Uterine Microbiota Progression from Calving until Establishment of Metritis in Dairy Cows

Soo Jin Jeon,[a] Achilles Vieira-Neto,[b] Mohanathas Gobikrushanth,[a] Rodolfo Daetz,[a] Rodolfo D. Mingoti,[b] Ana Carolina Brigolin Parize,[c] Sabrina Lucas de Freitas,[c] Antonio Nelson Lima da Costa,[d] Rodrigo C. Bicalho,[e] Svetlana Lima,[e] K. Casey Jeong,[b,f] Klibs N. Galvão[a,g]

Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, Florida, USA[a]; Department of Animal Sciences, University of Florida, Gainesville, Florida, USA[b]; Escola de Veterinária e Zootecnia, Universidade Federal de Goiás, Goiânia, Goiás, Brazil[c]; Departamento de Zootecnia, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil[d]; Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, New York, USA[e]; Emerging Pathogens Institute, University of Florida, Gainesville, Florida, USA[f]; D. H. Barron Reproductive and Perinatal Biology Research Program, University of Florida, Gainesville, Florida, USA[g]

The objective of this study was to evaluate the progression of the uterine microbiota from calving until establishment of metritis. Uterine swabs ($n = 72$) collected at 0, 2, and 6 ± 2 days postpartum (dpp) from 12 metric and 12 healthy cows were used for metagenomic sequencing of the 16S rRNA gene on the Illumina MiSeq platform. A heat map showed that uterine microbiota was established at calving. The microbiota changed rapidly from 0 to 6 ± 2 dpp, with a decrease in the abundance of *Proteobacteria* and an increase in the abundance of *Bacteroidetes* and *Fusobacteria*, which were dominant in metric cows. Uterine microbiota composition was shared; however, metric and healthy cows could be discriminated using relative abundance of bacterial genera at 0, 2, and 6 ± 2 dpp. *Bacteroides* was the main genus associated with metritis because it was the only genus that showed significantly greater abundance in cows with metritis. As the abundance of *Bacteroides* organisms increased, the uterine discharge score, a measure of uterine health, worsened. *Fusobacterium* was also an important genus associated with metritis because *Fusobacterium* abundance increased as *Bacteroides* abundance increased and the uterine discharge score worsened as the abundance increased. The correlation with uterine discharge score and the correlation with *Bacteroides* or *Fusobacterium* showed that other bacteria, such as *Helcoccocus*, *Filifactor*, and *Porphyromonas*, were also associated with metritis. There were also bacteria associated with uterine health, such as "*Candidatus* Blochmannia," *Escherichia*, *Sneathia*, and *Pedobacter*.

Metritis is a huge concern for the dairy industry worldwide because it is highly prevalent (25 to 40%) and negatively affects the productivity, survival, and welfare of dairy cows (1). Diverse bacteria, including anaerobes and facultative anaerobes, were observed in the uteri of dairy cows within the first 2 weeks postpartum, but they were naturally cleared out within 60 days postpartum (dpp) (1). Culture-based studies observed that *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Bacteroides* spp. (e.g., *Prevotella melaninogenica*, formerly *Bacteroides melaninogenicus*) were commonly associated with endometritis or pyometra (1–3).

Although culture-based studies have laid out the foundation of our understanding of the uterine microbiota, previous studies might have underestimated the microbial complexity of the intrauterine environment of cows postpartum, given that less than 1% of the microorganisms in many environments are readily cultured under standard laboratory conditions (4). In recent years, culture-independent techniques such as clone library sequencing (5, 6) and pyrosequencing (7, 8) have been used to characterize the uterine microbiota of cows with metritis (5–7) and endometritis (7, 8). Sequencing using the Illumina platform allows for deeper sequencing than has previously been feasible even with pyrosequencing (9). Indeed, evaluating the rarefaction curves from previous 16S rRNA sequencing studies (5–7), we can state that a larger number of sequences would be required to reflect the complete diversity of the microbiota of several samples.

Therefore, although the uterine microbiota of metric and healthy cows have previously been compared, use of a more powerful sequencing platform may allow us to have more complete coverage and a better understanding of the uterine microbiota. Furthermore, no one has evaluated the uterine microbiota at the time of calving. Our hypothesis was that uterine microbiota would have a different progression in healthy cows than in cows that develop metritis. Our objective was to evaluate the progression of the uterine microbiota composition from calving until establishment of metritis using the Illumina MiSeq platform to sequence the 16S rRNA gene.

MATERIALS AND METHODS

Animals. All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC protocol no. 201207405). Holstein cows from a commercial dairy in central Florida that milks 5,000 cows were used in this study.

Sampling. Uterine swab samples were collected from 92 cows at 0 (within 60 min of calving; mean time, 20 min; standard deviation, 14 min), 2, 4, 6, and 8 dpp as previously described (7). Swab samples were collected in March and April 2013. Briefly, cows were restrained and the perineum area was disinfected with 70% ethanol. Then, a sterile swab

(McCullough double-guarded uterine culture swab; Jorgensen Labs Inc., Loveland, CO) covered by a sterile pipette (inside a plastic sheath) was introduced into the cranial vagina. To avoid vaginal contamination of the swab, the plastic-sheath-covered pipette was directed into the cervix; inside the cervix, the plastic sheath (first layer of protection) was ruptured and the pipette was then manipulated through the cervix into the uterus. Once inside the uterus, the swab was advanced through the sealed plastic pipette (second layer of protection), exposing the sterile cotton swab to uterine secretion. The swab was pulled inside the pipette and removed while the pipette was maintained inside the uterus to avoid contamination by vaginal fluid. Swabs were transferred to a 15-ml conical sterile polypropylene centrifuge tube, placed on ice, transferred to the laboratory within 4 h, and then frozen at −80°C. Uterine discharge was scored at 4, 6, and 8 (6 ± 2) dpp using a 5-point scale as previously described (10): 1, not fetid normal lochia, viscous, clear, red, or brown; 2, cloudy mucoid discharge with flecks of pus; 3, not fetid mucopurulent discharge with <50% pus; 4, not fetid mucopurulent discharge with ≥50% pus; 5, fetid redbrownish, watery discharge. Cows with a discharge score of ≤4 were classified as healthy, and cows with a score of 5 and a fever (≥39.5°C) were classified as metritic, as previously described (11). For metagenomic sequencing, 72 uterine swab samples collected at 0, 2, and 6 ± 2 dpp from 12 cows that developed metritis and 12 randomly selected healthy cows were used. Samples from healthy cows matched the day a cow was diagnosed with metritis.

