Intrauterine infusion of a pathogenic bacterial cocktail is associated with the development of clinical metritis in postpartum multiparous Holstein cows

J. C. C. Silva,1* L. C. Siqueira,1 M. X. Rodrigues,1,2 M. Zinicola,3 P. Wolkmer,1 B. Pomeroy,3 and R. C. Bicalho1,2
1Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, NY 14853
2FERA Diagnostics and Biologicals, College Station, TX 77845
3Zoetis Animal Health, Kalamazoo, MI 49001

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*Corresponding author: jd975@cornell.edu

ABSTRACT

Metritis is a uterine disorder common in dairy cattle caused by bacterial infection, with greater incidence in the early postpartum period. The disease causes delayed uterine involution, with a fetid, watery, red-brown discharge, with animals presenting different clinical signs including fever, dullness, inappetence, decreased milk yield, and dehydration. We developed an in vivo model of clinical metritis in Holstein multiparous cows using a pathogenic cocktail of Escherichia coli, Fusobacterium necrophorum, and Trueperella pyogenes. A total of 36 multiparous cows were randomly allocated to 1 of 3 treatment groups of 12 animals each. Cows assigned to the control group received an intrauterine administration of sterile saline solution, those in the low-dose group received a bacterial inoculum containing 10⁶ cfu of Escherichia coli, Trueperella pyogenes, and Fusobacterium necrophorum; and those in the high-dose group received 10⁹ cfu of these same organisms, all within 24 h of parturition. Based on clinical signs, milk yield; dry matter intake; serum concentration of acute phase proteins, metabolites, and cytokines; and bacterial counts in vaginal discharge were measured from parturition until 14 d in milk. Based on clinical signs, cows in the low-dose group had a greater incidence of metritis (83.3%) compared with the control (9%) or high-dose (25%) group. The low-dose group also had lesser dry matter intake compared with controls and an acute phase protein profile that typified metritis. The low-dose group presented greater relative abundance of bacteria from the genus Fusobacterium in the vaginal discharge compared with control cows, and the high-dose group was similar to the control group. Unexpectedly, intrauterine infusion of cows with a lower dose of bacteria was more effective than a higher dose, indicating that the bacterial load may affect metritis incidence in a non-linear manner. These findings demonstrate that clinical metritis can be experimentally induced in postpartum multiparous Holstein cows, creating a relevant model for the study of uterine diseases.

Key words: metritis, challenge, microbiota, multiparous, dairy cow

INTRODUCTION

Metritis is a systemic illness caused by bacterial infection of the uterus that most commonly affects cows within 21 d postpartum. Animals that develop metritis present an abnormally enlarged uterus with a fetid, watery, red-brown discharge, and in some cases present inappetence, decreased milk production, dullness, toxemia, and rectal temperature ≥39.5°C (Williams et al., 2005; Sheldon et al., 2006). Metritis is classified as a multifactorial disease that can be associated with risk factors such as primiparity, dystocia, stillbirth, twin calves, male calves, cesarean section, retained placenta, ketosis, and hypocalemia. The disease occurs typically during the first 10 d postpartum (Markusfeld, 1984; Drillich et al., 2001; Sheldon et al., 2006; Galvão, 2018). The reported incidence of metritis ranges from 8% to >50% (LeBlanc, 2008; Galvão, 2012; Vieira-Neto et al., 2016). When comparing cows diagnosed with metritis to cows without metritis, previous studies have reported a reduction in milk production (Dubuc et al., 2011; Pérez-Báez et al., 2019, 2021), an increased interval to first service by 7 d, and a 20% decrease in pregnancy per AI at first service, which resulted in an increase of 19 d in the calving to conception interval (Fourichon et al., 2000; Carneiro et al., 2016). Based on a stochastic analysis the cost associated with a single case of metritis was estimated to be US$513, with 95% of cases ranging from $240 to $884 (Pérez-Báez et al., 2021).

The primary microorganisms associated with the development of metritis have been Escherichia coli, Trueperella pyogenes, Fusobacterium necrophorum, and other bacteria within the genera Prevotella and Bacte-
The development of metritis depends on both the pathogenicity and number of bacteria that invade the uterine tract, and on the immune response of the cow. Pathogenic bacteria must overwhelm the uterine defenses to cause an infection (Sheldon et al., 2006). In vivo models of uterine diseases such as endometritis and pyometra have been created previously (Farin et al., 1989; Piersanti et al., 2019; Dickson et al., 2020) using Escherichia coli, Trueperella pyogenes, or both. None of the models was able to induce the expected metabolic changes associated with uterine diseases, such as an increase in serum levels of acute phase proteins or cytokines. Therefore, we tested an in vivo model for metritis induction in healthy postpartum dairy cows by the infusion of a low ($10^6$ cfu) or a high ($10^9$ cfu) dose of E. coli, T. pyogenes, and F. necrophorum to test whether the model would induce classic clinical signs associated with the natural occurrence of metritis. Blood was analyzed, and the microbiome was assessed to determine metabolic and inflammatory changes, and the dynamics of microbial colonization during the development of metritis.

**MATERIALS AND METHODS**

**Ethics Statement**

The research protocol was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol number 2019–0040). The methods were carried out in accordance with the approved guidelines.

**Sample Size Estimation**

The commercial farm where animals were purchased had a metritis incidence ranging from 8 to 16%. The maximal expected incidence of metritis in control cows (not receiving challenge) was approximately 16%. Assuming a desired type I error rate of 5%, a power of 80%, a 2-sided statistical test, and a baseline probability of metritis of 16% (level of metritis in control group), a sample size of 12 cows per group was required to detect 75% effectiveness in inducing metritis in challenged cows.

**Animals, Facilities, and Management**

Cows enrolled in this study, which was conducted between September 2019 and February 2020, were purchased from a commercial farm located in Scipio Center, New York. Initial experimental procedures until the day of calving were conducted at the commercial farm. Thereafter, cows were transported to the Dairy Unit of the Cornell University Ruminant Center (CURC). The commercial farm used to source cows had approximately 4,100 lactating cows in freestall facilities bedded with manure solids. Dry and lactating cows had free access to ad libitum water and a TMR to meet nutritional requirements according to stage of lactation. Cows presenting signs of parturition were moved to a maternity pen assisted by trained farm staff. Calving data (i.e., date and time of calving, cow and calf ID, calf sex and weight, calving ease score, and personnel present at the time of parturition) were recorded by farm personnel.

