Effect of pegbovigrastim administration on the microbiome found in the vagina of cows postpartum

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ABSTRACT

The objective of this study was to evaluate the effect of pegbovigrastim (PEG) treatment of peripartum Holstein cows on the microbiome found in the vagina postpartum using sequencing of the 16S rRNA gene. A subset of cows was randomly sampled from a larger study where cows had been randomly assigned to 1 of 2 treatments: pegbovigrastim (PEG) or untreated control (CTR). The PEG-treated cows received a subcutaneous injection containing 15 mg of pegbovigrastim 7 d before expected calving and a second injection within 24 h of calving. Vaginal samples from 97 PEG-treated and 98 CTR cows were collected at calving, 7 ± 3 , and 35 ± 3 d in milk (0, 7, and 35 DIM). Metritis was diagnosed at 7 ± 3 DIM and purulent vaginal discharge (PVD) at 35 ± 3 DIM. The PEG treatment did not alter the vaginal microbiome. Principal coordinate analysis (PCoA) showed that metritic cows had a dissimilar vaginal microbiome compared with cows that did not develop metritis, particularly at 7 but also at 35 DIM. This difference was characterized by higher relative abundance of *Porphyromonas* and *Bacteroides* and a lower relative abundance of *Ureaplasma*, *Ruminococcaceae*, and *Clostridiales* at 7 DIM, and a higher relative abundance of *Ureaplasma* and a lower relative abundance of *Pasteurellaceae* at 35 DIM. Based on PCoA, we observed that cows that developed PVD had a dissimilar vaginal microbiome compared with cows that did not develop PVD, particularly at 35 DIM but also at 7 DIM. This difference was characterized by a higher relative abundance of *Bacteroides* at 7 DIM and higher relative abundance of *Fusobacterium* and *Bacteroides* at 35 DIM. Cows that developed metritis and PVD also had higher relative abundance of *Fusobacterium* and *Bacteroides* at 0 DIM. Furthermore, the Chao1 and Shannon indices were decreased in metritic

cows at 7 DIM and in PVD cows at 7 and 35 DIM. In summary, PEG treatment had no effect on the vaginal microbiome, and uterine disease was associated with major changes in the microbiome found in the vagina postpartum.

Key words: pegbovigrastim, microbiome, mastitis, metritis

INTRODUCTION

Improvements in genetics, housing, nutrition, and management have resulted in dramatic increases in milk production in the last 70 yr, from ~2,000 kg/yr in 1944 to over 10,000 kg/yr in 2016 (Capper et al., 2009; Santos et al., 2010; USDA-NASS, 2017). Nonetheless, the improvements in milk production have not been accompanied by improvements in health. Approximately 45% of dairy cows experience a disease or disorder in the first 60 d postpartum (Santos et al., 2010), and metritis and mastitis are two of the most prevalent and costly diseases to the dairy industry (Goshen and Shpigler, 2006; Overton and Fetrow, 2008; Cha et al., 2011).

Innate immunity is the most important defense system of the bovine uterus and mammary gland, and the neutrophil is the main cell type involved in innate immunity in those organs (Hussain, 1989; Paape et al., 2003). However, during the transition into lactation, dairy cows experience a reduction in leukocyte function, including a reduction in neutrophil phagocytosis and killing capacity, which is believed to be a predisposing factor to infectious diseases such as metritis and mastitis (Goff and Horst, 1997; Hammon et al., 2006; Galvão and Santos, 2014).

Granulocyte colony-stimulating factors (G-CSF) are cell-signaling glycoproteins involved in hematopoiesis and inflammation (Nicola et al., 1983; Souza et al., 1986; Zsebo et al., 1986; Groopman et al., 1989). Nickerson et al. (1989) first identified a role for G-CSF in prevention of clinical mastitis. They observed that treatment with recombinant G-CSF led to an increase in circulating neutrophils, an increase in migration of neutrophils to

Received October 1, 2018.

Accepted December 15, 2018.

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the mammary gland after an experimental challenge with *Staphylococcus aureus*, and a numerical reduction in new infections in challenged quarters (Nickerson et al., 1989). Later, Kimura et al. (2014) showed that treatment of periparturient dairy cows with pegylated recombinant bovine G-CSF (pegbovigrastim, Imrestor, Elanco Animal Health, Greenfield, IN) led to a dramatic increase in the number of circulating neutrophils and an increase in myeloperoxidase release after stimulation *in vitro*, which indicated a possible increase in neutrophil extracellular killing ability (Kimura et al., 2014).

The administration of pegbovigrastim (**PEG**) 7 d before expected calving and on the day of calving reduced the number of naturally occurring clinical mastitis cases (Hassfurth et al., 2015; Ruiz et al., 2017). However, in a recent study conducted by our group, PEG treatment did not prevent the occurrence of clinical mastitis (Zinicola et al., 2018). It has been reported that PEG treatment resulted in a small but significant increase in the incidence of metritis, although the negative effects of metritis on milk yield were less severe in multiparous PEG-treated cows that developed metritis (Ruiz et al., 2017). Similar results on milk yield in multiparous cows diagnosed with metritis and treated with PEG were observed (Zinicola et al., 2018); however, in that study, the administration of PEG did not alter the incidence of metritis.