Metagenomic DNA extraction. Frozen swabs were soaked in 1 ml of phosphate-buffered saline (PBS) and incubated on ice for 2 h to release uterine bacteria from swabs. The PBS suspension was used to isolate bacterial genomic DNA (gDNA) using a QIAamp DNA minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, with a minor modification: before AL buffer was added, samples were incubated with 500 μg of lysozyme for 1 h at 37°C to maximize bacterial DNA extraction. The purity and concentration of gDNA were measured by a spectrophotometer (Nanodrop 1000; Thermo Scientific, Waltham, MA, USA). Genomic DNA was isolated in March 2014. All samples had an A_{260}-to-A_{280} absorbance ratio between 1.7 and 2.0 (average of 1.82).

PCR amplification of the V4 hypervariable region of bacterial 16S rRNA genes. Sequencing of 16S amplicons was performed as previously described (12). The DNA template was tagged with 12-bp error-correcting Golay barcodes, and the V4 hypervariable region of the bacterial 16S rRNA gene was amplified using 10 μM primer 515F and 806R, 1× GoTaq Green master mix (Promega, Madison, WI, USA), 1 mM MgCl2, and the 5′-barcoded amplicons in triplicate. PCR was carried out as follows: an initial denaturing step at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 90 s, and a final elongation step at 72°C for 10 min. Replicate amplicons were pooled and purified with a QIAquick PCR purification kit (Qiagen). The purified amplicons were quantified and standardized to the same concentration using a Qubit 3.0 fluorometer (Thermo Fisher Scientific), and sequencing was performed on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) using the MiSeq reagent kit v2 at 300 cycles. Eleven samples (7 from calving [4 healthy and 3 metritic cows], 3 from 2 dpp [all healthy], and 1 from 6 ± 2 dpp [healthy]) failed to amplify and therefore were not sequenced. Additionally, one sequenced sample (metritic at 6 ± 2 dpp) was excluded (12 total) because of a low number of reads (42 reads); therefore, 60 samples (8 healthy and 9 metritic at 0 dpp, 9 healthy and 12 metritic at 2 dpp, and 11 healthy and 11 metritic at 6 ± 2 dpp) were used for data analysis.

Data analysis. Taxonomy (phylum, class, order, family, genus, and species) and relative abundance were identified using the MiSeq Reporter proprietary metagenomics workflow based on the Greengenes database (http://greengenes.lbl.gov).

Statistical analysis. Rarefaction curves of 60 samples based on the number of species were built using the Metagenomics RAST server (MG-RAST, http://metagenomics.anl.gov). Chao1 and Shannon indices were calculated using the R software (http://www.r-project.org). All continu-
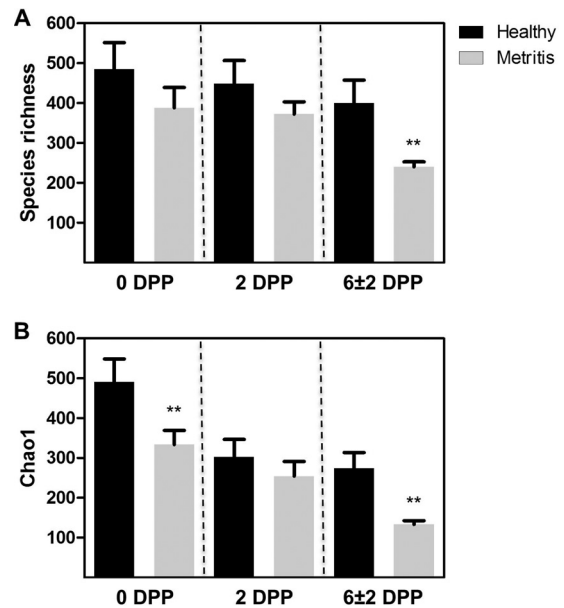

FIG 1 Estimates of alpha diversity. (A) Species richness calculated as the number of different species. (B) Species richness calculated as the Chao1 index. Data are presented as means ± SEM. **, significant difference between the healthy and metritic cows at $P < 0.01$ using ANOVA.

ous variables, such as relative abundance of bacteria, number of reads and number of species per sample, and species richness and diversity, were analyzed by analysis of variance (ANOVA) for repeated measures using the Mixed procedure of SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Models included the fixed effects of health status, time (0, 2, and 6 ± 2 dpp), and interaction between health status and time. Metritic and healthy cows at each time point were compared when there was a main effect of health status or an interaction between health status and time. Data are presented as means and standard errors of the means (SEM). Heat maps and hierarchical clustering of microbial communities at the phylum and genus level were generated based on Euclidean distance using the R software. To identify shared and unique bacterial genera, Venn diagrams were built using the Bioinformatics & Evolutionary Genomics tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). Discriminant analysis was performed using JMP Pro 11. The relative abundance of the 25 most abundant genera found at 0, 2, or 6 ± 2 dpp was used to discriminate cows that did or did not have metritis at 6 ± 2 dpp. A stepwise selection method was performed, and genera that did not contribute to the discrimination between groups were excluded. Microbial genera with canonical loadings of ≤−0.3 or ≥0.3 were considered important predictors of health status. Correlations were examined using Spearman's rank in SAS. For all statistical comparisons, differences at a P of ≤0.05 were considered significant. P values for comparisons between healthy and metritic cows were adjusted for multiple comparisons using the step-down Bonferroni adjustments available in the Multitest procedure of SAS. Both unadjusted and adjusted (adj) P values are presented.

Metagenome sequence accession numbers. Metagenome sequences were deposited in the MG-RAST server under the identification numbers listed in Table S1 in the supplemental material (4566112 to 4566156 and 4566158 to 4566172).

