Laboratory (Fargo, ND, USA), and sample collection took place on the harvest floor as well as a designated sample processing area that was connected to the harvest floor. While this creates a limitation to this study, samples were collected after euthanasia to minimize handling and potential discomfort to the animal prior to harvest.

Of note, the sampling time points d−2 and d 35 were chosen in the present study as the heifers were brought to the chute for CIDR removal (day −2), and pregnancy determination (day 35 post breeding). Sampling on d 63 was performed when the dams were euthanized for fetal harvesting. Day 63 of gestation was determined for fetal harvesting as it was considered a timepoint of importance for other major response variables in the experiment. Primary myogenesis in bovine fetus peaks at approximately 2 months of gestation [29].

DNA extraction from ruminal fluid, vaginal swabs, and blood

DNA was extracted from the ruminal fluid using a Qiagen DNeasy PowerLyzer PowerSoil kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's protocol with some modifications as outlined by our previous publications [6, 107]. Briefly, ruminal fluid samples were removed from the −80 °C freezer and thawed in a 37 °C water bath. Working with a single sample at a time, the 50-mL centrifuge tube (VWR International, Radnor, PA, USA) of ruminal fluid was thoroughly vortexed, and an aliquot was immediately transferred to a sterile 2-mL microfuge tube and stored on ice until all samples had been aliquoted. Then, the 2-mL microfuge tubes were centrifuged at 20,000×*g* for 10 min at 4 °C to pellet the sample. The supernatant was removed, and the sample was resuspended in 750 µl of PowerBead solution from the DNeasy PowerLyzer PowerSoil Kit. All subsequent steps were performed according to the manufacturer instructions. Negative extraction controls were included each time a new extraction kit was opened and included in the 16S rRNA gene sequencing.

Genomic DNA from vaginal swabs were extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Germantown, MD, USA) according to manufacturer's instruction with modifications as outlined previously [105, 107]. DNA from the environmental control swabs were extracted alongside the vaginal swabs. Negative extraction controls of sterile molecular biology grade water (Corning, Corning, NY, USA) were included each time a new extraction kit was opened and were included in the 16S rRNA gene sequencing.

DNA was extracted from whole bovine blood samples using a Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Germantown, MD, USA) using the following modifications. Blood samples were removed from the −80 °C freezer and thawed in a 37 °C water bath. Once thawed, samples were kept on ice. The blood sample was thoroughly vortexed and approximately 100 mg of blood was transferred into the 2-mL microfuge tube on the scale. Weight rather than volume was used for measurement due to the clotting of untreated blood. Once all samples were aliquoted, enzymatic lysis buffer (20 mM Tris–HCl [pH 8.0], 2 mM sodium EDTA, and 1.2% Triton X-100), also containing 100 mg/mL lysozyme and 25,000 U/mL mutanolysin, was added to the sample in the microfuge tube. Samples were thoroughly vortexed and incubated

at 37 °C for 1 h with agitation at 800 rpm. Following this incubation step, 25 µL of proteinase K and 400 µl of Buffer AL (without added ethanol) were added to the sample and vortexed. Samples were then incubated at 56 °C for 30 min with agitation at 800 rpm. Following this incubation, approximately 400 mg of sterile 0.1-mm zirconia-silica beads (BioSpec Products, Bartlesville, OK, USA), were added to each sample, which were then placed in a FastPrep-24 Classic bead beater (MP Biomedicals, Irvine, CA, USA). Samples were mechanically lysed at 6.0 m/s for 40 s for two cycles. Samples were centrifuged at 13,000×*g* for 5 min and 400 µL of supernatant was transferred to a new sterile microfuge tube. To this new tube, 400 µL of 100% molecular ethanol was added and vortexed. The remaining steps were followed according to the DNeasy Blood and Tissue kit protocol.

The concentration of all extracted DNA was measured using a NanoDrop One^{C} Microvolume UV–Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) followed by further quantification using the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). DNA was stored at −20 °C until 16S rRNA gene sequencing.

16S rRNA gene sequencing and analysis

Amplification and sequencing of the 16S rRNA gene in ruminal fluid, vaginal, and blood samples was performed at Novogene (Tianjin, China). A total of 205 samples, including 5 environmental and negative DNA extraction controls were sequenced. The V3-V4 region of the bacterial 16S rRNA gene was amplified using the 341F (5'-CCTAYGGGRBGCASCAG-3′) and 806R (5'-GGACTACNNGGGTATCTAAT-3′) primers [16]. The 16S rRNA gene libraries were sequenced on an Illumina Novaseq 6000 instrument (Illumina Inc., San Diego, CA, United States) with a SP flow cell (2×250 bp) as previously described [6, 105, 107]. The resulting 16S rRNA gene sequences were processed using DADA2 v. 1.26.0 [14] in R (v. 4.2.3). Primer sequences were removed from the 16S rRNA gene using Cutadapt v. 4.3 [66]. The forward reads were truncated at 224 bp and merged with a minimum overlap of 12 bp. Chimeric sequences were removed, and taxonomy was assigned to the generated amplicon sequence variants (ASVs) using the naïve Bayesian RDP classifier [103] and the SILVA SSU release 138.1 database [82]. Amplicon sequencing variants (ASVs) were considered to be likely contaminants and removed if they were more abundant in the negative controls on average than within the ruminal and vaginal samples. These negative controls included DNA extraction controls as well as unused swabs left open in the room/farm where sample collection took place. ASVs classified as chloroplasts, mitochondria, or eukaryote in origin were removed prior to downstream analysis as they were considered contamination. To account for uneven sequence depth, samples were randomly subsampled to prior to 57,832, 5615, and 6000 for ruminal fluid, vaginal, and blood samples, respectively, prior to calculation of microbial diversity measures. Richness (number of ASVs per sample), Shannon diversity, inverse Simpson's diversity, and Bray–Curtis dissimilarities were calculated in R using Phyloseq v. 1.44.0 [69] and vegan v. 2.6.4 [77]. All 16S rRNA gene sequences can be found in the NCBI sequence read archive under BioProject PRJNA1033356.

Statistical analysis

The effect of dietary restriction and OCM supplementation on microbial community structure was assessed using the Bray–Curtis dissimilarities and PERMANOVA (adonis2 function) using vegan in R. The R package pairwiseAdonis v. 0.4 [67] was utilized to compare the Bray–Curtis dissimilarities within sampling times for the ruminal fluid and vaginal samples and the *P* values were corrected for multiple comparisons using the Benjamini–Hochberg procedure. Differentially abundant phyla and genera between the two levels of gain were identified using MaAsLin2 v. 1.8.0 [65] in R. Only genera with a relative abundance of 0.1% or greater were included. Data on the microbial richness (number of ASVs) and diversity indices of the ruminal fluid and vaginal microbiota were analyzed as repeated measurements in 2×2 factorial treatment arrangement using the generalized liner mixed model estimation procedure (PROC GLIMMIX) in SAS (v. 9.4, SAS Institute Inc., Cary, NC, United States). The data was first analyzed with fixed effects of gain, OCM supplementation, day, gain×OCM, gain×day, OCM×day, gain×OCM×day. After observing no significant OCM effects on microbial richness and diversity of both ruminal and vaginal microbiota, the data was compared between RES and CON heifers over time. For this, the OCM was removed from the model and the fixed effects of gain, day, and gain×day were included in the model. Means between different treatment groups were compared using LSMEANS statement. Statistical significance was considered at $P < 0.05$.

Results

16S rRNA gene sequencing results summary

After processing and quality filtering, the average number of sequences per sample were 83,715 ± 1267 (SEM), 65,968 ± 4528, and 31,115 ± 6590 for the ruminal, vaginal, and blood samples, respectively. These sequencing reads were classified into 30 phyla (1 archaeal and 29 bacterial phyla).

Effects of dietary restriction and OCM supplementation on the ruminal microbiota

To determine the effects of feeding a restricted intake diet with and without OCM supplementation on the ruminal microbiota in early gestating heifers, we investigated the changes in microbial community structure, richness, diversity, and composition on d −2 (prior to breeding), 35, and 63. There was a significant effect of restricted dietary intake on the ruminal microbial community by d 35 ($R^2=0.05$, $P=0.03$) and d 63 ($R^2=0.15$, $P<0.0001$; Fig. 2A), as well as a significant effect due to day ($R^2=0.2$, $P<0.0001$) and interactions ($R^2=0.08$, $P=0.0004$; Supplementary Fig. 1A). Significant alterations in microbial richness (ASVs) associated with time ($P=0.0007$) and restricted gain ($P=0.005$) were detected. Overall microbial richness was greater in RES heifers compared to CON heifers (mean ASVs 2900 vs 2737; $P=0.005$). Mean ASVs were greater in RES heifers than CON heifers on d 63 ($P<0.05$; Fig. 2B). Shannon and Inverse Simpson diversity indices did not differ between RES or CON heifers ($P\geq0.20$; Fig. 2B), nor did supplementation with OCM affect microbial community richness or diversity ($P>0.05$; Supplementary Fig. 1B).