The dairy unit of the CURC (located in Harford, NY) is structured to house approximately 600 lactating cows in naturally ventilated freestalls and tiestall barns. Cows for this experiment were housed in the tiestall barn with deep-bedded sawdust. Forced ventilation was provided by fans placed on the ground in the feed and manure lane, and exhaust fans were placed on one of the lateral barn walls.

Cows had free access to water and TMR in individual water bowls and feed bins. Feed was offered individually once a day, during the morning (between 0700 and 0800 h), at 110% of their expected daily consumption. Individual feed intake was measured by weighing the amount of feed offered and refused daily. The DM analyses of TMR were performed once weekly (aliquots were dried in 55°C for 48 h). Daily feed consumption and average weekly DM were used to calculate the DMI. The TMR was ground once a week to pass a 2-mm screen of a Wiley mill (Arthur H. Thomas), and once a month the composites of dried TMR were sent to a commercial laboratory for analysis (Cumberland Valley...
Analytical Services, Waynesboro, PA). Ingredients and chemical composition (DM basis) of the postpartum diet are presented in Supplemental Tables S1 and S2 (https://data.mendeley.com/datasets/yv6j6xtyj7/2; Silva, 2022).

**Experimental Design**

The experiment was a randomized complete block design with a one-way treatment structure. The blocking factor was based on numerical order (order of enrollment). Briefly, after selecting eligible cows, groups of 3 cows were allocated in treatments according to ear tag number in ascending order. The treatment randomization was generated using the SAS statistical package (version 9.4; SAS/STAT, SAS Institute Inc.) that uses a random number generator function created by the Biometrics Representative from Zoetis Animal Health (Kalamazoo, MI).

After parturition (4 to 22 h), eligible cows were infused at the maternity pen at the commercial farm by research personnel with the bacterial cocktail corresponding to the experimental treatment group and immediately transported to the dairy unit of the CURC, where cows stayed until the end of the experimental period (14 DIM). Cows were eligible for enrollment in the experiment if they were in their second through sixth lactations, had a gestation length of 270 to 285 d, gave birth to a single calf without assistance, did not receive antibiotic treatment 30 d before calving, did not present the placenta at the time of challenge, and did not receive antibiotic treatment 30 d before calving. Also, cows were enrolled only if they had no clinical signs of disease, including lameness, dehystation, respiratory distress, empty rumen, recumence, dullness, depression, displaced abomasum, mastitis, or vaginal tear. A total of 36 cows were selected and assigned to one of the following treatment groups: high-dose group (n = 12), which received an intrauterine infusion with 120 mL of an inoculum containing 10⁹ cfu of each bacterial strain (E. coli, T. pyogenes, and F. necrophorum); low-dose group (n = 12), which received an intrauterine uterine infusion with 120 mL of an inoculum containing 10⁶ cfu of each bacterial strain (E. coli, T. pyogenes, and F. necrophorum); and control group (n = 12), which received an intrauterine infusion with 120 mL of sterile saline solution (TEKnova). One cow enrolled in the control group was excluded from the experiment due to aggressive behavior. Thirty-five cows, therefore, completed the experiment.

Milking was performed 3 times a day at approximately 8-h intervals. Milk weights were recorded at each milking for each cow using milk meters that transferred data to the dairy herd management software (Dairy Comp 305, Valley Ag Software).

**Inoculum Preparation**

The bacterial challenge used for metritis induction was composed of E. coli, T. pyogenes, and F. necrophorum. All strains were obtained from a well-characterized bacterial collection belonging to the Bicalho Laboratory from Cornell University (Ithaca, NY). Strains were previously isolated from uterine contents of cows diagnosed with metritis and selected according to virulence genes presence (Bicalho et al., 2010, 2012b; Santos et al., 2010; Machado and Bicalho, 2014).

_Escherichia coli_ was cultured under aerobic conditions, with agitation (3 × g), in Luria-Bertani (LB) broth (Sigma-Aldrich) at 37°C for 24 h. Following the 24-h incubation, the culture was harvested by centrifugation (2,500 × g for 10 min, room temperature). The cell pellet was resuspended in LB broth supplemented with 25% (vol/vol) of glycerol to a final count of 10⁶ cfu/mL and aliquoted in 2-mL cryogenic vials. Vials were flash frozen in liquid nitrogen and stored at −80°C.

_Trueperella pyogenes_ was cultured in VersaTrek Redox 1 Media broth (Thermo Fisher Scientific) in 5% CO₂ supplemented incubator, at 37°C for 48 h. Following the 48-h incubation, the culture was harvested by centrifugation (2,429 × g for 10 min, room temperature) and the cell pellet resuspended in microbial freeze-drying buffer (OPS Diagnostics) to a final count of 10⁶ cfu/mL and aliquoted in 2-mL cryogenic vials. The bottled culture was lyophilized in Advantage Pro Lyophilizer (SP Scientific) according to manufacturer instructions, sealed, and stored at 4°C. _Trueperella pyogenes_ was lyophilized as a strategy to achieve the concentration needed for challenge.

_Fusobacterium necrophorum_ was cultured in VersaTrek Redox 2 Media broth (Thermo Fisher Scientific), in a Bactron 300 anaerobic incubator (Sheldon Manufacturing Inc.) for 48 h at 37°C. Following the 48-h incubation, the culture was harvested by centrifugation (2,429 × g for 10 min, room temperature). The resuspension of the cell pellet was performed using VersaTrek Redox 2 Media broth (Thermo Fisher Scientific) supplemented with 25% (vol/vol) of glycerol to a final count of 10⁶ cfu/mL and aliquoted in 2-mL cryogenic vials. Vials were flash frozen in liquid nitrogen and stored at −80°C.

Within an interval of 2 to 4 h before use in the field, each stock was transferred to 40-mL bottles containing the transport medium, and dilutions were carried to achieve the specific concentration for each challenge. The inoculum containing _E. coli_ and _T. pyogenes_ was transported in the VersaTrek Redox 1 Media, whereas _F. necrophorum_ was transported in VersaTrek Redox 2 Media. Once diluted, challenge material was transported to the farm on ice and used upon arrival.
**Evaluation of Bacterial Culture Purity**

Before the field trial study, bacterial stocks were produced and quality control assessments performed between September 2019 and February 2020 to ensure the purity and bacterial count (cfu/mL) of each strain. DNA was extracted from the pure bacterial stocks, the 16S rRNA gene was amplified and submitted to Sanger sequencing (Cornell University Biotechnology Institute) to confirm the bacterial species cultured according to the methods previously described (Rodrigues et al., 2016).