Recently, we observed that metritic cows treated with PEG had a higher proportion of neutrophils in the vagina at 7 DIM compared with metritic control cows (Zinicola et al., 2018). In addition, we observed a greater reduction in blood neutrophils in PEG-treated cows diagnosed with metritis compared with metritic control cows, indicating that more neutrophils were migrating to the reproductive tract in PEG-treated cows (Zinicola et al., 2018). Neutrophil migration and function is believed to be critical for prevention or elimination of infection in the reproductive tract. Thus, one way to better understand the effects of PEG on disease establishment in the reproductive tract is to study the microbiome. We have shown that metritis is associated with a dysbiosis of the normal uterine microbial flora (Jeon et al., 2015). This dysbiosis is characterized by an increased in the relative abundance of gram-negative anaerobes such as *Bacteroidetes* and *Fusobacteria* and a decrease in the relative abundance of *Firmicutes* (Jeon et al., 2015). Therefore, we hypothesized that periparturient dairy cattle treated with PEG would have a greater migration of neutrophils to the uterus, which would prevent the microbial dysbiosis associated with metritis. Therefore, our primary objective was to characterize the microbiota found in the vagina postpartum in cows treated with PEG 7 d from the expected calving date and within 24 h of calving. A secondary

objective was to compare the microbiome in the vagina postpartum in cows that developed uterine disease with that of cows that did not develop uterine disease.

MATERIALS AND METHODS

Cows, Housing, and Feeding

The study was conducted in a large commercial dairy farm located near Ithaca, New York, from September 2016 to December 2016. All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocol number 2016-0028). The farm milked ~3,800 Holstein cows 3 times daily in a rotary parlor with integrated milk meters that record individual production at every milking (DeLaval, Tumba, Sweden). The cows were housed in freestall barns, with concrete stalls bedded with composted manure solids treated with quicklime (CaO). After calving, all cows were offered a TMR with feed pushed up 6 to 8 times a day and consisting of approximately 55% forage (corn silage, haylage, and wheat straw) and 45% concentrate (corn meal, soybean meal, canola, cottonseed, and citrus pulp) on a DM basis. The diet was formulated to meet or exceed National Research Council nutrient requirements for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% FCM (NRC, 2001). As cows demonstrated signs of calving, they were moved to individual maternity pens for delivery, where trained farm personnel assisted with parturition as needed. Calves were removed from their dams immediately after birth.

Study Cows

The cows used for this study are a subset of cows enrolled in a larger experiment that evaluated the effect of PEG on periparturient diseases, milk production, and reproductive performance of Holstein cows (Zinicola et al., 2018). For the larger experiment, 840 cows were randomly assigned within parity group (primigravid or multigravid) to 1 of 2 treatments using a treatment allocation sheet built using the random number generator formula in Excel (Microsoft Corp., Redmond, WA): PEG (n = 417) or untreated control (**CTR**; n = 423). The PEG-treated cows received a subcutaneous injection containing 15 mg of pegbovigrastim in 2.7-mL prefilled syringes (Imrestor, Elanco Animal Health) 7 d before expected calving and a second injection within 24 h of calving. The CTR group did not receive any treatment. Treatments were administered by our research group. To avoid biases, treatment identification was not listed in the data collection forms or in the on-farm herd management software program. A subset of PEG (n =

102; 37 primiparous and 65 multiparous) and CTR ($n = 103$; 39 primiparous and 64 multiparous) cows was randomly selected (every fourth cow enrolled) to evaluate the reproductive tract microbiome postpartum by collecting swab samples from the vagina at 0, 7 ± 3 , and 35 ± 3 DIM. Five vaginal swabs were not collected from each of the treatment groups in multiparous cows. Therefore, a subset of 97 PEG-treated (37 primiparous and 60 multiparous) and 98 CTR cows (39 primiparous and 59 multiparous) cows were sampled for the evaluation of the microbiome found in the vagina postpartum.

Sample Collection and DNA Extraction

Vaginal discharge was sampled from the caudal portion of the vagina by a member of the research team at calving (hereafter 0 DIM), 7 ± 3 , and 35 ± 3 DIM ($n = 580$) using individually wrapped, sterile, cotton-tipped swabs as previously reported (Bicalho et al., 2017a). Samples were collected immediately before evaluation of vaginal discharge using the Metrichick device (Simcro, Hamilton, New Zealand). Briefly, the vulva was cleaned with alcohol wipes, the vulvar lips were opened, and the swab was introduced approximately 7.5 cm into the vagina, where the swab was rolled against the ventral portion of the vagina to sample the discharge draining from the uterus. The swabs were placed in sterile tubes, and the tubes were placed on ice and transported to the laboratory within 4 h, where the swabs were stored at -20°C until further processing. Metagenomic DNA was isolated from the swab samples using the BiOstic Bacteremia DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) following the manufacturer's recommendations.