At a compositional level, a total of 23 different bacterial phyla were detected in the ruminal fluid with the seven most relatively abundant phyla accounting for over 99% of the 16S rRNA gene sequences. At each sampling timepoint (d −2, 35, and 63) the phyla Bacteroidota, Firmicutes, and Euryarchaeota were the most abundant, respectively. Other phyla such as Proteobacteria, Actinobacteriota, Thermoplasmatota, Fibrobacterota, and Patescibacteria made up the remaining fraction of the top 7 predominant phyla (Fig. 2C). The relative abundance of Firmicutes, Actinobacteriota, and Chloroflexi was increased in RES heifers on d 63 ($P<0.05$; Fig. 2D). There were 34 differentially abundant bacterial genera identified in the rumen microbiota between RES and CON heifers on d 63 with 25 genera enriched in the RES heifers and 9 more relatively abundant in the CON heifers (Fig. 3). Among the genera enriched in the RES heifers were *Butyrivibrio, Eubacterium hallii* group, *Ruminiclostridium,* and *Christensenellaceae R7* and *Ruminococcus, Prevotella,* and *Lachnospiraceae* AC2044 in the CON heifers.

Ecological network modeling based on the interactions between genera was performed to evaluate the impact of restricted diet on the overall interaction network of the ruminal microbiota. As shown in the network plots of CON and RES (Fig. 4), a distinctive genera-genera interaction network structure was observed between CON (Fig. 4A) and RES (Fig. 4B) heifers. Compared to heifers on the control intake diet, more intensified interactions occurred among the bacterial genera in RES heifers with the majority of these interactions centering mainly on two hubs (Fig. 4B). Despite the overall interaction network structure being less intense in CON heifers, the total number of hubs connecting the interactions between the genera were significantly greater than that of RES heifers. The overall proportion of positive and negative interactions between genera was found to be equal in both network models.

Effects of dietary restriction and OCM supplementation on the vaginal microbiota

The vaginal microbiota also underwent significant changes over the course of 63 days of gestation. While there was no distinction in microbial community structure between CON or RES or OCM supplementation on d −2 and 35, by day 63 there was an effect due to gain ($R^2=0.02$, $P=0.02$), day ($R^2=0.34$, $P<0.0001$), and interaction ($R^2=0.06$, $P>0.05$; Supplementary Fig. 2A; Fig. 5A). Microbial richness in the vagina was significantly affected by sampling time ($P<0.0001$), but not by restricted gain ($P=0.46$) or OCM supplementation ($P=0.71$) (Supplementary Fig. 2B). Microbial diversity (Shannon and Inverse Simpson diversity) was also significantly altered during the first 63 days of gestation ($P<0.0001$; Fig. 5B). Both microbial richness and diversity increased steadily as the pregnancy progressed. However, no significant difference ($P>0.05$) was observed for microbial richness and diversity metrics between the RES and CON groups with or without OCM supplementation at any of the three timepoints (d −2, 35 and 63) evaluated.

Of the 30 different bacterial phyla identified, the top seven most relatively abundant phyla at each sampling time included Firmicutes, Actinobacteriota, Bacteroidota, Euryarchaeota, Proteobacteria, Campylobacterota, Fusobacteriota, and Deinococcota (Fig. 5C). Among these most predominant phyla, an increased relative abundance of Actinobacteriota, and a reduced relative abundance of Proteobacteria and Fusobacteria were detected in RES heifers as compared to CON heifers on d 63 ($P<0.05$; Fig. 5D). There were no significant differentially abundant genera identified in the vaginal microbiota between CON and RES heifers ($P>0.05$).

Ecological network modeling revealed a noticeable difference in the interaction network structure of vaginal microbiota between CON (Fig. 6A) and RES (Fig. 6B) heifers. Similar to the network structure observed in the ruminal microbiota of RES heifers, the genera-genera interactions in the vaginal microbiota of RES heifers were relatively more intense which can be observed by the greater number of genera retained in the model as compared to CON heifers. The total number of hubs observed in the CON model was slightly greater than

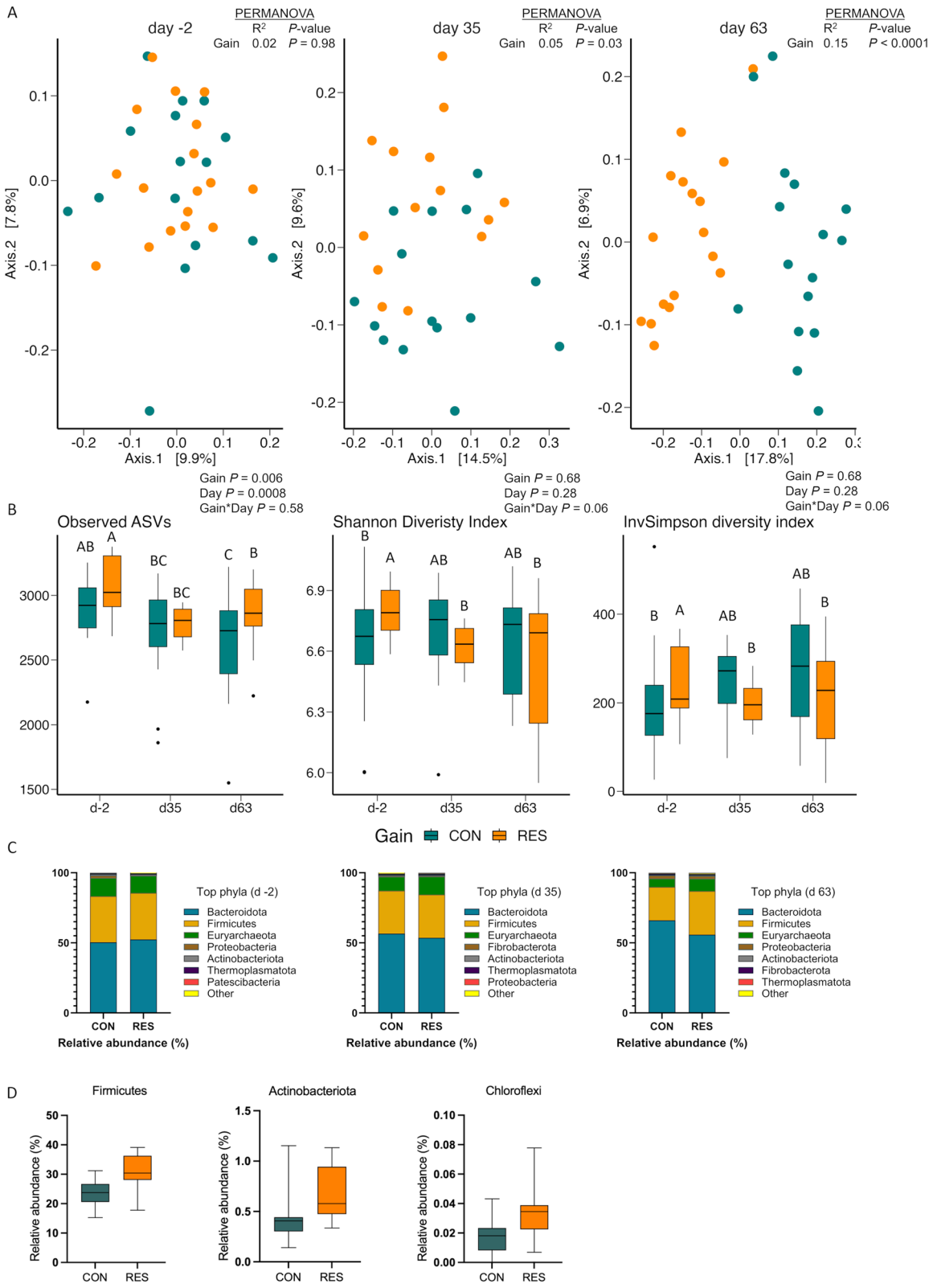

Fig. 2 **A** Principle coordinates analysis (PCoA) plot of the Bray Curtis dissimilarities for the ruminal fluid microbiota comparing gain across timepoints. **B** Alpha diversity indices of the ruminal fluid microbiota comparing gain across sample days. (InvSimpson diversity refers to Inverse Simpson diversity index). **C** The seven most relatively abundant phyla in the ruminal fluid microbiota across each timepoint. **D** Phyla whose relative abundance was significantly different between dietary gain ($P < 0.05$) CON (n = 16), RES (n = 17) groups on d 63