Bacterial counts were performed using the technique of agar droplets with some modifications. Briefly, for each bacterial strain, serial dilutions were prepared (10⁻¹ to 10⁻⁸) using sterile 1× PBS (pH 7.4). Next, 3 drops of each dilution were inoculated onto each quadrant of agar plates. All stocks were incubated in aerobic, anaerobic, and 5% CO₂–supplemented conditions, to ensure that stocks were not contaminated with any other microorganism. After incubation (for approximately 48 h at 37°C), the features of bacterial growth were assessed and the counts (cfu) were determined.

For *E. coli* culturing, blood agar and a selective medium plate (Mastitis GN, CHROMagar) were used, incubating at 37°C for 24 h in an aerobic incubator. Blood agar and LKV agar plates (Laked Brucella Blood Agar with Kanamycin and Vancomycin; Anaerobe Systems) were used for culturing *F. necrophorum* for 48 h at 37°C under anaerobic conditions. Last, blood agar plates were used to culture *T. pyogenes* in a 5% CO₂–supplemented incubator for 48 h at 37°C.

**In Vivo Experimental Challenge**

Bacterial challenge was administered by an assigned study member blinded to clinical observation. Cows were restrained in a headlock at the maternity pen; the perineal area and the vulva were cleaned with paper towels and then disinfected with 70% ethanol. The inoculum was administered using a sterile gilt foampoint catheter (QC Supply) attached to a 60-mL syringe (Air-tite Products Co. Inc.), introduced into the cranial vagina and manipulated through the cervix, into the uterine lumen. A total volume of 40 mL of each inoculum (total of 120 mL) was infused into the uterus. The catheter was flushed with 10 mL of sterile saline solution to ensure that all challenge material was infused. Control cows were infused with 120 mL of sterile saline solution using a single catheter. No adverse health effects other than metritis were expected as the bacteria used were previously found in healthy and diseased animals.

**Sample Collection**

Blood samples were collected from coccygeal vessels using 10-mL Vacutainer tubes with spray-coated silica (BD Vacutainer) for serum separation, and 3-mL Vacutainer K₂-EDTA (BD Vacutainer) blood collection tubes for complete blood cell count and genomic testing (Clarifide, Zoetis Animal Health). Samples were collected before challenge administration and daily after feeding from study d 1 to 14.

Complete blood cell count was performed using a veterinary hemogram instrument (HemaTrue, Heska). Serum was obtained after centrifugation of 10-mL blood collection tubes at 2,000 × g for 15 min at room temperature and frozen at −80°C. Serum concentrations of calcium, nonesterified fatty acids (FA), BHB, total protein, BSA, glucose, alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate were measured using an automated clinical chemistry analyzer (Daytona, Randox Laboratories Ltd.), using reagents provided by the same company.

Frozen serum samples were sent to a Zoetis Research and Development facility (Kalamazoo, MI) for measurement of acute phase proteins using commercial ELISA kits: LPS binding protein (LBP, Hycult Biotech), haptoglobin (Life Diagnostics), and serum amyloid A (SAA, Life Diagnostics). Cytokine concentrations in serum [tumor necrosis factor-α (TNFα), IL-6, IL-2, IL-8/CXCL8, and IL-10] were analyzed using 2 custom U-Plex assays [Meso Scale Diagnostics (MSD) LLC] validated for bovine. The TNFα, IL-2, IL-6, and IL-10 analysis was performed in a 4-plex assay format, whereas bovine IL-8/CXCL8 was performed in a 1-plex format. In brief, biotinylated antibodies were diluted with MSD diluent 100 to a final concentration of 10 µg/mL and individually linked to specific MSD linkers according to the manufacturer’s recommendations. Linked antibodies were diluted in MSD stop solution: 4-Plex: TNFα (1:20), IL-2 (1:40), IL-6 (1:10), and IL-10 (1:40); 1-plex: IL-8/CXCL8 (1:40). The 4-plex linked antibodies were combined into one antibody capture solution, and the 1-plex linked antibody was prepared as a separate antibody capture solution. A total of 50 µL/well of capture antibody solution was added to the respective U-Plex assay plates and incubated for 1 h at room temperature with shaking. Following incubation, plates were washed 3 times with 0.05% Tween-20 in PBS. Samples for 4-plex measurements were diluted to 1:2, whereas samples for 1-plex measurements were diluted to 1:100 in SeaBlock (Thermo Fisher Scientific) and duplicates were added to each plate. Plates were incubated with samples and standards at room temperature for an interval of 1 to 1.5 h with shaking. Following incubation,
Vaginal discharge was evaluated daily from enrollment to the end of the study period. Fever was defined as an RT of ≥39.5°C on at least 1 day from study d 1 to 14. The BW and BCS (Edmonson et al., 1989) were recorded at enrollment and study d 14 and used to calculate BCS loss and BW loss. The scale was 0 (normal lochia with viscous discharge or no discharge observed); 1 (clear mucus with <50% purulent or mucopurulent material), 2 (clear mucus with ≥50% purulent or mucopurulent material), or 3 (fetid, watery, red-brownish uterine discharge). Metritis was defined as a VDS of 3 regardless of the presence of fever. The scheme of VDS was originally described for endometritis and is not validated for diagnosis of metritis. Nonetheless, it was used in this study to orient the progression of the VDS until the development of metritis. The case definition of metritis, which is classified as VDS = 3, follows the classification proposed by Sheldon et al. (2006).

DNA Extraction and Metagenomic Analysis

DNA extraction was initiated by adding 1 mL of UltraPure distilled water (DNase- and RNase-free, Invitrogen) into a 2-mL microtube containing the swab and vortexed for 10 min. Swabs were then removed and the remaining liquid centrifuged for 5 min at 16,200 × g at room temperature. The pellet DNA was extracted using a DNeasy PowerFood Microbial Kit (Qiagen) following the manufacturer’s instructions.