Health Disorders Evaluated

Metritis was diagnosed by a member of the research team at 7 ± 3 DIM by evaluating the discharge recovered from the fundus of the vagina using the Metrichick device as previously reported (Jeon et al., 2015). The discharge was scored using a 5-point scale (1: not fetid, normal lochia, viscous, clear, red, or brown; 2: cloudy, pink, red, or brown mucoid discharge with flecks of pus; 3: not fetid, pink red or brown mucopurulent discharge with $<50\%$ pus; 4: not fetid, pink, red, or brown purulent discharge with $\geq 50\%$ pus; 5: fetid red-brownish, watery discharge). Cows with a discharge score of 5 were classified as having clinical metritis. Farm personnel performed their own diagnosis and treatment of metritis at 7 ± 3 DIM (hereafter 7 DIM); however, coordination with the research team ensured cows were sampled before administration of antibiotics.

Purulent vaginal discharge (PVD) was diagnosed by a member of the research team at 35 ± 3 DIM (hereafter 35 DIM) by evaluating the discharge recovered from the fundus of the vagina using the Metrichick device. The discharge was scored using a 0 to 3 scale (0: no secretion material retrieved and/or clear mucus; 1: flecks of purulent material within otherwise clear mucus; 2: $\leq 50\%$ purulent material in the vaginal discharge; 3: $>50\%$ purulent material with/without fetid discharge); cows with a score of ≥ 2 were diagnosed with PVD and cows with a score ≤ 1 were classified as not having PVD (LeBlanc et al., 2002).

16S rRNA Gene Amplification and Sequencing

The concentration and purity of DNA for all samples were evaluated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE) at wavelengths of 260 and 280 nm; the optical density ratio at 260/280 nm of DNA ranged between 1.7 and 2.0. For each metagenomic DNA sample, the V4 hypervariable region of the bacterial/archaeal 16S rRNA gene was amplified using the 515F and 806R primer set according to previously described methods and optimized for the Illumina MiSeq platform (Caporaso et al., 2012). The PCR products were tagged with 280 different 12-bp error-correcting Golay barcodes (<http://www.earthmicrobiome.org/>; Gilbert et al., 2010). The PCR was carried out in triplicate in 25- μL reactions using 12 to 300 ng of template DNA, 1 $\times$ EconoTaq Plus Green Master Mix (Lucigen, Middleton, WI), and 10 μmol of each primer. Thermal cycling consisted of an initial denaturing step of 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 of 72°C for 10 min. Replicate amplicons were visualized on a 1.2% agarose gel stained with 0.5 mg/mL ethidium bromide, and then pooled and purified using the Gel/PCR DNA Fragment Extraction kit (IBI Scientific, Peosta, IA).

The amplicon DNA concentration in each sample was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) at wavelengths of 260 and 280 nm. Amplicons were standardized to the same concentration and pooled into 3 sequencing runs according to individual barcode primers for the 16S rRNA gene. Final equimolar libraries were sequenced using the MiSeq reagent kit v2 (300 cycles) on the MiSeq platform (Illumina Inc., San Diego, CA). The generated 16S rRNA gene sequences were processed through the open source software pipeline Quantitative Insights into Microbial Ecology (QIIME; <http://qiime.org/>; version 1.9.0) as previously reported (Caporaso et al., 2010; Bicalho et al., 2017a). Sequences were fil-

tered for quality using established guidelines (Bokulich et al., 2013). Sequences were binned into operational taxonomic units (OTU) based on 97% identity using UCLUST (Edgar, 2010) against the Greengenes reference database (McDonald et al., 2012). Low-abundance clusters were filtered and chimeric sequences were removed using USEARCH (Edgar, 2010). We compared the representative sequences for each OTU against the Greengenes database for taxonomy assignment and used only full-length, high-quality reads ($r = 3$) for analysis, where r is the minimum number of consecutive bases with Q-scores lower than q from the beginning of the read to identify the location of quality trimming and q is the Q-score threshold that classifies a base as low quality. Illumina sequencing of the V4 region of the 16S rRNA gene provides excellent sequencing depth and coverage, but the shorter reads (~300 bp) may provide inaccurate species classification, especially for species with high 16S rRNA gene similarity such as *Escherichia* and *Shigella* species; therefore, only classification to the genus level was attempted and presented (Chakravorty et al., 2007).

Data Analysis

Samples from the vagina resulted in 21,411,210 high-quality reads after filtering for size, quality, and the presence of chimeras. Four vaginal samples, 1 from PEG on d 0, 2 from PEG on d 7, and 1 from CTR on d 7, did not pass the filtering criteria and were excluded from the study. One CTR cow died before metritis was evaluated, and 3 PEG-treated cows and 5 CTR cows died or were sold before PVD was evaluated. Therefore, 97 PEG-treated and 97 CTR cows were available for metritis analysis and only 94 PEG-treated and 93 CTR cows were available for PVD analysis.