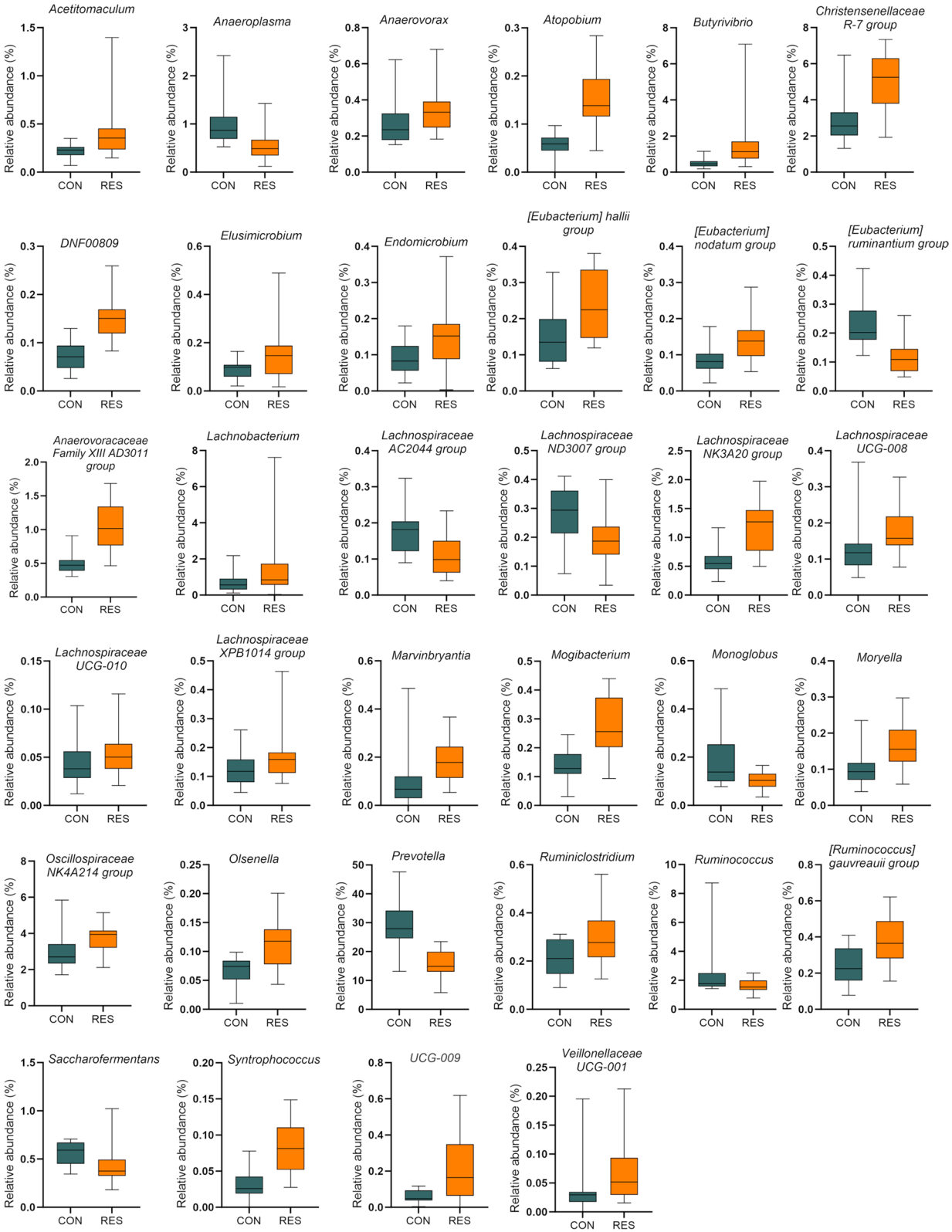

Fig. 3 Thirty-four genera within the ruminal fluid microbiota were differentially abundant between dietary gain on d 63 ($P < 0.05$)

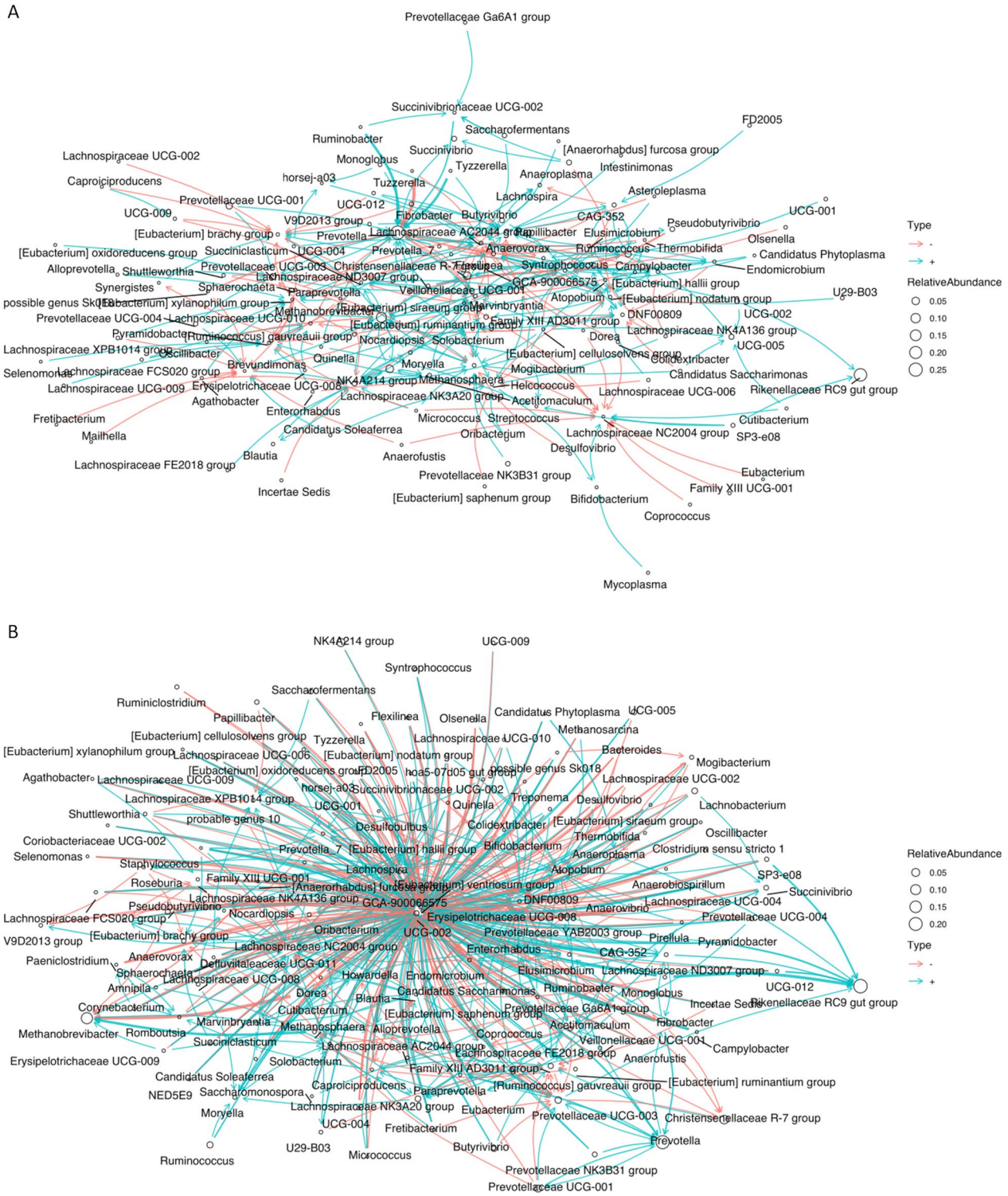

Fig. 4 Ecological network of observed bacterial genera in ruminal fluid microbiota of CON (**A**) and RES (**B**) heifers

in the RES model where a majority of the interactions between genera were centered on one hub. The overall proportion of positive and negative interactions between genera appeared to be equal in the RES model, whereas there appears to be more positive interactions than negative interactions between genera in the CON model.