For DNA sequencing, PCR was performed for amplification of the V4 hypervariable region of the bacterial/archaeal 16S rDNA gene using primers 515F and 806R, according to a previously optimized method for the Illumina MiSeq platform (Caporaso et al., 2012).
All DNA samples were amplified using different 12-bp error-correcting Golay barcodes for 16S rDNA gene PCR (http://www.earthmicrobiome.org). The PCR was performed using 10 µM of each primer, EconoTaq Plus Green 1× Master Mix (Lucigen), 10 to 100 ng of individual DNA, and UltraPure distilled water (DNase- and RNase-free, Invitrogen) to bring the final reaction volume to 50 µL. The PCR conditions were initial denaturing at 94°C for 3 min; 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 90 s; and final elongation at 72°C for 10 min. After amplification, samples were loaded in agarose gel (1.2%, wt/vol) containing 0.5 µg/mL ethidium bromide to verify the presence of amplicons. Amplified DNA was purified using Mag-Bind Total Pure NGS (Omega Bio-Tek Inc.) according to the manufacturer’s instructions. Determination of DNA concentration was performed by spectrophotometric estimation [absorbance at 260 nm, A260, of 1.0 = 50 µg/mL pure double-stranded (ds)DNA]. The DNA samples were diluted to the same concentration and pooled for library preparation and sequencing, which was performed using Reagent MiSeq V2 300 cycles on the MiSeq platform (Illumina Inc.). Procedures of PCR, purification, normalization, and pooling were automated using OT-2 robot pipetting (Opentrons).

Operational taxonomic unit tables were generated through the MiSeq Reporter Metagenomics Workflow, which is based on the Greengenes database (http://greengenes.lbl.gov/). The output from this workflow is a classification of reads at multiple taxonomic levels (phylum, genus, and species).

**Data Analysis**

Descriptive statistics were performed using SAS version 9.4 (SAS Institute Inc.). General linear mixed models using the MIXED procedure of SAS version 9.4 (SAS Institute Inc.) were created to analyze continuous data collected over time (e.g., RT, milk production, bacterial count, molecular analyses, and blood cytokines and metabolites). Normality and homoscedasticity of residuals were assessed using residual plots. Models included the fixed effects of treatment (control, low dose, and high dose), day after challenge, and the interaction term between treatment and days after challenge, and random effects of block and animal within block.

Continuous data such as bacterial counts and concentrations of interleukins, such as IL-10 and IL-6, were log_{10}-transformed or transformed to natural logarithm, as residuals did not follow a normal distribution. Based on Akaike information criterion (AIC), the covariance structure with the lowest AIC was selected for each variable.

Multivariate logistic regression models were applied using the binary distribution of the GLIMMIX procedure (SAS, version 9.4) to evaluate dichotomous outcomes such as incidence of metritis and fever. The model included fixed effect of treatment and random effect of block.

For all models, variables were considered statistically significant when a P-value ≤ 0.05 was detected, and a trend was considered if the P-value was between 0.05 and 0.10. In all models, Fisher’s protected least significant difference was used for multiple comparisons such that pairwise treatment comparisons were performed if the treatment effect or treatment by time point effect (only for repeated measures models) was significant at the 0.05 level.

**RESULTS**

**Metritis Incidence, RT, DMI, and Milk Production**

Treatment groups were similar prepartum for BCS, BW, and RT at enrollment; days dry; days of gestation; and results of genomic tests (Table 1). The average time from calving to intrauterine inoculation was also similar. Cows in the control group received sterile saline solution at a mean interval of 11.9 h (SD = 5.6; ranging from 3.5 to 19.7 h) after calving, whereas cows in the low-dose and high-dose groups received the bacterial inoculum at a mean interval of 10.3 h (SD = 4.6; ranging from 3.8 to 18.8 h) and 13.7 h (SD = 5.1; ranging from 5.6 to 22 h) after calving, respectively.

The incidence of metritis for the low-dose group was 83% (10/12), and that in the control and high-dose groups had incidences of 8% (1/11) and 25% (3/12), respectively. There was an effect of challenge dose on the incidence of metritis (P = 0.009). The low-dose group had a greater proportion of cows with metritis than the control (P = 0.006) and the high-dose (P = 0.01) groups. There was no difference (P = 0.30) between control and high-dose cows.

Based on survival analysis, there was an effect of challenge dose (P = 0.005) on the hazard ratio (HR) of metritis (Figure 1). Cows that received the low-dose challenge had a 16-fold (P = 0.01) or 5.2-fold (P = 0.03) greater risk of being diagnosed with metritis during the study period than cows in the control or high-dose groups, respectively (low-dose vs. control HR = 16, 95% CI = 1.9–13.4; low-dose vs. high-dose HR = 5.2, 95% CI = 1.1–23.8). Cows in the high-dose group had a similar (P = 0.38) HR of being diagnosed with metritis as the control cows (high-dose vs. control HR = 3, 95% CI = 0.3–38.3). The time from inoculation to the first diagnosis of metritis for control was 5 d,
whereas low-dose cows were diagnosed between study d 4 and 10, and high-dose cows were diagnosed between d 2 and 10.

Challenge administration did affect RT (effect of treatment: $P = 0.008$; control = 38.7 ± 0.1, low dose = 38.8 ± 0.1, high dose = 38.5 ± 0.1°C; Figure 2A) but did not affect the incidence of fever ($P = 0.20$; control = 26.8, low dose = 58.6, high dose = 24.4%).

There was an effect of challenge on DMI ($P = 0.02$; Figure 2B). Cows that received the low dose consumed, on average, 1.4 ($P = 0.09$) and 2.3 ($P = 0.006$) kg/d less DM compared with the control and high-dose groups, respectively (control = 16.2 ± 0.9, low dose = 14.8 ± 0.9, high dose = 17.1 ± 0.9 kg/d). No differences were observed between control and high-dose cows ($P = 0.25$).

There was no effect of challenge dose on milk production ($P = 0.26$; Figure 2C). The low-dose group produced approximately 3.0 kg/d less on average compared with the other 2 groups (control = 33.7 ± 1.9, low dose = 30.7 ± 1.8, high dose = 33.3 ± 1.8 kg/d). Bacterial challenge had no effect on BCS ($P = 0.12$) or BW loss ($P = 0.50$) during the first 14 d of lactation.

**Serum Acute Phase Proteins, Metabolites, and Cytokines**

There was a tendency for an effect of bacterial challenge on SAA ($P = 0.06$; Figure 3A) and LBP ($P = 0.08$; Figure 3B). There was no effect of challenge on serum haptoglobin concentration ($P = 0.30$; Figure 3C).

Bacterial challenge did not affect serum metabolites, total protein, or calcium concentration (Table 2, Supplemental Figure S1; [https://data.mendeley.com/datasets/yv6j6xtyj7/2; Silva, 2022]), and had no effect on serum TNF-α, IL-2, IL-6, and IL-8 concentrations among groups (Table 3, Supplemental Figure S2; [https://data.mendeley.com/datasets/yv6j6xtyj7/2; Silva, 2022]). Bacterial challenge affected IL-10 concentration ($P = 0.01$), with low-dose animals presenting greater serum concentration of IL-10 ($P \leq 0.05$) compared with high-dose or control.