Principal coordinates analysis (PCoA) of Bray-Curtis dissimilarity was used to compare treatment groups, time points, and health categories (e.g., metritis vs. no metritis) using the lowest taxonomic classification available using PAST version 3.18 (https://palaeo-electronica.org/2001_1/past/issue1_01.htm). The effects of treatment, health category, time (0, 7, 35 DIM), and the interaction between treatment or health category and time on the microbiome structure found in the vagina were evaluated with 2-way permutational multivariate ANOVA (PERMANOVA) with 9,999 permutations based on Bray-Curtis dissimilarity using the lowest taxonomic classification available using PAST version 3.18. The effect of antibiotic treatment of metritis, time (7 and 35 DIM), and the interaction between antibiotic treatment and time on the microbiome structure found in the vagina of cows that developed

metritis was evaluated with 2-way PERMANOVA with 9,999 permutations based on Bray-Curtis dissimilarity using the lowest taxonomic classification. Continuous outcomes such as the relative abundances of bacterial taxa, Chao1 richness index, and Shannon diversity index were compared using ANOVA for repeated measures for vaginal samples using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Normality and homoscedasticity of residuals were assessed using residual plots. For repeated measures, unstructured, autoregressive 1, and compound symmetry covariance structures were tested, and the one with the smallest Akaike information criterion was chosen. For all models, Tukey-Kramer adjustment for multiple comparisons was performed.

Because PEG has been shown to cause a slight increase in the incidence of metritis (Ruiz et al., 2017), we evaluated the effect of PEG treatment without including uterine disease as an independent variable. The independent variables offered to the ANOVA model were treatment (PEG vs. CTR), time (0, 7, 35 DIM), parity (primiparous vs. multiparous), and 2-way interactions between treatment and other variables. Then, we evaluated the association between the vaginal microbiome and the development of uterine disease postpartum controlling for any effect of treatment in 2 separate models. Two models were run for each uterine disease because metritis is correlated with PVD (Spearman $\rho = 0.22$; $P < 0.001$) and because there were cows with missing diagnoses of metritis or PVD. The independent variables offered to the ANOVA models were treatment (PEG vs. CTR), time (0, 7, 35 DIM), parity (primiparous vs. multiparous), uterine disease (metritis vs. no metritis, PVD vs. no PVD), and 2-way interactions between variables.

The effect of PEG treatment on uterine disease was evaluated using logistic regression, and the model included the fixed effects of treatment (PEG vs. CTR) and parity (primiparous vs. multiparous). For all models, the effect of treatment was forced in, but other variables were removed according to the Wald statistics criterion when $P > 0.10$. Significance was considered when $P \leq 0.05$.

RESULTS

Descriptive Data

Twenty-six cows (13.4%) were diagnosed with metritis, and 25 cows (13.4%) were diagnosed with PVD. Of the 26 cows diagnosed with metritis by our research team, only 12 (46.2%) were treated with antibiotics by the farm personnel.

Comparison of Microbiome in the Vagina Between Treatment Groups over Time

Microbial community richness and diversity postpartum were analyzed using the Chao1 and Shannon indices, respectively. We detected no effect of treatment ($P > 0.42$) or interaction between treatment and time ($P > 0.65$) on richness or diversity indices (Figure 1A, 1B). There was an effect of time on richness and diversity ($P < 0.01$). The Chao1 richness index was significantly ($P < 0.01$) lower at 7 than at 0 and 35 DIM (Figure 1A). The Shannon diversity index was significantly higher ($P < 0.05$) at 0 than at 7 DIM (Figure 1B), and at 35 than at 0 and 7 DIM (Figure 1B).

We used PCoA paired with PERMANOVA based on the Bray-Curtis dissimilarity of relative abundance using the lowest taxonomic classification available to evaluate the effect of PEG treatment, time, and the interaction between treatment and time on the structure of the microbiome found in the vagina postpartum. There was no effect of PEG treatment ($P = 0.47$) or interaction between treatment and time ($P = 0.97$). For ease of visualization, the effect of treatment on the microbiome structure found in the vagina postpartum is presented separately at 0, 7, and 35 DIM (Figures 2A, 2B, and 2C). Despite not finding a significant effect of PEG treatment on the microbiome structure, we compared the relative abundance of the 15 most

Figure 1. Diversity indices for the reproductive tract microbiota at 0, 7, and 35 DIM. (A) Chao1 richness and (B) Shannon diversity indices for cows treated (PEG) and untreated (control, CTR) with pegbovigrastrim. Pegbovigrastrim-treated cows received a subcutaneous injection containing 15 mg of pegbovigrastrim in 2.7-mL prefilled syringes (Imrestor, Elanco Animal Health, Greenfield, IN) 7 d before expected calving and a second injection within 24 h of calving. (C, E) Chao1 richness and (D, F) Shannon diversity indices for cows diagnosed with and without metritis at 7 DIM (C, D) or with and without purulent vaginal discharge (PVD) at 35 DIM (E, F). Letters (a, b, c) indicate significant ($P \leq 0.05$) differences between time points; *significant difference ($P \leq 0.05$) between groups within a time point; **significant ($P \leq 0.01$) difference between groups. Error bars represent SEM.

abundant taxa found in all vaginal samples between treatments. These taxa had an average prevalence $\geq 2\%$ and represented 65% of all assigned taxa. Likewise, there was no effect of PEG treatment ($P > 0.10$) or interaction between treatment and time ($P > 0.15$) on the 15 most abundant taxa found in all vaginal samples (Figure 3A). Finally, we evaluated the effect of PEG treatment on the relative abundance of the 6 most prevalent phyla in all vaginal samples: *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Proteobacteria*, *Tenericutes*, and *Actinobacteria*. These phyla had an average prevalence $\geq 2\%$ and represented 98% of all phyla. The remaining phyla were combined into a single category termed “others.” There was no effect of PEG treatment ($P > 0.25$) or interaction between treatment and time ($P > 0.50$) on the 6 most abundant phyla or “others” in vaginal samples (Supplemental Figure S1A; <https://doi.org/10.3168/jds.2018-15783>).