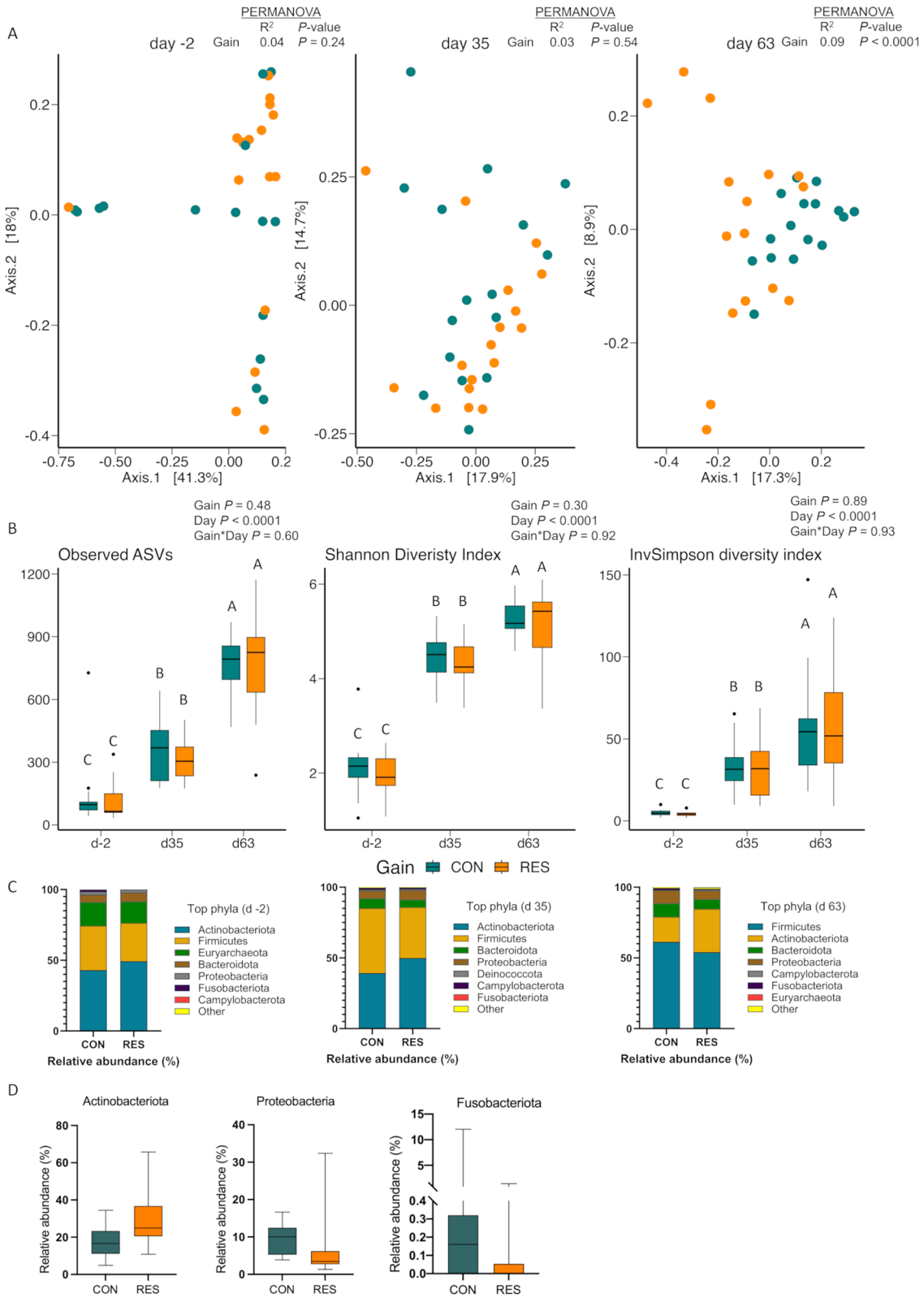

Fig. 5 **A** PCoA plot of the Bray Curtis dissimilarities for the vaginal microbiota comparing gain across timepoints. **B** Alpha diversity indices of the vaginal microbiota comparing gain across timepoints. **C** Top 7 most relatively abundant phyla in the vaginal microbiota across sample days. **D** Phyla whose relative abundance was significantly different between dietary gain ($P < 0.05$) CON (n = 16), RES (n = 17) on d 63

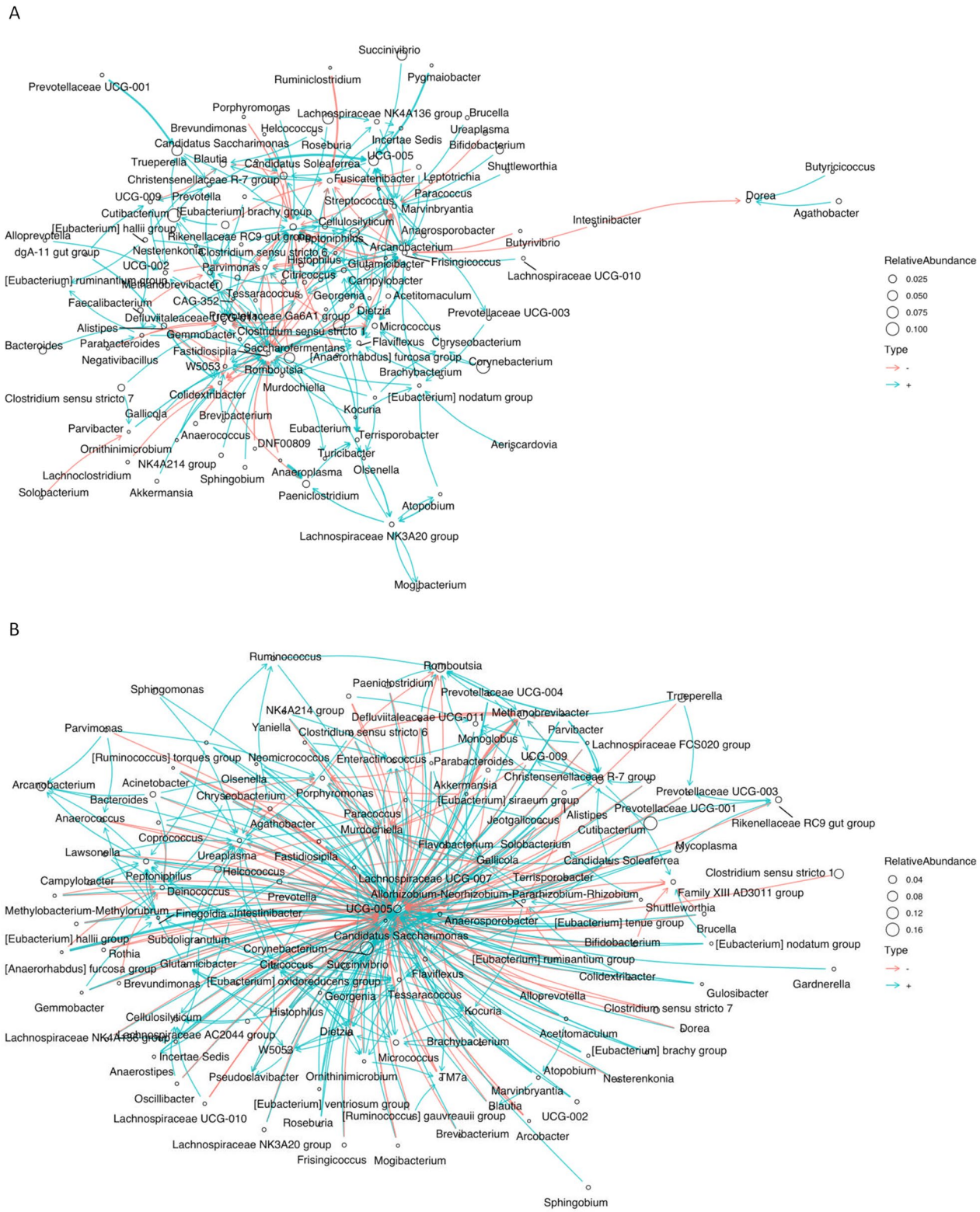

Fig. 6 Ecological network of observed microbial genera in the vaginal microbiota of CON (**A**) and RES (**B**) heifers

Effects of dietary restriction and OCM supplementation on the blood microbiota

Only 22 out of 32 blood samples collected on d 63 were successfully sequenced and 23 different bacterial phyla were detected from these sequenced samples. The dominant phyla present in the blood samples were Actinobacteriota (38.7%), Firmicutes (35.3%), Proteobacteria (18.6%), Bacteroidota (5.5%), Cyanobacteria (0.5%), Fusobacteriota (0.4%), and Verrucomicrobiota (0.2%) (Fig. 7C). At a genus level, a total of 358 genera were identified, with *Mycoplasma, Cutibacterium, Sphingomonas, Corynebacterium,* and *Escherichia-Shigella* being the relatively most dominant genera (Fig. 7D). The microbial community structure ($R^2 = 0.05$, $P = 0.67$; Fig. 7A), species richness and microbial diversity (Fig. 7B) of the blood microbiota were not different ($P > 0.05$) between the CON and RES heifers. We did not identify any differentially abundant phyla or genera between the two group of heifers ($P > 0.05$).