Other serum constituents, including AST ($P = 0.77$), ALP ($P = 0.10$), ALT ($P = 0.26$), and lactate ($P = 0.21$), were not affected by challenge (Supplemental Figure S3, Supplemental Table S3; [https://data.mendeley.com/datasets/yv6j6xtyj7/2; Silva, 2022]). We detected a treatment effect on serum GGT concentration ($P = 0.04$), where control cows had greater GGT concentration compared with low- and high-dose cows (Supplemental Table S3).

**Table 1.** Descriptive data (LSM ± SEM) of cows challenged with a low dose (n = 12) or high dose (n = 12) of bacterial inoculum or unchallenged (control; n = 11)

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Low dose</th>
<th>High dose</th>
<th>$P$-value2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median parity</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>BW at enrollment, kg</td>
<td>683.5 ± 24.1</td>
<td>718.2 ± 23.1</td>
<td>671.8 ± 23.1</td>
<td>NS</td>
</tr>
<tr>
<td>RT at enrollment, °C</td>
<td>38.5 ± 0.12</td>
<td>38.6 ± 0.12</td>
<td>38.5 ± 0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Days of dry period</td>
<td>59.5 ± 2.7</td>
<td>58.3 ± 2.6</td>
<td>55.4 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Days of gestation</td>
<td>280.3 ± 2.1</td>
<td>279.4 ± 2.0</td>
<td>280.0 ± 2.0</td>
<td>NS</td>
</tr>
<tr>
<td>GMILK</td>
<td>277.5 ± 167.9</td>
<td>291.4 ± 160.3</td>
<td>323.5 ± 153.6</td>
<td>NS</td>
</tr>
<tr>
<td>Zoetis Metritis</td>
<td>100.6 ± 1.6</td>
<td>101.5 ± 1.4</td>
<td>100.8 ± 1.4</td>
<td>NS</td>
</tr>
<tr>
<td>DWP</td>
<td>229.2 ± 42.2</td>
<td>206.7 ± 38.2</td>
<td>202.3 ± 36.6</td>
<td>NS</td>
</tr>
<tr>
<td>TPI</td>
<td>2.073 ± 53.6</td>
<td>2.026 ± 51.1</td>
<td>2.085 ± 48.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

1RT = rectal temperature; GMILK = genomic-enhanced PTA for milk yield; Zoetis Metritis = genomic standardized transmitting ability for metritis risk (Zoetis); DWP = Dairy Wellness Profit Index (Zoetis); TPI = Total Performance Index (Holstein Association, USA).

2$P$-value indicates the overall group effect.
Blood Cell Counts

Total white blood cell \((P = 0.22)\) and lymphocyte \((P = 0.31)\) counts were similar among groups (Table 4, Supplemental Figure S4; https://data.mendeley.com/datasets/yv6j6xtyj7/2; Silva, 2022). There was an effect of challenge on granulocyte \((P = 0.04)\) and monocyte counts \((P = 0.02;\) Table 4, Supplemental Figure S3). Challenge cows had fewer circulating monocytes and granulocytes compared with control (Table 4).

Uterine Microbiology

No difference was observed in counts (cfu) of total anaerobes \((P = 0.10;\) Figure 4A), whereas the bacterial challenge tended to increase the counts of \(F.\) necrophorum \((P = 0.07;\) Figure 4B) in the vaginal discharge. Furthermore, the bacterial challenge increased the relative abundance of 16S sequence reads for the genus Fusobacterium (metagenomic analyses; \(P = 0.004)\). Low-dose cows had greater relative abundance of 16S sequence reads of the genus Fusobacterium compared with control \((P = 0.01)\) or high-dose \((P = 0.07)\) cows (Figure 4C). In general, the counts and sequence reads for \(F.\) necrophorum and Fusobacterium, respectively, increased with DIM \((P < 0.001,\) Figures 4B and 4C).

Challenge administration did not affect total aerobes \((P = 0.58;\) Figure 5A) or \(E.\) coli counts \((P = 0.38;\) Figure 5B), or the relative abundance of the genus Escherichia \((P = 0.53;\) Figure 5C). Counts of \(E.\) coli and Escherichia 16S sequences decreased over time (DIM, \(P < 0.01,\) Figures 5B and 5C). Also, there was no effect of challenge on total facultative bacteria \((P = 0.37;\) Figure 6A) or \(T.\) pyogenes count \((P = 0.67;\) Figure 6B), or the relative abundance of 16S sequences for the genus Trueperella \((P = 0.55;\) Figure 6C) among challenge groups. The counts of \(T.\) pyogenes and Trueperella 16S sequences increased over time (DIM, \(P < 0.05,\) Figure 6B and 6C).

To identify the microorganisms most associated with metritis in infected cows regardless of study group, we stratified the cows into 2 groups: metritic and nonmetritic. The counts (cfu) of \(F.\) necrophorum \((P < 0.001)\) and the 16S relative abundance of the genus Fusobacterium \((P < 0.001)\) were greater in cows diagnosed with metritis compared with cows without metritis (Supplemental Figure S5; https://data.mendeley.com/datasets/yv6j6xtyj7/2; Silva, 2022). Metritic cows also harbored more \(T.\) pyogenes \((P = 0.05)\) and had a greater relative abundance of 16S sequences of the genus Trueperella \((P = 0.02)\) compared with nonmetritic cows (Supplemental Figure S6; https://data.mendeley.com/datasets/yv6j6xtyj7/2; Silva, 2022). Counts of \(E.\) coli were higher in cows

Figure 2. (A) Rectal temperature, (B) DMI, and (C) milk production during the first 14 d of lactation of cows challenged intrauterine with a bacterial inoculum containing \(10^6\) cfu (low dose; \(n = 12)\) or \(10^9\) cfu (high dose; \(n = 12)\) of \(E.\) coli, \(T.\) pyogenes, \(F.\) necrophorum, or unchallenged (control; \(n = 11)\). Results are presented as LSM ± SEM. TRT = treatment.
with metritis compared with cows without metritis ($P = 0.04$), but there was an increase in the relative abundance of 16S sequences of the genus *Escherichia* in cows without metritis compared with cows diagnosed with metritis ($P = 0.05$; Supplemental Figure S7; https://data.mendeley.com/datasets/yv6j6xtv7j7/2; Silva, 2022).