We detected an effect of time ($P < 0.01$) on the microbiome structure (Figure 2D). The microbiome structure was significantly different between d 0 and 7 ($P < 0.01$), d 0 and 35 ($P < 0.01$), and d 7 and 35 ($P < 0.01$). Comparison of the relative abundance of the 15 most abundant taxa found in all vaginal samples between time points revealed an effect of time ($P < 0.01$) on the relative abundance of family *Ruminococcaceae*, genus *Streptococcus*, genus *Fusobacterium*, genus *Porphyromonas*, order *Clostridiales*, genus 5–7N15, order *Bacteroidales*, family *Leptotrichiaceae*, genus *Aggregatibacter*, genus *Helcococcus*, family *Lachnospiraceae*, genus *Corynebacterium*, and genus *Bacteroides* (Figure 3B). There was also a significant effect of time ($P < 0.05$) on the relative abundance of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Tenericutes*, *Actinobacteria*, and “others” (Supplemental Figure S1A; <https://doi.org/10.3168/jds.2018-15783>).

Comparison of the Microbiome Found in the Vagina Between Cows that Did and Did Not Develop Uterine Disease

The Chao1 richness index of the microbiome in the vagina was decreased in cows that developed metritis postpartum compared with cows that did not ($1,708.4 \pm 75.0$ vs. $1,867.1 \pm 29.8$; $P = 0.05$), mostly because of a significant decrease in metritic cows at 7 DIM ($1,388.8 \pm 106.8$ vs. $1,688.4 \pm 44.9$; $P = 0.01$; Figure 1C). The Shannon diversity index of the microbiome found in the vagina was also decreased in cows that developed metritis postpartum compared with those that did not (5.5 ± 0.3 vs. 6.1 ± 0.1 ; $P = 0.05$), mostly because of a significant decrease in metritic cows at 7 DIM (4.9 ± 0.3 vs. 5.6 ± 0.1 ; $P = 0.05$; Figure 1D). There was no interaction ($P > 0.50$) between PEG treatment and metritis development on the Chao1 and Shannon indices.

By using PCoA paired with PERMANOVA, we observed that the vaginal microbiome structure was different ($P < 0.01$) in cows that did and did not develop metritis, mostly because of a significant difference at 7 DIM ($P < 0.01$; Figure 4B) but also at 35 DIM ($P = 0.03$; Figure 4C). There was no association between antibiotic treatment of metritis at 7 DIM and vaginal microbiome structure at 7 and 35 DIM, as evidenced by the lack of an effect of antibiotic treatment ($P = 0.07$) and the lack of an interaction between antibiotic treatment and time ($P = 0.24$). Comparison of the relative abundance of the 15 most abundant taxa found in all vaginal samples between cows that did and did not develop metritis showed that cows that developed metritis had higher relative abundance of *Fusobacterium* (9.0 ± 1.2 vs. 6.2 ± 0.5 ; $P = 0.04$), *Porphyromonas* (7.8 ± 1.1 vs. 4.4 ± 0.4 ; $P < 0.01$), *Bacteroides* (5.6 ± 0.6 vs. 1.4

Figure 2. Assessment of the effect of pegbovigrastim treatment (PEG) on the reproductive tract microbiome using principal coordinate analysis of Bray-Curtis dissimilarity using the lowest taxonomic classification available at 0 (A), 7 (B) and 35 (C) DIM, and the assessment of the effect of time on the reproductive tract microbiome (D). There was no effect of treatment ($P > 0.35$), but there was an effect of time ($P < 0.001$) on the reproductive tract microbiome. CTR = control.

± 0.2 ; $P < 0.01$), and lower relative abundance of genus 5-7N15 (*Bacteroidaceae* family; 2.0 ± 0.6 vs. 2.8 ± 0.1 ; $P = 0.02$). Interactions between metritis and time ($P \leq 0.05$) also showed that cows that developed metritis had higher relative abundance of *Fusobacterium* and *Bacteroides* at 0 DIM, higher relative abundance of *Porphyromonas* and *Bacteroides* at 7 DIM, and higher relative abundance of *Ureaplasma* at 35 DIM (15.7 ± 3.0 vs. 6.1 ± 1.2 ; $P < 0.01$) compared with cows that did not develop metritis (Figure 5A, 5B, 5C). In contrast, cows that developed metritis had lower relative abundance of *Ureaplasma* (3.8 ± 3.9 vs. 12.2 ± 1.6 ; $P = 0.05$), *Ruminococcaceae* (3.3 ± 1.4 vs. 6.5 ± 0.6 ; $P = 0.03$) and *Clostridiales* at 7 DIM (1.2 ± 0.6 vs. 2.8 ± 0.2 ; $P = 0.01$), and lower abundance of *Pasteurellaceae*