Similarities and differences among the ruminal fluid, vaginal, and blood microbiota

Given the possibility that the gut microbiota may supply some of the members of the female reproductive microbiota, and that blood may be a source of transport microbes in the body, we next evaluated the similarity among the ruminal, vaginal and blood microbiota. For this, we identified shared and unique ASV's among the three sample types as shown in the Venn diagram (Fig. 8A). Ruminal fluid harbored the highest number of ASVs (90,548 ASVs), followed by the vagina (15,159 ASVs), and blood (1974 ASVs). A very small portion (286 ASVs) of the total ASVs was shared among all three sample types (Fig. 8A). Interestingly, the blood and ruminal fluid shared a greater number of ASVs than the vagina and ruminal fluid (845 vs 599) and even fewer were shared between blood and the vagina (519; Fig. 8A). According to the heatmap of the 100 most abundant ASVs (Fig. 8B), there is clear variation in the frequency and abundance of taxa present in the blood, ruminal fluid, and vaginal swab samples (Fig. 8B). Only ASV8 (*Corynebacterium xerosis*) was present in the majority of the three sample types with relatively similar abundance and frequency.

Two taxa including ASV1 (*Cutibacterium acne*) and ASV6 (*Clostridium* sensu stricto 1) were more exclusively present in the blood and vagina. The vast majority of the ASVs present in ruminal fluid samples were absent in vaginal and blood samples, and these ASVs were within the *Prevotella*, *Rikenellaceae* RC9, and *Methanobrevibacter* genera. Among the 26 ASVs identified in at least 50% of all blood, ruminal fluid, and vaginal swab samples across all sampling timepoints (Table 1), 5 ASVs were found in at least 55% of all samples. Only one ASV – ASV62 (*Lachnospiraceae NK3A20 group*) and ASV31 (unidentified *Ruminococcus* species) were identified in at least 70% and 65% of all samples, respectively. Overall, bacterial taxa found in blood samples are distinct from ruminal and vaginal sample associated taxa at higher taxonomic resolution.

Discussion

Perturbed maternal nutrition during critical developmental windows in gestation can have long-term impacts on postnatal calf growth and production efficiency via negatively influencing in utero fetal programming [18, 26, 85]. Recent developments derived from rodent animals and humans suggest that the maternal gut microbiota is involved in metabolic [51] and neurological [97, 102] programming of offspring beginning at the embryonic stage. These developments further support the notion of the microbiome involvement in the developmental origins of health and disease (DOHaD) [4, 95, 96]. Despite the known intimate relationship between the diet and gut microbiome and host health [92], the impact of perturbed nutrition on the gut microbiota and gut microbiota-mediated fetal programming in cattle remains elusive. In this study, we evaluated the effects of maternal nutrition (restricted dietary intake) and OCM supplementation from breeding to d 63 of gestation on maternal ruminal, vaginal and blood microbiota in beef cattle.

Our 16S rRNA gene sequencing results revealed that the ruminal microbiota underwent changes during the first 63 days of pregnancy due to time and restricted gain. While the impact of pregnancy on the ruminal microbiota in cattle has less been characterized, there is an ample evidence from human research showing that the maternal gut microbiota undergoes profound changes over the course of pregnancy [22, 55, 76, 93]. As pregnancy progresses from the 1st to the 3rd trimester, the maternal gut microbiota of women becomes less diverse [55], but increases in microbial cell density [22]. In the present study, ruminal microbiota diversity remained stable while overall community structure and species richness changed from pre-breeding to day 63 of pregnancy. Overall species richness decreased as pregnancy progressed during the course of this study. Such changes in the ruminal microbiota following pregnancy might partially be driven by the increased metabolic demands from the developing fetus [21, 93].

Diet is the most important factor shaping the ruminal microbiota [43]. Diet composition and eating patterns have profound impact on the composition and function of the gut microbiota [54] including ruminal microbiota [104]. The impact of caloric restriction on the gut microbiota in humans has been well documented [50, 89]. Thus, the significant impact of restricted diet on

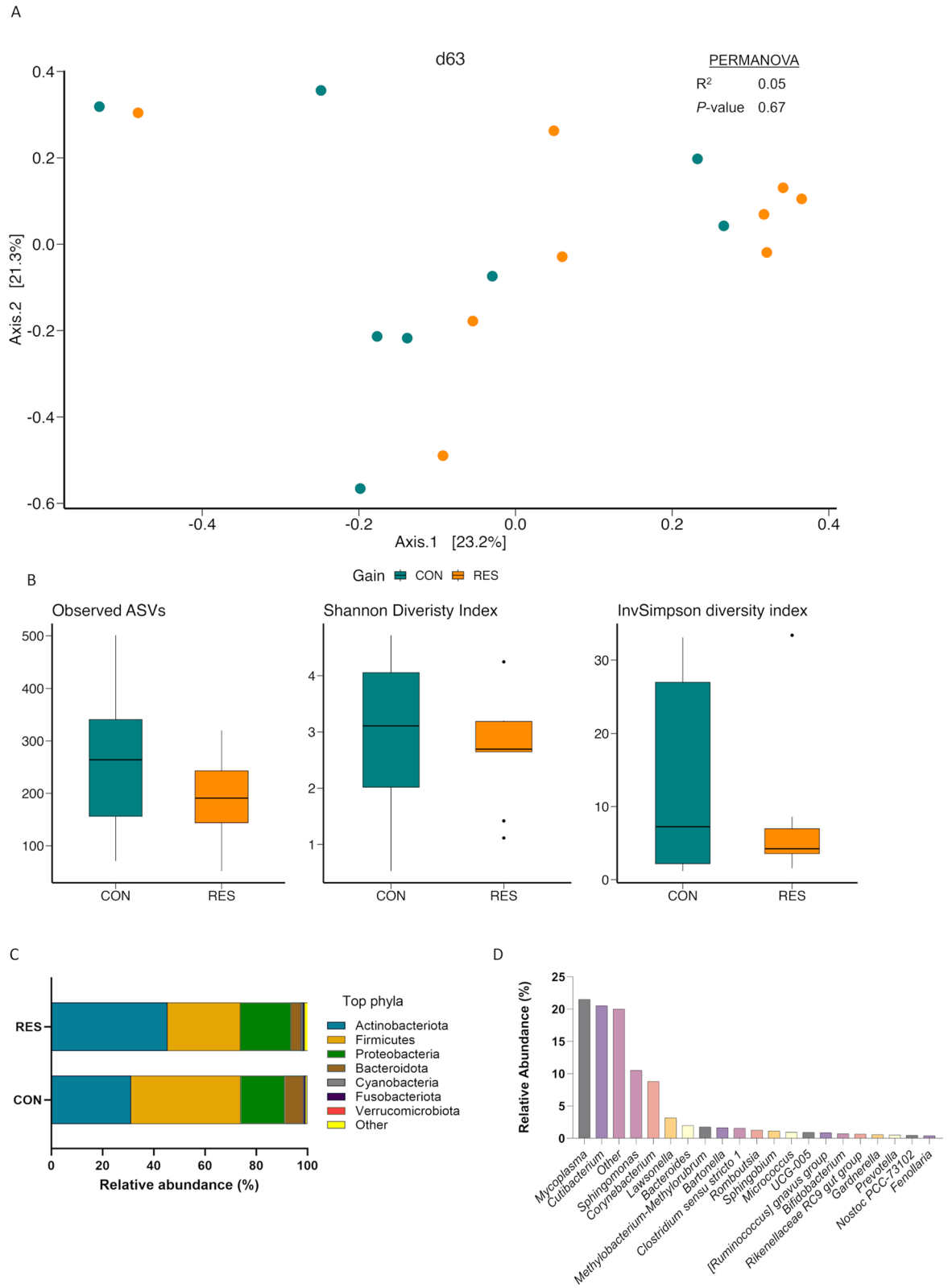

Fig. 7 Blood microbiota of pregnant heifers receiving diets of either a restricted (RES) or control (CON) level of gain (n = 22) on day 63 of gestation. **A** Principal coordinates analysis **(**PCoA) plot of the Bray Curtis dissimilarities for the blood microbiota of heifers comparing gain (RES: n = 9; CON: n = 8). **B** Alpha diversity indices of the blood microbiota of heifers comparing gain (RES: n = 9; CON: n = 8). **C** The seven most relatively abundant microbial phyla in blood compared by gain. **D** The 20 most relatively abundant genera in all blood samples irrespective of gain or treatment

