Running Title: Chitosan microparticles affect the uterine microbiome

Effect of chitosan microparticles on the uterine microbiome of dairy cows with metritis

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ABSTRACT

The objective was to evaluate the effect of chitosan microparticles on the uterine microbiome of cows with metritis. Dairy cows with metritis (n = 89) were assigned to 1 of 3 treatments: chitosan microparticles (n = 21) = intrauterine infusion of chitosan microparticles at metritis diagnosis (D0), D2 and D4; ceftiofur (n = 25) = subcutaneous injection of ceftiofur on D0 and D3; Untreated (n = 23) = no treatment. Non-metritic (n = 20) = healthy cows matched by days postpartum at metritis diagnosis. Uterine swabs collected on D0, D3, D6, D9, and D12 were used for 16S rRNA gene sequencing and qPCR quantification. Principal coordinate analysis showed that the microbiome of the ceftiofur-treated and metritic untreated groups progressed towards the non-metritic group by D3, whereas the chitosan microparticles-treated remained unchanged. The differences on D3 were mainly due to a greater relative abundance of *Fusobacteria*, particularly *Fusobacterium* in the chitosan microparticles-treated compared with the ceftiofur-treated and metritic untreated groups. Furthermore, the microbiome of the ceftiofur-treated group became similar to the non-metritic group by D9; whereas the chitosan microparticles-treated and metritic untreated groups only became similar to the non-metritic group by D12. The chitosan microparticles treatment resulted in greater total bacterial 16S rRNA gene counts than the metritic untreated controls on D6 and D9, whereas the ceftiofur treatment was the only group that became similar to the non-metritic group by D12. In summary, chitosan microparticles slowed the progression towards a healthy uterine microbiome, whereas ceftiofur hastened the progression towards health.

52 **IMPORTANCE**

53 Third-generation cephalosporins such as ceftiofur are commonly used to treat metritis in
54 dairy cows. Chitosan microparticles has been shown to have a broad spectrum of
55 activity *in vitro* and to be effective against uterine pathogens *in vivo*; therefore, it has
56 been hailed as a possible alternative to traditional antibiotics. Nonetheless, herein we
57 saw that chitosan microparticles slowed the progression of the uterine microbiome of
58 cows with metritis towards a healthy uterine microbiome, whereas ceftiofur treatment
59 hastened the progression towards a healthy uterine microbiome. Given the lack of an
60 effective alternative to traditional antibiotics and an increased concern about
61 antimicrobial resistance, a greater effort should be devoted to the prevention of metritis
62 in dairy cows.

63

64 **INTRODUCTION**

65 Improvements in genetics, housing, nutrition, and management have produced dramatic
66 increases in milk production in the last 70 years, going from ~2000 kg/y in 1944 to over
67 10,000 kg/yr in 2016 (1-3). Nonetheless, the improvements in milk production have not
68 been accompanied by improvements in health. Approximately 45% of dairy cows
69 experience a disease or disorder in the first 60 days postpartum (2), and metritis, the
70 inflammation of all layers of the uterus, is one of the most prevalent and costly diseases
71 to the dairy industry (4, 5).

72 Metritis is associated with polymicrobial infection with both Gram-negative (e.g.
73 *Fusobacterium*, *Bacteroides*, and *Porphyromonas*) and Gram-positive bacteria (e.g.
74 *Trueperella*, *Peptostreptococcus*, and *Helcococcus*) (6-8); therefore, metritis is

commonly treated with broad-spectrum antibiotics. Ceftiofur, an approved third-generation cephalosporin for treatment of metritis, is the broad-spectrum antibiotic of choice for treating metritis because it does not incur in milk withdrawal (9-11). Nonetheless, because third generation cephalosporins are an important class of antimicrobials in human medicine, The Food and Drug Administration has banned its use for disease prevention in food animals. This highlights the need for alternatives to traditional antibiotics for prevention and treatment of diseases in food animals.

Chitosan microparticles are derived from chitosan, which is a linear polysaccharide produced by deacetylation of chitin. Chitosan has antimicrobial properties at acidic pH, whereas chitosan microparticles maintains broad-spectrum of antimicrobial activity even at neutral pH (12). Chitosan microparticles exerts its antimicrobial activity by binding to the outer membrane protein A (OmpA) and by electrostatic interactions (12). Preliminary studies with a limited number of cows showed that intrauterine infusion of chitosan microparticles was as effective as ceftiofur in reducing *Escherichia coli* (*E. coli*) counts (12) and the relative abundance of *Fusobacterium necrophorum* (*F. necrophorum*), and appeared to be even more effective than ceftiofur in reducing the relative abundance of Bacteroidetes (13). *E. coli*, *F. necrophorum* and *Bacteroides* spp. are believed to be important pathogens associated with the development of metritis (8); therefore, chitosan microparticles seemed to be a promising alternative to traditional antibiotics. The ultimate goal of antibiotic treatment is to return the organ and organism to a healthy state; therefore, we hypothesized that chitosan microparticles would be as effective as ceftiofur in returning the uterine microbiome of metritic cows to a healthy state. Health is the state of being