at 35 DIM (0.7 ± 2.0 vs. 5.8 ± 0.8 ; $P = 0.02$) compared with cows that did not develop metritis (Figure 5A, 5B, 5C). Phylum-level data were evaluated, and cows that developed metritis had higher relative abundance of *Bacteroidetes* compared with cows that did not develop metritis (21.0 ± 1.6 vs. 15.8 ± 0.6 ; $P < 0.01$). Interactions between metritis and time ($P \leq 0.05$) also showed that cows that developed metritis had higher relative abundance of *Bacteroidetes* at 7 DIM (39.1 ± 3.6 vs. 20.5 ± 1.5 ; $P < 0.01$), and higher relative abundance of *Tenericutes* (18.2 ± 3.1 vs. 7.1 ± 1.2 ; $P < 0.01$), *Actinobacteria* (8.6 ± 1.6 vs. 5.1 ± 0.6 ; $P = 0.04$), and “others” at 35 DIM (3.7 ± 0.5 vs. 2.3 ± 0.2 ; $P = 0.02$) compared with cows that did not develop metritis (Supplemental Figure S2B, S2C; <https://doi>

Figure 3. Overall mean relative abundance of the 15 most prevalent bacterial taxa (letters preceding name indicate taxon: g = genus, f = family, o = order) found in the reproductive tract at 0, 7 and 35 DIM in cows treated (PEG) and untreated (CTR) with pegbovigrastrim (top panel), and the mean relative abundance of the 15 most prevalent bacterial taxa found in the reproductive tract at 0, 7 and 35 DIM (bottom panel). Pegbovigrastrim-treated cows received a subcutaneous injection containing 15 mg of pegbovigrastrim in 2.7-mL prefilled syringes (Imrestor, Elanco Animal Health, Greenfield, IN) 7 d before expected calving and a second injection within 24 h of calving. Treatment had no effect ($P > 0.10$) on the most prevalent bacterial taxa. Letters (a, b, c) indicate significant ($P \leq 0.05$) differences between time points. Error bars represent SEM.

Figure 4. Evaluation of the association between the reproductive tract microbiome at 0 (A), 7 (B) and 35 (C) DIM and the diagnosis of metritis at 7 DIM using principal coordinate analysis of Bray-Curtis dissimilarity using the lowest taxonomic classification available. There was no association between metritis diagnosis at 7 DIM and the reproductive tract microbiome at 0 DIM ($P = 0.12$), but there was an association between metritis diagnosis at 7 DIM and the reproductive tract microbiome at 7 ($P < 0.001$) and 35 ($P = 0.03$) DIM.

.org/10.3168/jds.2018-15783). In contrast, cows that developed metritis had lower relative abundance of *Firmicutes* (22.9 ± 3.6 vs. 31.4 ± 1.5 ; $P = 0.03$), *Tenericutes* (5.0 ± 4.1 vs. 14.9 ± 1.6 ; $P = 0.03$), and “others” at 7 DIM (0.6 ± 0.3 vs. 1.4 ± 0.1 ; $P = 0.02$), and lower relative abundance of *Proteobacteria* at 35 DIM (4.5 ± 3.2 vs. 17.3 ± 1.3 ; $P < 0.01$; Supplemental Figure S2B, S2C; <https://doi.org/10.3168/jds.2018-15783>). There was no interaction ($P > 0.15$) between PEG treatment and metritis on the relative abundance of the 15 most abundant taxa or the 6 most abundant phyla.

The Chao1 richness index of the microbiome found in the vagina was decreased in cows that developed PVD compared with cows that did not ($1,671.7 \pm 76.6$ vs. $1,870.6 \pm 30.4$; $P = 0.02$), mostly because of a significant decrease in cows with PVD at 7 DIM ($1,349.4 \pm 116.0$ vs. $1,674.3 \pm 45.6$; $P = 0.01$) and 35 DIM ($1,682.6 \pm 111.9$ vs. $2,000.1 \pm 44.9$; $P < 0.01$; Figure 1E). The Shannon diversity index of the microbiome found in the vagina was also decreased in cows that developed PVD postpartum compared with cows that did not (5.5 ± 0.3 vs. 6.1 ± 0.1 ; $P = 0.04$), mostly because of a significant decrease in cows with PVD at 7 DIM (4.8 ± 0.4 vs. 5.6 ± 0.1 ; $P = 0.04$) and 35 DIM (5.6 ± 0.4 vs. 6.7 ± 0.1 ; $P < 0.01$; Figure 1F). There was no interaction ($P > 0.35$) between PEG treatment and PVD on the Chao1 and Shannon indices.