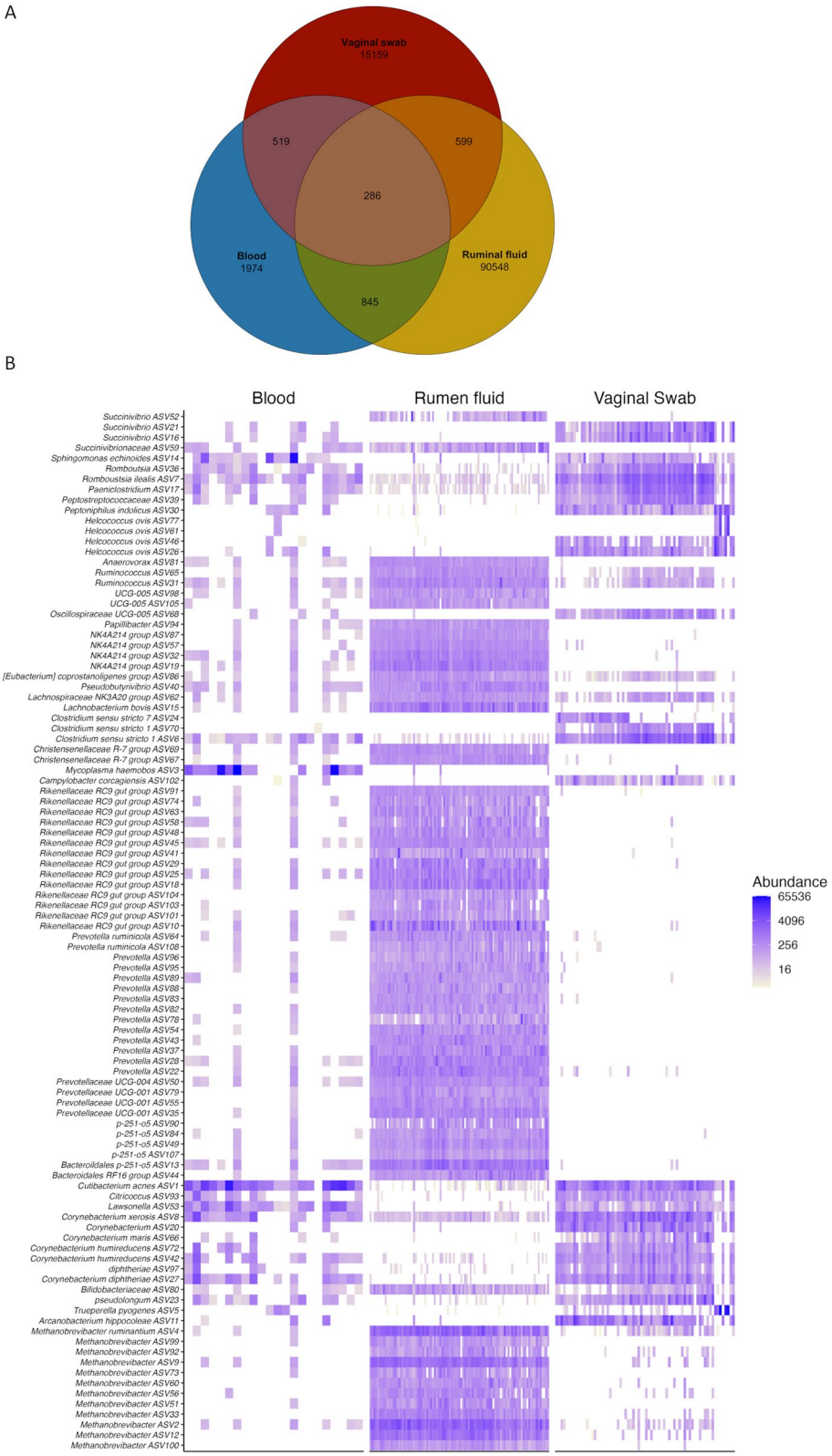

Fig. 8 **A** Venn diagram depicting the number of shared and unique ASVs in the ruminal fluid, vagina, and blood independent of dietary treatment. **B** Heat map showing the 100 most abundant ASVs overall by sample type

Table 1 Amplicon sequence variants (ASVs) identified in at least 50% of ruminal fluid, vaginal, and blood microbiota samples obtained from heifers on d −2, 35, and 63

ASV	Taxonomic Assignment	50%	55%	60%	65%	70%
ASV62	k_Bacteria; p_Firmicutes; c_Clostridia; o_Lachnospirales; f_Lachnospiraceae; g_Lachnospiraceae NK3A20 group; s_NA					
ASV31	k_Bacteria; p_Firmicutes; c_Clostridia; o_Oscillospirales; f_Ruminococcaceae; g_Ruminococcus; s_NA					
ASV65	k_Bacteria; p_Firmicutes; c_Clostridia; o_Oscillospirales; f_Ruminococcaceae; g_Ruminococcus; s_NA					
ASV2	k_Archaea; p_Euryarchaeota; c_Methanobacteria; o_Methanobacteriales; f_Methanobactiaceae; g_Methanobrevibacter; s_NA					
ASV4	k_Archaea; p_Euryarchaeota; c_Methanobacteria; o_Methanobacteriales; f_Methanobacteriaceae; g_Methanobrevibacter; s_ruminantium					
ASV9	k_Archaea; p_Euryarchaeota; c_Methanobacteria; o_Methanobacteriales; f_Methanobacteriaceae; g_Methanobrevibacter; s_NA					
ASV12	k_Archaea; p_Euryarchaeota; c_Methanobacteria; o_Methanobacteriales; f_Methanobacteriaceae; g_Methanobrevibacter; s_NA					
ASV13	k_Bacteria; p_Bacteroidota; c_Bacteroidia; o_Bacteroidales; f_p-251-o5; g_NA; s_NA					
ASV15	k_Bacteria; p_Firmicutes; c_Clostridia; o_Lachnospirales; f_Lachnospiraceae; g_Lachnobacterium; s_bovis					
ASV18	k_Bacteria; p_Bacteroidota; c_Bacteroidia; o_Bacteroidales; f_Rikenellaceae; g_Rikenellaceae RC9 gut group; s_NA					
ASV19	k_Bacteria; p_Firmicutes; c_Clostridia; o_Oscillospirales; f_Oscillospiraceae; g_NK4A214 group; s_NA					
ASV22	k_Bacteria; p_Bacteroidota; c_Bacteroidia; o_Bacteroidales; f_Prevotellaceae; g_Prevotella; s_NA					
ASV25	k_Bacteria; p_Bacteroidota; c_Bacteroidia; o_Bacteroidales; f_Rikenellaceae; g_Rikenellaceae RC9 gut group; s_NA					
ASV28	k_Bacteria; p_Bacteroidota; c_Bacteroidia; o_Bacteroidales; f_Prevotellaceae; g_Prevotella; s_NA					
ASV32	k_Bacteria; p_Firmicutes; c_Clostridia; o_Oscillospirales; f_Oscillospiraceae; g_NK4A214 group; s_NA					
ASV33	k_Archaea; p_Euryarchaeota; c_Methanobacteria; o_Methanobacteriales; f_Methanobacteriaceae; g_Methanobrevibacter; s_NA					
ASV35	k_Bacteria; p_Bacteroidota; c_Bacteroidia; o_Bacteroidales; f_Prevotellaceae; g_Prevotellaceae UCG-001; s_NA					
ASV37	k_Bacteria; p_Bacteroidota; c_Bacteroidia; o_Bacteroidales; f_Prevotellaceae; g_Prevotella; s_NA					