**DISCUSSION**

The lack of a reliable and effective animal model of metritis in postpartum dairy cows makes it difficult to study and establish the efficiency of certain intervention strategies. Here we presented a successful in vivo model associated with metritis induction in postpartum cows through intrauterine infusion of *E. coli*, *T. pyogenes*, and *F. necrophorum*. Our model produced a metritis incidence of 83% in multiparous cows receiving an intrauterine challenge containing $10^6$ cfu of each bacterial species. Clinical signs of illness included a decrease in milk production and DMI and an increase in RT (Figure 2), all of which might be associated with the increase in serum acute phase proteins (Figure 3) and proinflammatory interleukins (Table 3). The use of culture-dependent (bacterial count) and culture-independent methods (16S rRNA gene sequencing) allowed us to describe the importance of bacterial species including *F. necrophorum* in the development of metritis (Figures 4 to 6).

Previous reports have described induction of endometritis or pyometra in primiparous and multiparous cows during the luteal phase through the intrauterine infusion of *T. pyogenes* with or without anaerobic bacteria (*F. necrophorum* and *Bacteroides melaninogenicus*), or *T. pyogenes* combined with *E. coli* (Rowson et al., 1953; Farin et al., 1989; Piersanti et al., 2019; Dickson et al., 2020). Although all models were able to change the VDS in challenged cows, no clinical signs of illness were observed. Our model relied upon evidence from recent reports that characterized resident microorganisms of a metritic uterus, taking advantage of the techniques of bacterial culture and metagenomic analysis. These previous reports suggested that *F. necrophorum* is a relevant microorganism for the development of metritis (Sheldon et al., 2009; Machado et al., 2012; Jeon et al., 2015, 2016; Galvão et al., 2019). Thus, we administered well-characterized strains of *E. coli*, *T. pyogenes*, and *F. necrophorum* into the uterus of postpartum cows in 1 of 2 doses, $10^6$ (low dose) and $10^9$ (high dose) cfu, within 24 h after parturition. Surprisingly, the low-dose group had the greater incidence of metritis (83%), whereas the high-dose group had a 25% incidence of metritis and did not differ from controls (Figure 1). Although the reasons for this are unknown, we propose some plausible
hypotheses. It is possible that a high concentration of bacteria limited their ability to colonize the uterus due to competition with other microorganisms that were already present in the uterus at the time of infusion. Alternatively, communication among gram-positive and gram-negative bacteria, called “quorum sensing” (or cell-to-cell communication), allows bacteria to produce, detect, and respond to extracellular signaling molecules called autoinducers. Expression of autoinducers allows bacteria to share information and adjust growth and gene expression accordingly (Nealson et al., 1970; Miller and Bassler, 2001; Hawver et al., 2016). Virulence factor expression appears to be controlled by this mechanism, and a bacteria will only express virulence factors under precisely defined conditions. It is thus possible that the expression of virulence factors by pathogenic bacteria might have been repressed by the high concentration of bacteria (Ng and Bassler, 2009). Also, the potential of this mechanism of communication has been explored by scientists as a therapeutic strategy for controlling infections, as it is less prone to resistance than the use of antibiotics (Rutherford and Bassler, 2012). There is also an interesting possibility that a high bacterial dose can prevent metritis when the cow is infected with specific bacteria.

The model we created accurately reproduced the clinical signs associated with naturally occurring metritis, including increased RT (Figure 2A), reduced DMI (Figure 2B), and reduced milk production (Figure 2C). Cows in the low-dose group had greater RT compared with high-dose cows. Fever results from the communication of immune system and the brain in response to infection, inflammation, trauma, or a combination of these (Leon, 2002). Sammann et al. (2012) discussed the reliability of the use of fever to diagnose metritis. In our study, we observed that cows had an increase in RT before the diagnosis of metritis. The same was reported by Benzaquen et al. (2007), who observed that the increase in RT in metritic cows with or without fever was more evident around 3 d before diagnosis of disease (Benzaquen et al., 2007). The same authors also observed that most cows did not present fever at the time of diagnosis of metritis; cow attitude and uterine conditions were more relevant. In a literature search published by Sammann et al. (2012), the authors present a series of papers showing that using RT as a diagnostic criteria for metritis increases type I and type II errors; they conclude that fever is less reliable than evaluating the uterine discharge.

In addition to increased RT, low-dose cows had reduced DMI; they consumed, on average, 1.4 and 2.2 kg/d less DM compared with control and high-dose cows, respectively. This is in agreement with previous observational studies (Urton et al., 2005; Bell and Roberts, 2007; Wittrock et al., 2011). Cows in the low-dose group also presented a numeric reduction of approximately 3 kg/d in milk production during the study period, which was in agreement with reports for

### Table 2

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Low dose</th>
<th>High dose</th>
<th>P-value1</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA, mmol/L</td>
<td>0.60 ± 0.11</td>
<td>0.67 ± 0.10</td>
<td>0.54 ± 0.10</td>
<td>0.29</td>
</tr>
<tr>
<td>BHB, mmol/L</td>
<td>1.28 ± 0.30</td>
<td>1.28 ± 0.20</td>
<td>0.98 ± 0.11</td>
<td>0.24</td>
</tr>
<tr>
<td>Albumin, g/dL</td>
<td>3.55 ± 0.11</td>
<td>3.45 ± 0.10</td>
<td>3.51 ± 0.10</td>
<td>0.51</td>
</tr>
<tr>
<td>Total protein, mg/dL</td>
<td>7.23 ± 0.25</td>
<td>6.86 ± 0.24</td>
<td>7.33 ± 0.27</td>
<td>0.16</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>74.31 ± 3.16</td>
<td>75.16 ± 3.03</td>
<td>76.94 ± 3.03</td>
<td>0.80</td>
</tr>
<tr>
<td>Calcium, mg/dL</td>
<td>9.23 ± 0.26</td>
<td>8.88 ± 0.24</td>
<td>9.22 ± 0.25</td>
<td>0.13</td>
</tr>
</tbody>
</table>

1P-value indicates the overall group effect.