RESULTS

Sequencing results, number of reads and species, and Chao1 and Shannon indices. Sequencing of the V4 region of the bacterial 16S rRNA gene of 60 swab samples resulted in 4,979,168 reads, with an average of 82,986 ± 3,674 (SEM) reads per sample after
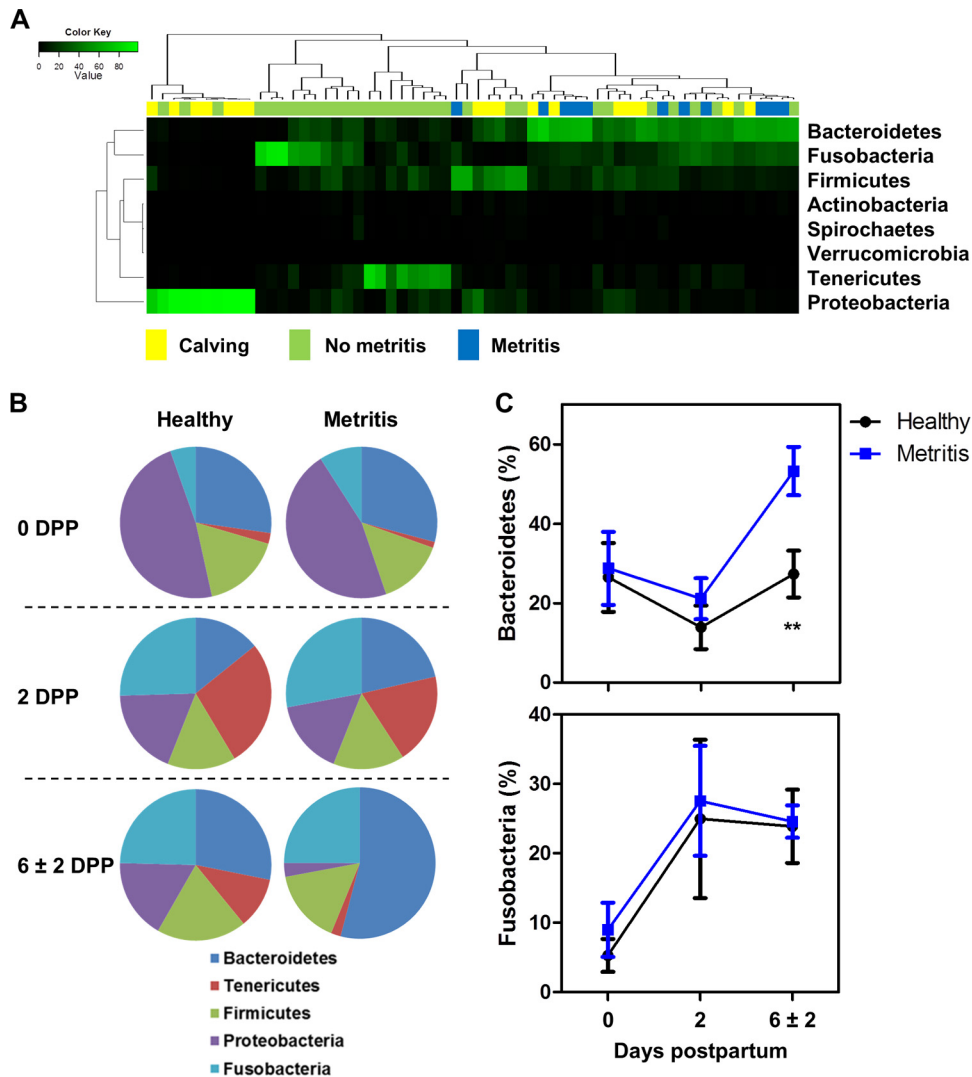
FIG 2 Bacterial composition and abundance at phylum level. (A) Heat map analysis using the Euclidean distance. Columns represent 60 dairy cows, and rows represent the eight most abundant bacterial phyla. The color of each cell indicates the relative abundance of bacterial phyla. The heat map shows the shift of uterine microbiota from calving (Calving; 0 dpp) until establishment of metritis (Metritis) at 6 ± 2 dpp. "No metritis" shows samples from healthy and metric cows at 2 dpp and healthy cows at 6 ± 2 dpp. (B) Distribution of the five most abundant phyla in healthy and metric cows at 0, 2, and 6 ± 2 dpp. (C) Relative abundances of *Bacteroidetes* and *Fusobacteria* in healthy and metric cows from 0 to 6 ± 2 dpp. **, significant difference between healthy and metric cows at $P < 0.01$ using ANOVA.

quality filtering. Rarefaction curves reached plateau in all but one of the samples sequenced, indicating that sampling depth was sufficient to describe uterine microbiota (see Fig. S1 in the supplemental material). Species richness, the mean number of different species, was lower ($P = 0.01$) for metric than for healthy cows at 6 ± 2 dpp (Fig. 1A). The Chao1 index, a measure of species richness based on the number of rare species (singletons and doublets), was also significantly lower ($P < 0.01$) in metric cows at 0 and 6 ± 2 dpp (Fig. 1B). The mean number of reads, a measure of species abundance, and the Shannon index, a measure of species diversity which combines species abundance and evenness, were not affected ($P > 0.15$) (see Fig. S2 in the supplemental material). Taken together, these data indicate that uterine microbiota from metric cows had decreased richness compared with that from healthy cows but the two had similar levels of diversity.

Comparisons at the phylum level. We found 28 phyla in all

samples; *Bacteroidetes* (28.9%), *Proteobacteria* (22.5%), *Fusobacteria* (20.2%), *Firmicutes* (15.8%), *Tenericutes* (10.7%), *Actinobacteria* (1.0%), *Spirochaetes* (0.5%), and *Verrucomicrobia* (0.1%) were the eight most abundant phyla in the uteri of all dairy cows, accounting for 99.7% of bacterial communities. A heat map analysis showed a change in uterine microbiota from calving until establishment of metritis, with an abundance of *Proteobacteria* at calving and an abundance of *Bacteroidetes* and *Fusobacteria* at metritis diagnosis (Fig. 2A). To identify the differences in uterine microbiota between healthy and metric cows, we compared the relative abundances of the five most abundant phyla between metric and healthy cows across time (Fig. 2B). *Bacteroidetes* were more abundant in metric cows than in healthy cows at 6 ± 2 dpp (53.1 versus 27.3%; $P < 0.01$; adj $P = 0.03$) (Fig. 2C). *Bacteroidetes* increased from 0 to 6 ± 2 dpp in metric cows (27.5 versus 53.1%; $P < 0.01$), whereas the abundance was unchanged in healthy cows
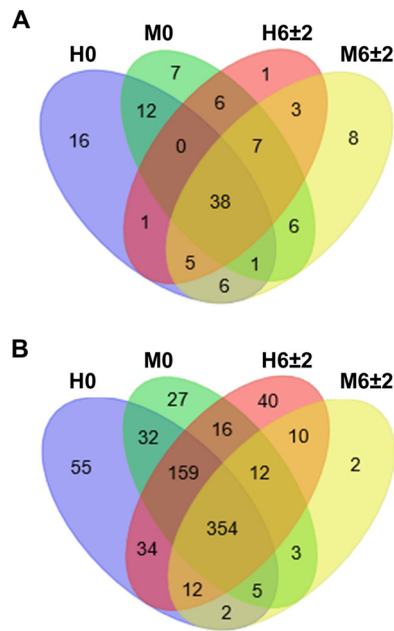
FIG 3 Venn diagrams showing numbers of shared and unique genera among uterine bacterial communities. (A) Number of genera shared by all of the dairy cows within each health status group. (B) Number of genera found in any of the dairy cows within each group. H, healthy; M, metritis; 0, calving; 6 ± 2, 6 ± 2 dpp.