Blue shaded color represents the presence of the ASV at % of total samples

the ruminal microbiota of RES heifers was expected. The effects associated with restricted diet on the ruminal microbiota structure and composition were the most evident on d 63 of gestation. On d 63, beta diversity of the ruminal microbiota diverged between RES and CON heifers, and microbial richness was increased in RES heifers. Likewise, species richness of the ruminal microbiota in lambs fed a diet with low (9.2 MJ/kg) metabolizable energy was higher than the lambs fed a diet containing a higher metabolizable energy (10.4 MJ/kg; [104]). In the present study, composition of the diet given to RES and CON heifers was the same, but the daily energy intake resulting from the diet restriction may have promoted an increase in richness of the ruminal microbiota in RES heifers in order to extract more energy from the diet to meet the energy demand by both ruminal microbes and the host. Another factor that could attributed to the difference in species richness of the ruminal microbiota observed between RES and CON heifers might be due to the difference in the ruminal fluid volume resulting from the different amounts of feed ingested into the rumen between the two groups. Caloric restricted diet [94] and time-restricted eating (TRE; [81] have been reported to be associated with increased alpha diversity in the human gut microbiota. A meta-analysis done by Pieczyńska-Zając also observed that TRE and fasting did not influence the alpha diversity of the gut microbiota in rodent animals, but enhanced microbial fluctuation [81]. We did not observe the impact of restricted diet on Shannon diversity indices of the ruminal microbiota.

The alterations in the relative abundance of phylum and genera observed in RES heifers on d 63 highlights significant modulation of the taxonomic composition of the ruminal microbiota due to restricted intake. Firmicutes, Actinobacteriota and Chloroflexi were enriched following 63 days of restricted dietary intake. Firmicutes is one of the most dominant phyla found in the rumen [44] and human gut [45], and it is the phylum most frequently reported to be affected by diet restriction and eating patterns [50]. In the mouse gut, the abundance of Firmicutes increased following intermittent fasting [13] while it was reduced in both the rodent and human gut when the hosts subjected to caloric intake reduced ad libitum intake by 10 to 30% [50]. Increased abundance of Firmicutes in the rumen has been associated with increased average daily gains

in beef steers [74] and milk-fat yield in dairy cattle [47], suggesting its positive correlation with feed efficiency [74]. The change in Firmicutes abundance results in change to the Firmicutes-to-Bacteroidetes ratio, which has previously been correlated with enhanced feed efficiency in sheep [110] and cattle [47]. There is little evidence showing either positive or negative correlations of phyla Actinobacteriota or Chloroflexi in cattle or other ruminant species. Nevertheless, Firmicutes enrichment induced by restricted gain may reflect taxonomic changes in the ruminal microbiota that might indicate re-assembly towards a more efficient energy extraction state to maximize limited feed ingestion. This notion is further supported by the alterations of the abundance of 34 bacterial genera, in which 25 of them become more abundant in RES heifers on d 63. Many of these bacterial genera enriched in RES heifers have been reported to have positive associations with animal health and feed efficiency. Among the enriched genera were SCFA producers including *Butyrivibrio* [the main butyrate-producing genus in the rumen [78]], *Christensenellaceae R-7 group* [acetate and butyrate producers in the rumen [9] that are also associated with increased feed efficiency [9, 33, 79]], and the acetate producing genus *Acetitomacum* [39]. The SCFAs produced from the gut microbiota have many important roles such as serving as an energy source to the host, and acting as signaling molecules between the gut and extraintestinal organs [15] and regulating the central appetite [37]. Potentially, RES heifers harbored greater abundance of SCFA producers in their rumen as compared to CON heifers as to modulate their appetite to adapt to the restricted caloric intake and/or to reduce fat accumulation. Acetate can reduce appetite via a central homeostatic mechanism [37], and butyrate can suppress insulin-mediated fat accumulation by SCFA receptor GPR43 (G protein coupled receptor) [52]. Another important factor driving the enrichment of SCFA producing bacteria in the rumen of RES pregnant heifers might be due to increased demand for SCFAs by the growing fetus. In rodent animal models, it has been demonstrated that the SCFAs produced from the maternal gut microbiota are provided to embryos via maternal circulation where they involve in regulation of fetal glucose homeostasis via the SCFA-GPR41/ 43 axis and imparting resistance to obesity in the offspring [51]. The maternal gut microbiota derived SCFAs are also involved in the regulation of fetal neurodevelopment [102]. In the present study we did not measure the SCFA production in the rumen of these pregnant heifers. However, our results point out that the ruminal bacteria associated with SCFAs might be key members affected by restricted dietary intake and maternal nutrition perturbations in early gestation. The implications of SCFA production in early gestation on fetal programming and maternal health warrants further investigation.

Some of the genera whose relative abundance was altered by restricted dietary intake on d 63 of gestation including *Lachnospiraceae, Prevotella,* and *Ruminococcus* are often positively or negatively associated with feed efficiency in cattle [33, 60, 61]. In addition to the changes observed in the relative abundance of 34 genera, overall genera-genera interaction network structure was also influenced by 63 days of restricted dietary intake (Fig. 4, resulting in more intense interactions that are centered around fewer hubs as compared to CON heifers. It is challenging to make inferences on the biological implications of the altered interaction network structure observed in RES heifers; however, active interactions between different microbial species are important for maintaining the stability and functional features of the microbiota associated with the gastrointestinal [25, 35] and respiratory tract [7]. Intensive interactions with balanced positive (cooperation) and negative (competition) proportions are positively associated with the functional activities and stability of the gut microbiota [31, 34, 101]. Accordingly, the intensified interactions between the ruminal genera of RES heifers may be an indication of a positive shift in the ruminal ecology in response to dietary intake restriction. Taken all together, restricted diet intake from breeding to 63 days of gestation resulted in significant alterations in maternal ruminal microbiota, which are characterized by the community structure, species richness, and composition at the phyla and genera level, and overall interaction network structure. How such microbial compositional and interaction network structural alterations that accompanied restricted dietary intake during the first trimester, which as a reminder, is a critical window of developmental programming events relating to skeletal muscle formation, organogenesis, and metabolic and neurodevelopment [10, 18, 23, 68, 86], should be the focus of feature research.

Supplementation of OCM from breeding to d 63 of gestation had minimal effect on the ruminal microbiota. Supplementation of OCM in this study was designed to test its ability to mitigate the undesired impact of the restricted dietary intake on fetal programming events. Immediately following fertilization, major epigenetic modifications including demethylation of paternal and maternal DNA, and embryonic genome re-methylation takes place [59, 70, 73]. These epigenetic events require adequate amount of OCM as they are essential for synthesis of the methyl donor S-adenosylmethionine (SAM), used for DNA and histone methylation [2, 56]. One-carbon metabolites such as folate, butyrate, and vitamin B_{12}

can be produced by microbial fermentation in the gut [49, 56, 75]; however, three essential B vitamins [folate (B_9), B_{12}, and B_6) utilized in the folate cycle are not supplied in sufficient amounts in the diet and must be supplied through de novo synthesis by the gut microbiota [56]. To the best of the author's knowledge, this is the first study to evaluate the impact of OCM supplementation on maternal ruminal microbiota in cattle. While the impact on the dam's ruminal microbiota was not evaluated, one study reported that rumen-protected methionine supplementation during the last 28 days of gestation resulted in alterations of the fecal microbiota of Holstein dairy calves, which were characterized by the enrichment of butyrate-producing bacteria, and microbial functional genes associated with antibiotic biosynthesis pathways [30]. There are several factors that could contribute to the resistance of ruminal microbiota modulation by OCM supplementation in the present study. One of which might be due to the rumen-protective coating of the choline and methionine, which limits microbial degradation of these OCMs in the rumen; thereby leading to negligible dietary influence. Another factor may be due to the robustness and resilient nature of the mature ruminal microbiota in these pregnant heifers (> 14 months old; [24, 106]. The dose of OCM supplementation may not have been high enough to induce changes in the ruminal microbiota, or noticeable alterations of the ruminal microbiota composition induced by OCM may take longer and be evident in the mid to late gestation periods.