By using PCoA paired with PERMANOVA, we observed that the vaginal microbiome structure was different ($P < 0.01$) in cows that did and did not develop PVD, mostly because of a significant difference at 7 DIM ($P = 0.03$; Figure 6B) and 35 DIM ($P = 0.02$; Figure 6C). Comparison of the relative abundance of the 15 most abundant taxa found in all vaginal sam-

ples between cows that did and did not develop PVD showed that cows that developed PVD had higher relative abundances of *Fusobacterium* (10.9 ± 1.2 vs. 5.9 ± 0.5 ; $P = 0.04$) and *Bacteroides* (5.0 ± 0.7 vs. 1.6 ± 0.3 ; $P < 0.01$). Interactions between PVD and time ($P \leq 0.05$) showed that cows that developed PVD had significantly ($P < 0.05$) increased relative abundance of *Fusobacterium* only at 0 and 35 DIM, not at 7 DIM (Figure 7A, 7B, 7C) compared with cows that did not develop PVD. Phylum-level data showed that cows that developed PVD had higher relative abundance of *Bacteroidetes* compared with cows that did not develop PVD (20.1 ± 16 vs. 16.2 ± 0.7 ; $P = 0.03$). Interactions between PVD and time ($P \leq 0.05$) showed that cows that developed PVD had higher relative abundance of *Fusobacteria* at 0 DIM, higher relative abundance of *Bacteroidetes* at 7 DIM, and lower relative abundance of *Firmicutes* at 7 and 35 DIM (Supplemental Figure S3A, S3B, S3C; <https://doi.org/10.3168/jds.2018-15783>). There was no interaction ($P > 0.15$) between PEG treatment and PVD on the relative abundance of the 15 most abundant taxa or the 6 most abundant phyla.

DISCUSSION

The use of PEG was previously shown to dramatically increase the number of neutrophils and their extracellular killing ability (Kimura et al., 2014; Zinicola et al., 2018). Nonetheless, PEG treatment results in a small but significant increase in the incidence of metritis (Ruiz et al., 2017). Little is known regarding the effect of PEG treatment on the microbiome of the postpartum reproductive tract. Therefore, in this study

we assessed the microbiome found in the vagina in response to PEG treatment. We also investigated the association between the vaginal microbiome and the development of uterine disease, adjusting for any effect of treatment.

Treatment with PEG did not alter the structure or composition of the microbiome found in the vagina postpartum. We expected that a greater proportion

of circulating neutrophils in PEG-treated cows would translate into a greater influx of neutrophils into the uterus shortly after calving, which would be expected to constrain the growth of gram-negative anaerobes such as *Bacteroidetes* and *Fusobacteria* (Jeon et al., 2015; Knudsen et al., 2016; Sicsic et al., 2018). Nonetheless, PEG treatment induced a reduction in circulating neutrophils combined with an increase in neutrophil counts

Figure 5. Mean relative abundance of the 15 most prevalent bacterial taxa (letters preceding name indicate taxon: g = genus, f = family, o = order) found in the reproductive tract at 0 (A), 7 (B) and 35 (C) DIM in cows diagnosed or not with metritis at 7 DIM. *Significant ($P \leq 0.05$) difference between groups; **significant ($P \leq 0.01$) difference between groups. Error bars represent SEM.

in the vagina only in cows diagnosed with metritis and only at 7 DIM, not in all cows and not at calving (Zinicola et al., 2018). This finding indicates an increased influx of neutrophil in PEG-treated cows after uterine microbiota dysbiosis but not before; therefore, PEG-treatment seems ineffective at preventing the excessive growth of gram-negative anaerobes such as *Bacteroidetes* and *Fusobacteria*. This increased influx of neutrophils into the uterus after uterine microbiota dysbiosis and the associated inflammation caused by neutrophil degranulation and oxidative burst activity might lead to more pronounced clinical signs of metritis such as watery uterine discharge, which may lead to slightly more diagnoses of metritis in PEG-treated cows (Ruiz et al., 2017; Zinicola et al., 2018).

Herein, we saw that cows that developed metritis had an altered vaginal microbial structure and composition, particularly at 7 DIM but also at 35 DIM. Based on PCoA using the lowest taxonomic assignment, we saw that metritic cows had a dissimilar vaginal microbial structure and composition compared with cows that did not develop metritis at 7 and 35 DIM, which was characterized by higher relative abundance of *Porphyromonas* and *Bacteroides* and a lower relative abundance of *Ureaplasma*, *Ruminococcaceae*, and *Clostridiales* at 7 DIM, and a higher relative abundance of *Ureaplasma* and a lower relative abundance of *Pasteurellaceae* at 35 DIM. Although at calving, the PCoA analysis showed no differences between cows that later developed metritis and cows that did not, cows that developed metritis had higher relative abundances of *Fusobacterium* and *Bacteroides*.

At the phylum level, we observed that cows that developed metritis had higher relative abundance of *Bacteroidetes* and lower relative abundances of *Firmicutes*, *Tenericutes*, and “others” at 7 DIM and higher relative abundance of *Tenericutes*, *Actinobacteria*, and “others” at 35 DIM. Furthermore, the Chao1 and Shannon indices were decreased in metritic cows at 7 DIM. These changes in metritic cows agree with the previous literature evaluating the uterine microbiome (Jeon et al., 2015; Knudsen et al., 2016; Bicalho et al., 2017b; Sicsic et al., 2018) or the vaginal microbiome (Bicalho et al., 2017a). The uterine microbiome has been shown to change rapidly from 0 to 6 DIM, mainly due to a decreasing abundance of *Proteobacteria* and increasing abundance of *Bacteroidetes* and *Fusobacteria*, which were dominant in metritic cows (Jeon et al., 2015; Knudsen et al., 2016; Bicalho et al., 2017b; Sicsic et al., 2018). Moreover, *Fusobacterium*, *Bacteroides*, and *Porphyromonas* work synergistically with other bacteria such as *Helcococcus*, *Filifactor*, and *Prevotella* to cause metritis in dairy cows (Jeon et al., 2015). To our knowledge, this is the first time that cows that developed metritis were shown to have a greater relative abundance of *Fusobacterium* at calving. We recently also showed that the absolute abundance of uterine total bacteria, *Fusobacterium necrophorum*, *Bacteroides pyogenes*, *Porphyromonas levii*, *Helcococcus ovis*, and *Prevotella melaninogenica* were increased at 6 DIM in cows with metritis compared with cows without metritis (Cunha et al., 2018). Interestingly, 2 common pathogens described as being involved in the pathogenesis of uterine diseases; namely, *Escherichia coli* and *Trueperella pyo-*