(26.5 versus 27.3%; $P = 0.93$). The abundances of *Fusobacteria* in metritic and healthy cows were similar ($P > 0.75$) at all time points, but there was an overall increase in abundance at 2 (26.2%) and 6 ± 2 (24.1%) dpp compared with that at 0 dpp (8.4%; $P < 0.02$) (Fig. 2C).

Comparisons at the genus level. We found 824 genera in all samples. Table S2 in the supplemental material shows the 30 most abundant genera, accounting for 91.1% of bacterial microbiota. *Fusobacterium* (15.7%) was the most abundant genus, followed by *Bacteroides* (13.9%), *Coxiella* (12.7%), *Porphyromonas* (9.9%), and *Ureaplasma* (5.2%). *Prevotella* (2.9%), *Escherichia* (2.1%), and *Arcanobacterium/Trueperella* (0.3%) were the 10th, 12th, and 27th most prevalent genera, respectively. The Illumina MiSeq Reporter metagenomics workflow lumps the genus *Trueperella* with the genus *Arcanobacterium*; therefore, we refer to it as *Arcanobacterium/Trueperella*.

We evaluated whether a unique bacterial microbiota was associated with the development of metritis by examining core genera shared by all of the dairy cows within each health status group (Fig. 3A) and genera that were found in any of the dairy cows within each group (Fig. 3B). Overall, most genera were shared between metritic and healthy cows at 0 and 6 ± 2 dpp. The similarity in bacterial genera between metritic and healthy cows was 48.6% at calving, 62.8% at 2 dpp, and 64.6% at 6 ± 2 dpp when only core genera were evaluated and 77.4% at calving, 78.6% at 2 dpp, and 59.8% at 6 ± 2 dpp when genera found in any of the cows were evaluated (see Fig. S3 in the supplemental material). Among bacterial genera that were shared are those that were prevalent and/or are considered important for the development of metritis, such as *Fusobacterium*, *Bacteroides*, *Escherichia*, and *Arcanobacterium/Trueperella*. Among genera that were not shared are those that were rare (<0.01% prevalence), such as *Pandoraea*, *Pseudonocar-*

dia, *Wautersiella*, *Ethanoligenens*, and others. Since most bacterial genera were shared among metritic and healthy cows at all times, it is likely that changes in bacterial abundance and interactions among shared bacteria are more important for the development of metritis than unique differences in bacterial communities.

To investigate this hypothesis, we evaluated the progression of the 15 most abundant genera (≥1% abundance; representing 84.3% of uterine microbiota) using a heat map analysis (Fig. 4A) and compared the relative abundances of the five most abundant genera (≥5% abundance) from 0 dpp to 6 ± 2 dpp (Fig. 4B). The overall relative abundances for the 30 most prevalent bacterial genera are presented in Table S2 in the supplemental material. Similarly to the results at the phylum level, the heat map (Fig. 4A) and pie chart (Fig. 4B) showed a change in uterine microbiota from calving until establishment of metritis. There was an overall increase in the relative abundances of *Bacteroides*, *Fusobacterium*, and *Porphyromonas* bacteria from 0 to 6 ± 2 dpp, whereas *Ureaplasma* increased from 0 to 2 dpp and then decreased from 2 to 6 ± 2 dpp and *Coxiella* decreased dramatically from 0 to 6 ± 2 dpp. *Bacteroides* was significantly more abundant in metritic than in healthy cows at 6 ± 2 dpp (28.3 versus 12.5%; $P < 0.01$; adj $P = 0.02$). *Ureaplasma* showed a lower abundance in metritic cows than in healthy cows at 2 dpp (5.8 versus 19.0%; $P = 0.04$), but that was not significant after adjustment for multiple comparisons (adj $P = 0.22$). *Fusobacterium*, *Coxiella*, and *Porphyromonas* were similar at all time points ($P > 0.05$; adj $P > 0.25$).

Furthermore, we evaluated the correlations between the 30 most prevalent bacterial genera and clinical signs of metritis at 6 ± 2 dpp (Fig. 5; also see Table S3 in the supplemental material). Although cows with a uterine discharge score of ≤4 were classified as healthy, the relative abundance of *Bacteroides* organisms increased as the uterine discharge score increased (Spearman $r_s = 0.51$; $P = 0.02$) (Fig. 5A). Likewise, the relative abundance of *Fusobacterium* bacteria increased as the uterine discharge score increased (Spearman $r_s = 0.49$; $P = 0.02$) (Fig. 5B). This indicates that *Bacteroides* and *Fusobacterium* are highly associated with metritis. On the other hand, *Sneathia*, *Mycoplasma*, *Escherichia*, *Pedobacter*, *Serratia*, "*Candidatus* Blochmannia," *Treponema*, *Oscillospira*, *Pasteurella*, and *Mannheimia* were all negatively correlated ($P ≤ 0.05$) with uterine discharge score (see Table S3). Interestingly, the abundance of *Fusobacterium* organisms was positively correlated with the abundance of *Bacteroides* organisms (Spearman $r_s = 0.51$; $P = 0.02$) (Fig. 5C). *Fusobacterium* was also positively correlated ($P = 0.01$) with *Helcococcus*, which showed a positive correlation ($P = 0.05$) with uterine discharge score. Meanwhile, *Bacteroides* was positively correlated ($P = 0.03$) with *Filifactor*, which was positively correlated ($P ≤ 0.05$) with *Porphyromonas*, *Peptoniphilus*, *Peptostreptococcus*, and *Campylobacter*. *Porphyromonas*, *Peptoniphilus*, and *Peptostreptococcus* were positively correlated ($P ≤ 0.05$) with *Prevotella*. *Escherichia*, *Ruminococcus*, *Blautia*, *Clostridium*, *Pedobacter*, *Serratia*, *Treponema*, *Oscillospira*, *Caloramator*, and *Pasteurella* were negatively correlated ($P ≤ 0.05$) with *Bacteroides* and/or *Fusobacterium*.