### Table 3

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Low dose</th>
<th>High dose</th>
<th>P-value1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α, pg/mL</td>
<td>47.7 ± 95</td>
<td>192 ± 100</td>
<td>109 ± 92</td>
<td>0.59</td>
</tr>
<tr>
<td>IL-2, pg/mL</td>
<td>185.7 ± 372</td>
<td>830 ± 384</td>
<td>354 ± 355</td>
<td>0.47</td>
</tr>
<tr>
<td>IL-6 (ln), pg/mL</td>
<td>3.2 ± 0.15</td>
<td>3.4 ± 0.16</td>
<td>3.3 ± 0.15</td>
<td>0.33</td>
</tr>
<tr>
<td>IL-8, pg/mL</td>
<td>1,952.6 ± 781.5</td>
<td>1,279.6 ± 526.84</td>
<td>2,612.5 ± 835.17</td>
<td>0.06</td>
</tr>
<tr>
<td>IL-10 (ln), pg/mL</td>
<td>2.4 ± 0.02</td>
<td>2.4 ± 0.02</td>
<td>2.4 ± 0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

1Means with different superscripts differ at P < 0.05.

1P-value indicates the overall group effect.
natural cases of metritis (Rajala and Grohn, 1998; Bell and Roberts, 2007; Dubuc et al., 2011; Wittrock et al., 2011; Giuliodori et al., 2013). Based on these data, we conclude that challenged cows presented clinical signs of disease similar to those reported for natural cases of metritis.

As we were successful in inducing metritis in cows challenged with the lower dose of inoculum, we investigated whether bacterial challenge also created signs of systemic disease. We assessed activation of the immune system by measuring acute phase proteins, production of proinflammatory cytokines, and changes in serum metabolites. Previously, others have described an increase in haptoglobin before the diagnosis of metritis (Smith et al., 1998; Sheldon et al., 2001; Chan et al., 2004, 2010; Dubuc et al., 2010; Galvão et al., 2010) and have proposed that SAA and haptoglobin are predictors of uterine diseases (Chan et al., 2010; Zhang et al., 2018). Herein, we observed a similar trend for increased haptoglobin before d 7 postpartum in challenged animals (Figure 3C) and the same for SAA (Figure 3A), which is in agreement with those reports.

Although we expected that systemic toxins, activation of the immune system, and release of acute phase proteins would be associated with liver damage, we did not observe changes in liver parameters. There was, however, a treatment effect on serum concentration of GGT (Supplemental Figure S3E). Cows enrolled in the control group had greater concentrations of GGT compared with nonmetritic cows during the first 12 d postpartum, but calcium concentrations compared with nonmetritic cows had lesser circulating concentrations of proinflammatory cytokines during the first 12 d postpartum, but calcium concentrations compared with nonmetritic cows during the first 12 d postpartum. Low-dose cows also had lesser feed intake compared with control cows, which may affect calcium availability and absorption in the gastrointestinal tract. Proinflammatory cytokines upregulate the calcium-sensing receptor gene (CaSR) expression in the parathyroid gland and the kidney, resulting in decreased serum parathyroid hormone and 1,25-dihydroxy vitamin D and calcium levels (Hendy and Canaff, 2015). In the present model, low-dose cows had greater circulating concentrations of proinflammatory cytokines. Thus, high levels of proinflammatory cytokines might have upregulated the expression of CaSR and reduced calcium levels.

With the present model, we answered important questions but also generated additional questions for further research. Several studies have proposed that low calcium concentration during early lactation is associated with an increased risk of metritis (Curtis et al., 1983; Goff and Horst, 1997; Martinez et al., 2012; Rodríguez et al., 2017; Neves et al., 2018). We observed a tendency for lesser serum calcium concentration in low-dose cows becoming evident on d 1 and persisting until study d 12 (Supplemental Figure S1F). Our model agrees with the finding of Martinez et al. (2012), who reported that metritic cows had lesser calcium concentrations compared with nonmetritic cows during the first 12 d postpartum, but calcium concentrations on the day of parturition were similar (metritic vs. nonmetritic cows; Martinez et al., 2012). We hypothesize that the reduction in blood calcium occurs as a consequence of parturition, contamination of the uterus, and disease. This hypothesis is different from the alternative hypothesis that periparturient hypocalcemia is a predisposing factor in the development of uterine disease postpartum. Low-dose cows also had lesser feed intake compared with control cows, which may affect calcium availability and absorption in the gastrointestinal tract. Proinflammatory cytokines upregulate the calcium-sensing receptor gene (CaSR) expression in the parathyroid gland and the kidney, resulting in decreased serum parathyroid hormone and 1,25-dihydroxy vitamin D and calcium levels (Hendy and Canaff, 2015). In the present model, low-dose cows had greater circulating concentrations of proinflammatory cytokines. Thus, high levels of proinflammatory cytokines might have upregulated the expression of CaSR and reduced calcium levels.

By monitoring cows daily, we were able to determine the dynamics of bacterial growth during colonization of the uterus. We observed that the low-dose cows had an increased relative abundance of 16S sequences of the genus Fusobacterium (Figure 4C), in addition to increases in counts (cfu) of F. necrophorum (Figure 4B). The E. coli (Figure 5B) and T. pyogenes (Figure 6B) counts (cfu) were not different among groups. Although

### Table 4.
Counts of white blood cells, lymphocytes, monocytes, and granulocytes during the first 14 d of lactation of cows challenged with a low dose (n = 12) or high dose (n = 12) of bacterial inoculum or unchallenged (control; n = 11)

<table>
<thead>
<tr>
<th>Item</th>
<th>Control (µL)</th>
<th>Low dose (µL)</th>
<th>High dose (µL)</th>
<th>P-value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC, ×10&lt;sup&gt;3&lt;/sup&gt;/µL</td>
<td>8.40 ± 0.65</td>
<td>7.48 ± 0.62</td>
<td>7.41 ± 0.62</td>
<td>0.22</td>
</tr>
<tr>
<td>Lymphocytes, ×10&lt;sup&gt;3&lt;/sup&gt;/µL</td>
<td>3.82 ± 0.42</td>
<td>3.97 ± 0.40</td>
<td>3.27 ± 0.10</td>
<td>0.31</td>
</tr>
<tr>
<td>Monocytes, ×10&lt;sup&gt;3&lt;/sup&gt;/µL</td>
<td>0.79 ± 0.039&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64 ± 0.037&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.67 ± 0.037&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.02</td>
</tr>
<tr>
<td>Granulocytes, ×10&lt;sup&gt;3&lt;/sup&gt;/µL</td>
<td>3.79 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.88 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.47 ± 0.29&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Means with different superscripts differ at P < 0.05.
<sup>1</sup>P-value indicates the overall group effect.
Figure 4. Bacterial counts of (A) total anaerobes and (B) *Fusobacterium necrophorum*, and (C) relative abundance of the genus *Fusobacterium* from vaginal contents during the first 14 d of lactation of cows challenged intrauterine with a bacterial inoculum containing $10^6$ cfu (low dose; n = 12) or $10^9$ cfu (high dose; n = 12) of *Escherichia coli*, *Trueperella pyogenes*, and *F. necrophorum*, or unchallenged (control; n = 11). Results are presented as LSM ± SEM. TRT = treatment.