Figure 6. Evaluation of the association between the reproductive tract microbiome at 0 (A), 7 (B) and 35 (C) DIM and the diagnosis of purulent vaginal discharge (PVD) at 35 DIM using principal coordinate analysis of Bray-Curtis dissimilarity using the lowest taxonomic classification available. There was no association between PVD diagnosis at 35 DIM and the reproductive tract microbiome at 0 DIM ($P = 0.28$), but there was an association between PVD diagnosis at 35 DIM and the reproductive tract microbiome at 7 ($P = 0.03$) and 35 DIM ($P = 0.02$).

Figure 7. Mean relative abundance of the 15 most prevalent bacterial taxa (letters preceding name indicate taxon: g = genus, f = family, o = order) found in the reproductive tract at 0 (A), 7 (B) and 35 (C) DIM in cows diagnosed or not with purulent vaginal discharge (PVD) at 35 DIM. **Significant ($P \leq 0.01$) difference between groups. Error bars represent SEM.

genes (Williams et al., 2005; Sheldon et al., 2009), were found to have low overall absolute abundance that was similar between metritic and healthy cows (Cunha et al., 2018). This indicates that *E. coli* and *T. pyogenes* are not a significant component of the mixed infection of the uterus at 6 DIM, which is in line with our findings herein and with other recent 16S metagenomic studies (Jeon et al., 2015; Knudsen et al., 2016; Bicalho et al., 2017b; Sicsic et al., 2018). It is possible that *E.*

coli with specific virulence factors such as *fimH* (Bicalho et al., 2010; Sheldon et al., 2010) play a more significant role in the development of metritis in the first few days after calving, when the uterine environment is less conducive to obligate anaerobe proliferation. As a facultative anaerobic organism, *E. coli* may colonize the uterine lumen early after calving, although at low abundance, and act as a pioneer organism until the changes in the uterine environment create a competi-

tive disadvantage that allows other anaerobes to thrive. Conversely, although the microbiome of endometritic cows is dominated by *Bacteroidetes* and *Fusobacteria*, *T. pyogenes* may be more important in the development of endometritis (Machado et al., 2012). Herein, we saw that metritic cows had a significant increase in the relative abundance of *Actinobacteria*, which includes *T. pyogenes*, at 35 DIM, whereas endometritic cows had a numeric increase compared with cows that did not develop uterine disease.

Decreased richness and diversity of uterine microbiota in metritic cows was also observed in our previous work (Jeon et al., 2015, 2016). Recent work evaluating the microbiome of the vagina observed a similar pattern of change in the microbiome of metritic cows as observed in uterine samples, such as a greater relative abundance of total bacteria, *Fusobacteria*, and *Bacteroidetes* in metritic cows compared with cows without metritis at 7 DIM (Bicalho et al., 2017a). The similarity between uterine and vaginal samples is likely because most of the discharge found in the vagina postpartum is likely derived from the uterus. Lower relative abundances of *Ureaplasma* and *Ruminococcaceae* at 7 DIM in metritic cows also agree with our previous observation that *Ruminococcus* and *Ureaplasma* were associated with uterine health (Jeon et al., 2015). Lower relative abundance of *Clostridiales* in metritic cows is a novel finding; however, we had observed that *Clostridium* was negatively correlated with *Bacteroides*, indicating, therefore, that it was not part of the dysbiosis observed in metritic cows. For the most part, the changes observed in cows that developed PVD were similar to those observed in cows that developed metritis, likely because of the high correlation between metritis and PVD.

Interestingly, antibiotic treatment of cows with metritis was not associated with their vaginal microbiome structure at 7 or 35 DIM. This finding agrees with a recent report in which we showed that the uterine microbiome structure was not markedly changed 6 d after treatment with antibiotics, although there were some minor differences depending on the antibiotic used (Jeon et al., 2018). Hence, it is likely that antibiotics affect the absolute number of microbes in the reproductive tract rather than their relative abundance.

CONCLUSIONS

In summary, PEG treatment had no effect on the microbiome found in the vagina but the vaginal microbiome was associated with the development of uterine disease. In general, cows that developed metritis and PVD had reduced richness and diversity of the vaginal microbiome and an increased relative abundance of the

phylum *Bacteroidetes* and the genera *Fusobacterium*, *Porphyromonas*, and *Bacteroides*.

ACKNOWLEDGMENTS

This study was funded by Elanco Animal Health (Greenfield, IN).

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