Because of the complex interactions among the main bacteria, we used discriminant analysis to identify the most important bacteria involved in the development of metritis. Discriminant analysis was performed using the 25 most abundant genera in the uteri of cows at 0, 2, or 6 ± 2 dpp. Abundance was evaluated at each time point separately. Discriminant analysis is a multivariate procedure used to predict to which group a case belongs. The discrim-
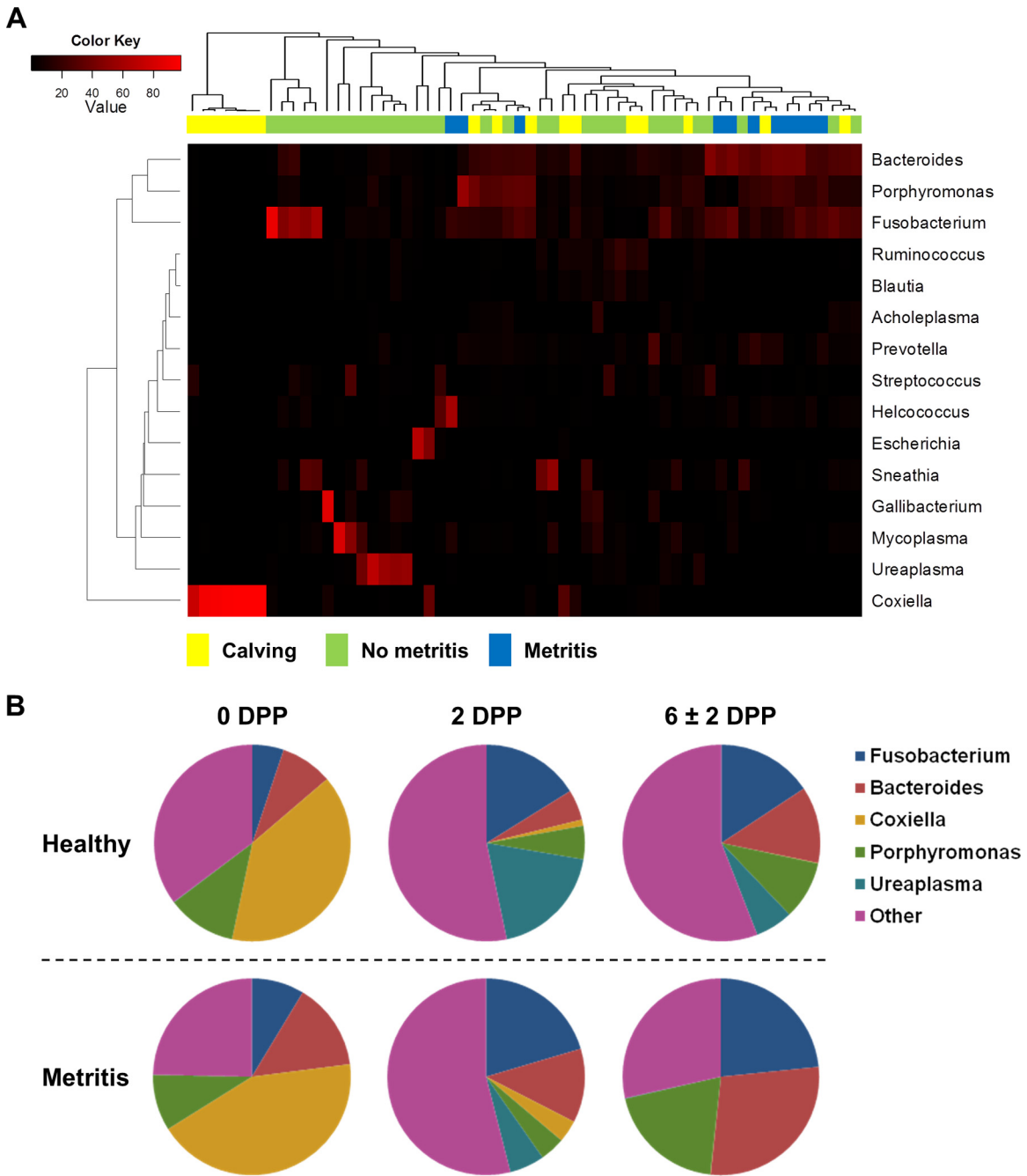
FIG 4 Bacterial composition and abundance at genus level. (A) Heat map analysis using the Euclidean distance. Columns represent 60 dairy cows, and rows represent the 15 most abundant bacterial genera. The color of each cell indicates the relative abundance of bacterial genera. The heat map shows the shift of uterine microbiota from calving (Calving; 0 dpp) until establishment of metritis (Metritis) at 6 ± 2 dpp. "No metritis" shows samples from healthy and metric cows at 2 dpp and healthy cows at 6 ± 2 dpp. (B) Distribution of the five most abundant genera in healthy and metric cows at 0, 2, and 6 ± 2 dpp.

inant function is used to calculate canonical scores, which are plotted to visualize the separation between groups. The goal is to have an equation that has strong discriminatory power between groups. The discriminant analysis showed that the discriminant function was highly significant ($P < 0.001$), and all cows were correctly discriminated at all time points (Fig. 6A; also see Table S4 in the supplemental material). Canonical structure coefficients (canonical loadings), which represent the correlation between

each variable (bacterial genus) and the discriminant function adjusted for intercorrelations among microbial genera, were used to evaluate the importance of each bacterial genus. Canonical structure coefficients for bacterial genera that contributed to the discriminant function are presented in Fig. 6B. A canonical loading cutoff of ± 0.3 was used. At 0 dpp, the top three genera that loaded most strongly for the metritis group did not reach the 0.3 cutoff (*Helcococcus* [0.27], *Bacteroides* [0.24], and *Fusobacterium* [0.20]);
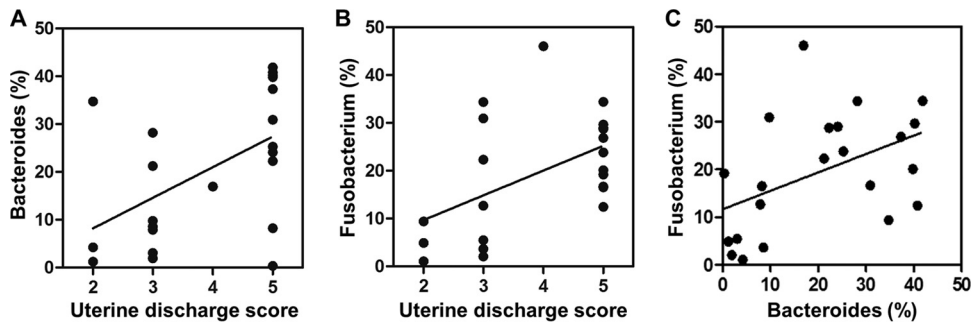
FIG 5 Associations of *Bacteroides* and *Fusobacterium* with metritis. (A) Correlation between uterine discharge score and *Bacteroides* (Spearman $r_s = 0.51$; $P = 0.02$). Cows were assigned to five groups according to uterine discharge score: 1, not fetid normal lochia, viscous, clear, red, or brown; 2, cloudy mucoid discharge with flecks of pus; 3, not fetid mucopurulent discharge with <50% pus; 4, not fetid mucopurulent discharge with ≥50% pus; 5, fetid red-brownish, watery discharge. Cows with a discharge score of ≤4 were classified as healthy, and cows with a score of 5 were classified as metritic at 6 ± 2 days postpartum. (B) Correlation between uterine discharge score and *Fusobacterium* (Spearman $r_s = 0.49$; $P = 0.02$). (C) Correlation between *Bacteroides* and *Fusobacterium* (Spearman $r_s = 0.51$; $P = 0.02$).