Figure 5. Bacterial counts of (A) total aerobes and (B) *Escherichia coli*, and (C) relative abundance of the genus *Escherichia* from vaginal contents during the first 14 d of lactation of cows challenged intrauterine with a bacterial inoculum containing $10^6$ cfu (low dose; n = 12) or $10^9$ cfu (high dose; n = 12) of *E. coli*, *Trueperella pyogenes*, and *Fusobacterium necrophorum*, or unchallenged (control; n = 11). Results are presented as LSM ± SEM. TRT = treatment; ln = natural log.
the role of pathogenic strains of *E. coli* for the development of metritis is well described (Sheldon et al., 2010; Goldstone et al., 2014), based on our data, we question whether *E. coli* and *T. pyogenes* are microorganisms needed before or during the development of metritis. When analyzing the progression of the microbiome from calving until establishment of metritis, Jeon et al. (2015) found that the genus *Fusobacterium* was the most abundant (15.7%) among 28 other genera found (Jeon et al., 2015). Similar findings were reported in 2 additional studies from the same group (Jeon et al., 2016; Cunha et al., 2018). Collectively, these data support the notion that *Fusobacterium* is one of the most relevant genera and that *F. necrophorum* is the most relevant species for the development of metritis. Nevertheless, it is still unclear whether *E. coli* or *T. pyogenes* is important for the development of the disease. In other words, do *E. coli* and *T. pyogenes* prepare the uterine environment for *F. necrophorum* infection or is *F. necrophorum* simply an opportunistic pathogen? Previous work has demonstrated that the damage caused to the uterine epithelium by *E. coli* and *T. pyogenes* is crucial to the subsequent colonization by *F. necrophorum* (Bicalho et al., 2012a; Galvão et al., 2019). Although the data for *E. coli* (Figure 5B), the genus *Escherichia* (Figure 5C), *T. pyogenes* (Figure 6B), and the genus *Trueperella* (Figure 6C) did not demonstrate a clear difference following the challenge dose, these same genus and species were clearly different for metritic and nonmetritic cows from this study (Supplemental Figures S5 to S7). When cows were grouped by disease, *F. necrophorum* increased early postpartum (Supplemental Figure S5), *T. pyogenes* increased a little later (Supplemental Figure S6), and *E. coli* decreased immediately postpartum (Supplemental Figure S7). Further investigation is needed therefore to determine whether the interaction among *E. coli*, *T. pyogenes*, and *F. necrophorum* is requisite for the development of the disease, or whether *F. necrophorum* individually is capable of inducing metritis.

A strength of the present model is the induction of metritis in a group of cows receiving intruterine infusion of a specific dose of a known bacterial cocktail. The disease was created in postpartum cows, where the disease is typically found. Our model is different from other models that aim to create metritis in heifers, later-lactation cows, or other species (Rowson et al., 1953; Farin et al., 1989; Piersanti et al., 2019; Dickson et al., 2020). These alternative models do not reproduce the metabolic challenges faced during the early postpartum (transition) period in high-production dairy cows. They also do not recapitulate the unique environment for bacterial growth created.
by the postpartum uterus undergoing involution. We observed that the intrauterine infusion of bacteria increased the incidence of metritis, from 8% in control cows to 83% in cows receiving the lower dose of inoculum, corroborating the idea that metritis is caused by a dysbiosis of the uterine microbiome, associated with an increase in specific uterine pathogens (Galvão et al., 2019).

We acknowledge that the present model has limitations. Control cows, for example, were not infused with the same medium used for bacterial culture but instead were infused with sterile saline solution. The rationale for sterile saline was that we did not want the sterile culture medium to create an ideal environment for bacterial growth in the control cows or introduce any potential source of contamination to the uterus of control cows. An alternative approach would be to resuspend all bacteria in saline solution before infusion. We elected to use the transport medium to resuspend bacteria before infusion so that ideal culture conditions were maintained up until the infusion. Also, we did not examine the potential impact of external factors such as stress, grouping, or diet formulation on the model and its effect on disease incidence. Metritis is a multifactorial disease associated with a dysbiosis of the uterine microbiome that is not necessarily limited to a change in 3 microorganisms, as was tested here. The model that we tested can lead to more refined disease models and knowledge about clinical disease development in vivo. Last, although a power sample calculation was performed to design the experiment, we acknowledge that only 12 cows were assigned per group in this field trial to achieve the expected incidence of disease in one of the challenged groups, respecting the principle of 3Rs: replacement, reduction, and refinement; thus, the results obtained in this disease model may not be generalized.

We propose that the present in vivo model could be used in the future to evaluate the viability of different tools used for prevention or treatment of metritis. A vaccine containing subunits of *E. coli*, *T. pyogenes*, and *F. necrophorum* was developed by our group and has been shown to reduce the incidence of metritis on dairy farms (Meira et al., 2020). A recent article has described the effect of ceftriaxone in the uterine microbiome (Jeon et al., 2021), characterized by a reduction in *Fusobacterium*, the main microorganism associated with the development of metritis in our model. With our model, we have established the expected incidence of metritis, and we can test the efficacy of vaccination and other preventative measures. We can also compare the efficacy of treatments used to reduce the severity of metritis and determine whether such treatments present practically and economically justified means to control this important disease.

**CONCLUSIONS**

We successfully induced metritis in postpartum dairy cows via intrauterine administration of *E. coli*, *T. pyogenes*, and *F. necrophorum*. We observed an increased incidence of disease in animals challenged with a dose of $10^6$ CFU of this combination of bacteria. The observation that the lower dose was the more efficaciously associated with metritis indicated that the development of metritis when using this particular bacterial cocktail depends on the dose of microorganisms used. We established the dynamics of microorganisms for the development of metritis and found that *F. necrophorum* plays an important role in the pathophysiology of the disease.

There were changes in blood components and blood cell counts that were related to the observed clinical signs of natural disease. This model may provide an effective clinical outcome that can be used to compare the effects of strategies to prevent or treat metritis, thus providing benefits to the dairy industry.

**ACKNOWLEDGMENTS**

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**REFERENCES**