Although the extent of dietary restriction from breeding to 63 days of gestation on vaginal microbiota is not as extensive as what was observed in the ruminal microbiota, it is interesting to detect a distinct community structure, altered phylum abundance, and different interaction network structure in the vaginal microbiota of RES heifers as compared to CON heifers on d 63. The effects of diet and eating patterns on vaginal microbiota had largely been underexamined in both humans [88] and livestock animals. However, considering the increased appreciation of the role of vaginal microbiota in protecting the pregnant uterus from pathogen invasion [3], from spontaneous preterm birth [36], as well as its role as a microbial seeding source of offspring perinatally [5, 40, 71], the dietary impact on the vaginal microbiota particularly during early gestation in cattle deserves full scale investigation. Diet could indirectly influence the vaginal microbiota through modulation of the immune system and the availability of micronutrients such as vitamins and minerals involved in overall host health [3, 11]. Additionally, changes in the gut microbiome due to dietary changes could alter the vaginal microbiome through the transfer of fecal microbiota to the vagina given the proximity of the anus to the vulva in cattle [57]. Our group recently observed significant alterations of the vaginal microbiota composition and diversity following 112 days of feeding two different high concentrate diets in beef heifers [107]. While we are unable to provide clear insights into the mechanisms of modulation of the vaginal microbiota by restricted dietary intake and OCM supplementation, our results show that maternal nutrition and caloric restriction during early pregnancy can influence vaginal microbiota of cattle.

Future research is warranted to investigate the effects of vaginal microbiota alterations on fetal programming due to restricted diet intake, and on feto-maternal crosstalk and offspring microbiome development. Focus should be given to the impact of altered relative abundance of the main bacterial phyla implicated in reproductive health, pregnancy maintenance, and offspring microbial seeding. In the present study, we observed changes in the vaginal microbiota characterized by the increase in the phyla Actinobacteriota and decrease of Proteobacteria and Fusobacteria of RES heifers. These three phyla are important members of the microbial communities in the vagina and uterus of cattle, and their presence has been reported in fetal samples [63, 71]. These phyla are also dominant phyla correlated with gut, reproductive, and respiratory tract-associated microbiota of newborn calves [62].

Our results also revealed that vaginal microbiota of pregnant heifers underwent significant changes immediately after fertilization which can be seen by the sharp increase in species richness, and diversity (Shannon and Inverse Simpson diversity) from pre-breeding to 35 days post-breeding, followed by further increase from d 35 to d 63. Vaginal microbiota in women has been reported to undergo significant changes over the course of pregnancy [1, 64, 84]. In contrast to our findings, other studies reported that species richness and diversity of vaginal microbiota reduced as pregnancy progressed in women [1, 64]. One of the explanations for such change is to protect both mother and the fetus from pathogen invasion by reducing the pH, which can be initiated by increased lactic acid production and immune modulation [28, 84]. A healthy vaginal microbiota in women is typically characterized by a low-diversity microbial community mainly dominated by lactic acid-producing *Lactobacillus* [12]. The increased richness and diversity of the vaginal microbiota have been associated with spontaneous preterm birth [36]. Our results suggest that vaginal microbiota in cattle increases in richness and diversity following impregnation and throughout the first 63 days of gestation. Whether the increased richness and diversity of the maternal vaginal microbiota remains throughout the 2nd and 3rd trimester is a question for future studies.

Increasing evidence derived from humans and sheep revealed the presence of microbial DNA signatures

in blood samples, suggesting the presence of blood-associated microbiota [19, 27, 80, 90]. Although the hypothetical presence of a unique microbiome specific to the blood is not supported by the results of recent large-scale study conducted to evaluate the blood microbiota of healthy individuals (n = 9770; [99], it was identified that the bloodstream of healthy individuals contains DNA from more than 100 different microbial species, and the bloodstream allows these microbes to move between different body sites including the gut, mouth, and urogenital tract. These identified microbial species were distinct from pathogens detected in hospital blood cultures. Replication rate analyses revealed that some of these microbes might be live and can replicate actively in the blood stream [99]. Yet, it is still debated if there is a self-sustaining and unique microbial community in the bloodstream of healthy animals. However, the presence of peripheral blood mononuclear cell-associated microbiota in goats has recently been reported [80]. Given that the blood may serve as a microbial transfer medium from the gut to extra-gastrointestinal microbial niches including the uterus, we were interested in characterization of the microbial DNA from whole blood of both RES and CON pregnant heifers at d 63. We identified microbial DNA signatures of bacterial species within 23 different phyla, and 358 different genera. All top 7 phyla and the majority of top 20 genera (Fig. 7C and D) are commonly present in the rumen (e.g. *Rikenellaceae RC9 gut group*), reproductive (e.g. *Cutibacterium)* and respiratory (e.g. *Mycoplasma*) tracts of cattle. While the phyla and genera level taxonomic composition of the blood microbiota supports the notion by Tan and colleagues [99] that the blood may not harbor blood specific microbiota, but instead harbors transient microbes using the bloodstream to translocate between the gut and extra-gastrointestinal microbial niches, our results presented in the Venn diagram (Fig. 8A), heatmap (Fig. 8B) and potential core ASVs table (Table 1) suggest otherwise. We identified over 1900 ASVs unique to the blood and not found in the ruminal and vaginal samples, and only 2 ASVs (ASV62 and 31) were shared by more than 65% of all ruminal, vaginal and blood samples. As shown in the heatmap, the ASVs found in blood are distinctively different in terms of frequency and relative abundance from the ASVs found in the ruminal fluid. This suggests that the ruminal microbiota may not be the only seeding source for the microbes present in the blood stream. Other microbial sources such as the hindgut [83], oral [53], and urogenital tract [32] associated microbial communities may contribute microbes. No effects of restricted dietary intake were observed on blood microbial community composition and diversity.

Conclusion

Significant effects of restricted gain from breeding to 63 days of gestation were observed on the community structure, microbial richness, composition, and interaction network structure of the ruminal microbiota in pregnant heifers, while the maternal ruminal microbiota remained unaffected by the supplementation of OCM. Overall community structure, diversity and composition of the maternal ruminal microbiota altered in response to pregnancy during the first trimester. Restricted gain affected the vaginal microbiota (community structure, phyla abundance, and interaction network structure) on d 63. Maternal vaginal microbiota underwent significant changes as pregnancy progressed and these changes included dramatic increase in microbial richness and diversity immediately following fertilization. Twenty-three different bacterial phyla and 358 different genera were identified from the whole blood samples collected on d 63, and the majority of the bacterial ASVs found in blood were unique and not shared by ruminal and vaginal microbiota. Blood associated microbiota did not differ between RES and CON heifers. Overall, these results indicate that maternal nutritional alterations during early gestation can not only impact the gut microbiome, but its effects could extend to the reproductive microbiota, calling for the further investigation of the impact of diet and intake restriction associated maternal microbiota alterations on developmental programming of offspring, as well as the maternal seeding of the fetal microbiota in utero.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-024-00335-2.

Additional file1

Acknowledgements

The authors gratefully acknowledge the support from the employees of the NDSU Animal Nutrition and Physiology Center and Meat Lab. Mention of a trade name, proprietary product, or specific agreement does not constitute a guarantee or warranty by the USDA and does not imply approval to the inclusion of other products that may be suitable. USDA is an equal opportunity provider and employer.

Author contributions

Conceiving the idea, designing the study, and providing supervision: AKW, CRD, JSC, MSC, and SA. Cattle management and sample collection: AKW, CRD, JSC, SA, KNS. Sample processing: SML and KNS. Data processing, bioinformatics, and statistical analysis: DBH, GA, SA, SML. Manuscript writing: SML and SA. Manuscript review, editing, and finalizing: SML, GA, DBH, KNS, MSC, AKW, CRD, JSC, SA. All authors have read and agreed to the published version of the manuscript.

Funding
The microbial work presented in this study was funded by the North Dakota Agricultural Experiment Station as part of a start-up package for S.A. Animal procurement and management was supported by the Agriculture Food and Resource Initiative [Award Number 2018-07055-2009] from the USDA National Institute of Food and Agriculture.

Availability of data and materials
Raw sequence data is available on the NCBI Sequence Read Archive under BioProject accession PRJNA1033356. Other data supporting the findings of this study are presented within the paper and in the supplementary information files.

Declarations

Competing interests
The authors declare no competing interests.

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Received: 20 December 2023 Accepted: 19 August 2024
Published online: 29 August 2024

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