however, *Escherichia* (−0.43) loaded significantly for the healthy group. At 2 dpp, *Bacteroides* (0.30) was the only important variable for the metritis group, whereas "*Candidatus* Blochmannia" (−0.50) and *Ureaplasma* (−0.31) were important variables for the healthy group. At 6 ± 2 dpp, *Bacteroides* (0.54), *Filifactor* (0.39), *Porphyromonas* (0.38), and *Fusobacterium* (0.32) were important variables to predict metritis, whereas *Sneathia* (−0.41), *Acholeplasma* (−0.41), "*Candidatus* Blochmannia" (−0.41), *Ruminococcus* (−0.31), and *Pedobacter* (−0.30) were important variables to predict the healthy group.

Taken together, our data indicate that *Bacteroides* is the main genus associated with metritis because it showed higher abundance in cows with metritis, it was positively correlated with uterine discharge score, and it loaded most strongly in the discriminant analysis at 6 ± 2 dpp. The correlation and discriminant analyses also indicate that other genera, such as *Fusobacterium*, *Helcococcus*, *Filifactor*, *Porphyromonas*, *Peptoniphilus*, *Peptostreptococcus*, *Campylobacter*, and *Prevotella*, may be important for the development of metritis. Figure 7 shows the microbial interactions among bacteria that may be important for the development of metritis, highlighting *Bacteroides* and *Fusobacterium*, which were positively correlated with uterine discharge score and loaded significantly for the metritis group in the discriminant analysis, but also showing bacteria that were positively correlated with these main bacteria, such as *Filifactor* and *Helcococcus*, and other bacteria that were positively correlated with *Filifactor* and among themselves, such as *Peptoniphilus*, *Porphyromonas*, *Peptostreptococcus*, and *Prevotella*. There were also several bacteria associated with uterine health, such as *Escherichia*, "*Candidatus* Blochmannia," *Sneathia*, *Pedobacter*, *Mycoplasma*, *Ureaplasma*, *Bifidobacterium*, *Acholeplasma*, *Ruminococcus*, *Serratia*, *Treponema*, *Oscillospira*, *Pasteurella*, and *Mannheimia*. Figure 8 shows the microbial interactions associated with uterine health, highlighting *Escherichia*, "*Candidatus* Blochmannia," *Sneathia*, and *Pedobacter*, which were negatively correlated with uterine discharge score and loaded significantly for the healthy group in the discriminant analysis, but also showing bacteria that were positively correlated with these main bacteria, such as *Ruminococcus*, *Treponema*, *Oscillospira*, *Pasteurella*, *Acholeplasma*, and *Mannheimia*, and other bacteria that were positively correlated with them and among themselves, such as *Serratia*, *Bifidobacterium*, *Ureaplasma*, and *Mycoplasma*.

DISCUSSION

Our study used high-throughput sequencing on the Illumina MiSeq platform, which is one of the most powerful tools to investigate the microbiome associated with health and disease (13, 14). The rarefaction curves confirmed that the MiSeq platform provided deeper sequencing and greater coverage than what has been previously reported using pyrosequencing (5, 7) or clone library sequencing (6). It is not clear why 11 samples failed to amplify and one sample resulted in a low number of reads, but samples at calving were particularly more likely not to amplify (7/11); therefore, low bacterial abundance at calving may be an important factor contributing to a lack of PCR amplification.

Our data showed that uterine microbiota was already established shortly after calving and that microbiota changed rapidly, favoring anaerobes such as *Bacteroidetes* and *Fusobacteria*. This change is probably a consequence of stoppage of blood flow through the placenta, low oxygen tension, and necrosis of caruncular epithelium, which, along with substrates such as remaining blood and amniotic and allantoic fluids, may create the ideal conditions for the growth of anaerobes (1, 15, 16). In this study, *Bacteroidetes* was the only phylum with significantly higher abundance in metritic than in healthy cows, therefore indicating that *Bacteroidetes* may be an important phylum for the development of metritis in dairy cows. Two recent studies using clone library sequencing found either an overwhelming predominance of *Fusobacteria* in metritic cows (5) or an overall predominance of *Bacteroidetes* in both metritic and healthy cows (6). One study using pyrosequencing observed a predominance of *Bacteroidetes* and *Fusobacteria* in both metritic and healthy cows (7). One study using pyrosequencing observed higher abundances of *Bacteroidetes*, *Tenericutes*, and *Fusobacteria* in cows with endometritis than in healthy cows (8). A recent study using MiSeq sequencing found a predominance of the families *Fusobacteriaceae*, *Bacteroidaceae*, *Porphyromonadaceae*, and *Pasteurellaceae* in the uterine fluid of cows with pyometra (17). Consistent with our findings, Santos et al. described that the microbiota of healthy cows was dominated by *Proteobacteria* and *Tenericutes* (5). Since metritis is a complex disease, some conflicting findings are expected; therefore, further studies in different parts of the country and/or the world are needed to further elucidate the role of each phylum in the development of metritis.
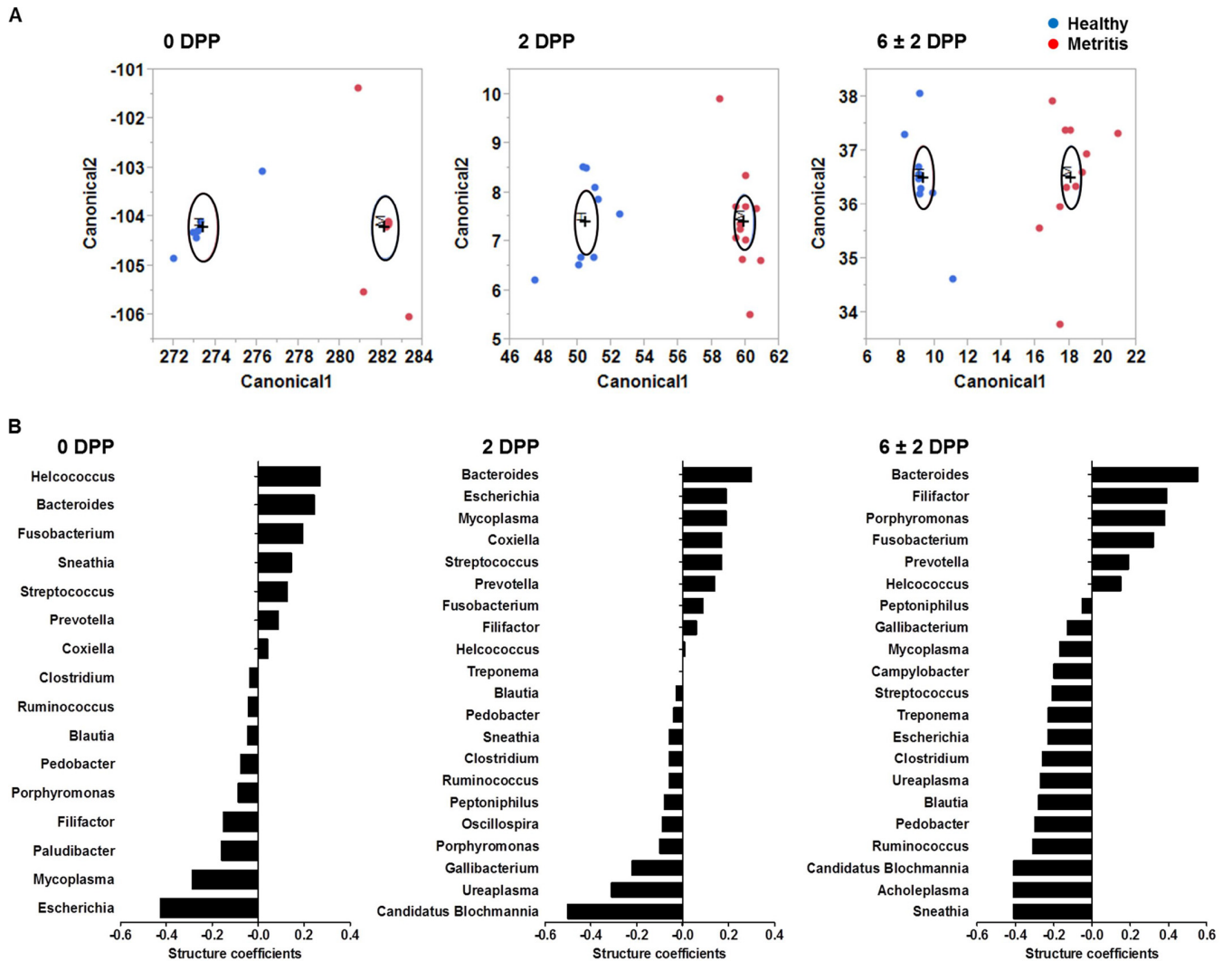
FIG 6 Discriminant analysis using the 25 most abundant genera. (A) Canonical discriminant analysis of uterine swab samples from cows at 0, 2, and 6 ± 2 dpp. Metritic cows were significantly discriminated from healthy cows from 0 to 6 ± 2 dpp. An ellipse indicates the 95% confidence region to contain the true mean of the group, and a plus symbol indicates the center (centroid) of each group. (B) Canonical structure coefficients representing the correlation between each bacterial genus and the discriminant function. Bacteria with canonical structure coefficients of ≤−0.3 or ≥0.3 are considered important to predict between healthy and metritic cows.

Differences in relative abundance, correlation with uterine discharge score, and discriminant analysis showed that *Bacteroides*, followed by *Fusobacterium*, was the most important genus associated with metritis. The correlation with uterine discharge score, the correlation with *Bacteroides* and *Fusobacterium*, and the discriminant analysis showed that other bacteria, such as *Helcococcus*, *Filifactor*, *Porphyromonas*, *Peptoniphilus*, *Peptostreptococcus*, *Campylobacter*, and *Prevotella*, may also be important for the development of metritis. Particularly, *Helcococcus*, *Filifactor*, and *Porphyromonas* were directly associated with metritis by either having a positive correlation with the uterine discharge score (*Helcococcus*) or being associated with the metritis group in the discriminant analysis (*Filifactor* and *Porphyromonas*). Therefore, it is possible that *Bacteroides* acts synergistically with *Fusobacterium* and other bacteria, such as *Helcococcus*, *Filifactor*, and *Porphyromonas*, to cause metritis in dairy cows. The pathogenicity of *Bacteroides* in mixed bacterial infections is believed to occur

through inhibition of phagocytosis and killing by polymorphonuclear leukocytes (PMN); *Bacteroides* was found to produce short-chain fatty acids that directly inhibited phagocytosis and killing of bacteria by PMN (18). In models of necrobacillosis, the lethal dose of *F. necrophorum* could be greatly decreased by coinfection with *Bacteroides* spp., *T. pyogenes*, or *E. coli* (19, 20). *Porphyromonas levii* has also been shown to produce an immunoglobulin protease that can cleave bovine IgG and reduce PMN phagocytosis (21).

Early culture-based studies found an association between *Bacteroides* spp. and uterine discharge score; however, *P. levii* (formerly *Bacteroides levii*) and *Prevotella melaninogenica* (formerly *Bacteroides melaninogenicus*) were all combined into *Bacteroides* spp., which now can be interpreted as the phylum *Bacteroidetes* (3). Early culture-based studies also found an association between *F. necrophorum* and endometritis (i.e., purulent uterine discharge after 21 dpp) or pyometra and a correlation between *Bacteroides* and *F. necrophorum* (3, 22, 23). Other studies found that cows with
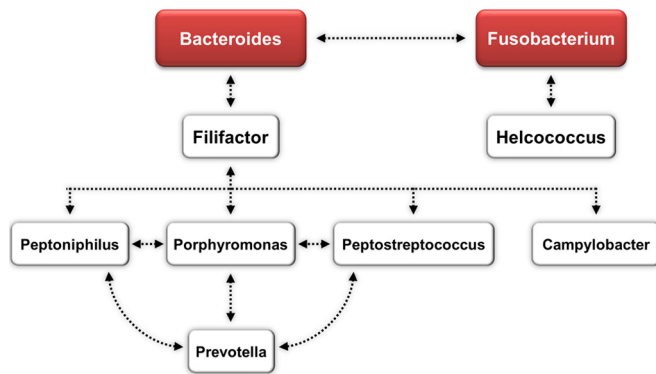
FIG 7 Microbial interactions associated with metritis. Bacteria in red boxes were positively correlated with uterine discharge score (higher score indicates worse uterine health) and loaded significantly for the metritis group in the discriminant analysis. Bacteria in the white boxes were positively correlated with the bacteria in the red boxes. Arrows indicate positive correlations ($P \leq 0.05$).
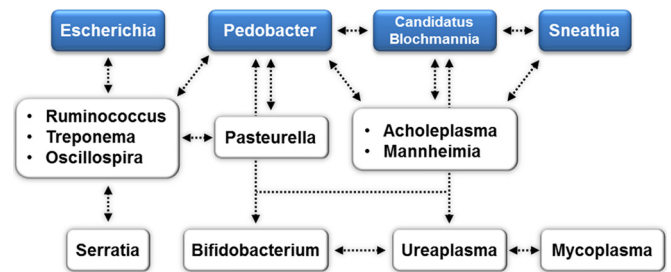

FIG 8 Microbial interactions associated with uterine health. Bacteria in blue boxes were negatively correlated with uterine discharge score (lower score indicates uterine health) and loaded significantly for the healthy group in the discriminant analysis. Bacteria in the white boxes were positively correlated with the bacteria in the blue boxes. Arrows indicate positive correlations ($P \leq 0.05$).

retained placenta, a major risk factor for metritis, usually had mixed bacterial infections containing *P. levii*, *F. necrophorum*, and *T. pyogenes* (24). These studies pointed to the importance of *Bacteroides* spp. and *P. levii* for causing endometritis and potentially metritis. Recent studies using clone library sequencing (5, 6) or pyrosequencing (7) focused on characterization of phylum but also tried to identify the main species in healthy and metric cows. An advantage of the clone library sequencing studies is the ability to sequence large (6) or multiple (5) regions of the 16S rRNA gene; however, a disadvantage is the low sequencing depth and coverage. Pyrosequencing and Illumina sequencing provide greater sequencing depth and coverage, but the shorter reads may provide inaccurate species classification, especially for species with high 16S rRNA gene similarity, such as *Escherichia* and *Shigella* species (25). Within the phylum *Bacteroidetes*, Peng et al. observed that *Bacteroides pyogenes*, *Bacteroides heparinolyticus*, and *P. levii* were the most prevalent species (6). Santos et al. reported that the genus *Porphyromonas* was the most prevalent in the phylum *Bacteroidetes*, but species like *B. heparinolyticus* and *Bacteroides denticanum* (also known as *B. denticanoris*) were also identified (5). Within the phylum *Fusobacteria*, recent studies using clone library sequencing (5, 6) or pyrosequencing (7) observed that *F. necrophorum* was the most prevalent species in metric cows (5) or in both healthy and metric cows (6, 7). Although sequencing of only the V4 region may be less accurate for assigning taxonomy at the species level, our observations at the species level (see Table S5 in the supplemental material) corroborate previous observations (5–7). A recent study using pyrosequencing observed that endometritic cows had higher relative abundances of *Ureaplasma*, *Fusobacterium*, *Bacteroides*, and *Porphyromonas* bacteria than healthy cows (8); therefore, apart from *Ureaplasma*, our observations in metric cows corroborate the findings in endometritic cows.

Members of the genus *Helcococcus*, found to be associated with metritis in this study, were also associated with endometritis in the study by Machado et al. (8). Herein, *Helcococcus* was positively correlated with the uterine discharge score, and *Helcococcus ovis* was identified as the most prevalent species for this genus. *H. ovis* was recently reported to be an important pathogen associated with bovine valvular endocarditis (26); therefore, it may be an impor-

tant species associated with uterine disease. In addition, we recognized *Filifactor* as an important genus because it was associated with the metritis group in the discriminant analysis and it was positively correlated with *Bacteroides*. *Filifactor villosus* was the main species from this genus (see Table S5 in the supplemental material). Therefore, further investigation of the role of *Helcococcus* and *Filifactor* and the main species from these genera in the development of metritis is warranted. *Peptoniphilus* was also associated with endometritis in the study by Machado et al. (8). The genus *Prevotella* was positively correlated with bacteria considered important, such as *Porphyromonas*, which indicates an indirect association with metritis. The most prevalent species from the genus *Prevotella* was *Prevotella albensis* (0.6%) (see Table S5). Previous culture-based studies have identified *P. melaninogenica* as an important pathogen for the development of endometritis (2, 3); however, none of the metric cows and only one healthy cow had *P. melaninogenica*. Similarly, Machado et al. identified *Prevotella* as an important genus in cows with endometritis; however, the analysis at the species level was not presented (8). Therefore, further investigation into the importance of *Prevotella* spp. in the development of metritis is warranted.

Intriguingly, the genera *Trueperella* and *Escherichia*, which contain species commonly associated with uterine disease, such as *Trueperella pyogenes* and *Escherichia coli* (1, 2, 27), were not associated with metritis in this study. In fact, *Escherichia* was associated with uterine health. Herein, we identified *T. pyogenes* as the most prevalent species of the genus *Trueperella* and *E. coli* as the second most prevalent species of the genus *Escherichia* (see Table S5 in the supplemental material). It is likely that species-intrinsic factors may be more relevant to the development of uterine disease than abundance. For instance, previous studies have observed that uterine disease is associated with the presence of specific virulence factor genes, such as *fimA* in *T. pyogenes* (28) or *fimH* in *E. coli* (29).

In conclusion, we observed that a uterine microbiota that was shared among metric and healthy cows was present in the uterus shortly after calving. The microbiota changed rapidly, favoring anaerobes such as *Bacteroidetes* and *Fusobacteria*. *Bacteroides* was the main genus associated with metritis, followed by *Fusobacterium*. Other bacteria, such as *Helcococcus*, *Filifactor*, *Porphyromonas*, *Peptoniphilus*, *Peptostreptococcus*, *Campylobacter*, and *Prevotella*, may also be important for the development of metritis. There were also bacteria associated with uterine health, such

as "*Candidatus* Blochmannia," *Escherichia*, *Sneathia*, and *Pedo-bacter*.

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