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98 free from disease or injury, and in our previous work we have shown that uterine
99 disease (i.e. metritis) is associated with a dysbiosis of the uterine microbiome
100 characterized by decreased bacterial richness, decreased heterogeneity, greater total
101 bacterial count, greater relative abundance of *Fusobacteria* and *Bacteroidetes*, and
102 lesser relative abundance of *Proteobacteria* and *Tenericutes* (6, 8). Hence, reversal of
103 those shifts characterize the microbiome of cows that cure from metritis (14). Therefore,
104 the objective of this study was to evaluate if chitosan microparticles would be as
105 effective as ceftiofur in returning the uterine microbiome of metritic cows to a healthy
106 state.
107
108 **MATERIAL AND METHODS**
109
110 **Cows, housing, and feeding**
111 All animal procedures were approved by the University of Florida Institutional Animal
112 Care and Use Committee (IACUC Protocol #: 201509189).
113 The study was conducted in a dairy herd located in North Central Florida from
114 May, 2016 to June, 2017. The herd milked ~4,400 Holstein cows three times daily, and
115 the rolling herd average milk yield was ~12,000 kg. Postpartum cows were housed in
116 tunnel-ventilated six row barns with sprinklers over the feeding areas that were
117 activated when the ambient temperature rose above > 21 °C. The stalls were deep-
118 bedded with sand. The postpartum diet was formulated to meet or exceed the dietary
119 nutrient requirements for a lactating cow weighing 680 kg and producing 45 kg of 3.5%

fat-corrected milk and 3.0% protein (15), and it was delivered as a total mixed ration twice daily.

Diagnosis of metritis

Cows were examined for diagnosis of metritis at 5, 7 and 9 days postpartum using the Metricheck® device (Simcro, Hamilton, NZ) as previously reported (6, 16). Discharge retrieved from the vagina was scored as: 1 = not fetid normal lochia, viscous, clear, red, or brown; 2 = cloudy mucoid discharge with flecks of pus; 3 = not fetid, mucopurulent discharge with < 50% pus; 4 = not fetid mucopurulent white, yellow or reddish-brownish discharge with ≥ 50% pus; and, 5= fetid, thin, serous, or watery, may have been reddish-brownish, with or without pieces of necrotic tissue present (10). Cows with a vaginal discharge score of 5 were classified as having metritis.

Treatment groups

The cows used for this study represent a subset of 89 cows with metritis that were randomly sampled from a larger study with 826 cows with metritis that had been randomly assigned to 1 of 3 treatments: chitosan microparticles = intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 ml of sterile distilled water at the time of diagnosis (D0), D2 and D4; ceftiofur = subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis, Parsippany, NJ, USA) in the base of the ear at D0 and D3; untreated = no intrauterine or subcutaneous treatment (17). A group of healthy non-metritic cows matched by days in milk at metritis diagnosis was added for comparison. The chitosan microparticles were prepared as previously reported (12).

143 Briefly, a 2% (wt/vol) chitosan (Molecular weight 50−190 kDa, deacetylation degree 75−
144 85%, 448869−250G, Sigma-Aldrich) solution was prepared with 2% acetic acid (v/v)
145 and 1% tween 80 (v/v). For cross-linking, the chitosan solution was stirred and 10% of
146 sodium sulfate (w/v) was added dropwise during 25 min of sonication. The chitosan
147 microparticles were collected by centrifugation (8200 g) and washed with sterile water.
148 The weight of chitosan microparticles was measured after freeze-drying. For Quality
149 control, each batch of chitosan microparticles were confirmed to have antimicrobial
150 activity against *E. coli in vitro* at a concentration of 0.2% chitosan microparticles. The
151 amount of chitosan microparticles infused in the uterus (24 g) was calculated to give a
152 final concentration of at least 0.6% (assuming a uterine content in cows with metritis of
153 4 L), which was the lowest concentration shown to effectively eliminate *E. coli* from
154 uterine fluid *ex vivo* (18). The sample size was estimated based on our previous studies
155 where a sample size of approximately 15 cows per group provided adequate power to
156 evaluate the uterine microbiome shift after antibiotic treatment of cows with metritis (14).
157 Hence, uterine swab samples were collected from chitosan microparticles-treated (n =
158 21), ceftiofur-treated (n = 25), metritic untreated (n = 23), and non-metritic (n = 20) cows
159 on D0, D3, D6, D9, and D12.

160

161 **Sample collection and DNA extraction**

162 Uterine swab samples were collected right before the first treatment on D0 in ceftiofur-
163 treated and chitosan microparticles-treated cows. The swab samples were collected
164 using a 30” double-guarded sterile culture swab (Continental Plastics Corporation,
165 Delavan, WI). Briefly, the instrument was gently passed through the cervix and

positioned in the uterine body where the internal sheath and the swab were exposed, and the swab was gently rolled against the uterine wall. The swab was retracted within the double sheath before removal from the cow. The swab was stored in a 15 mL conical sterile tube and placed on ice until return to the laboratory where the tip of the swab was cut-off, immersed in 1000 µL of phosphate-buffered saline, vortexed, then the swab tip was discarded and the fluid was stored at -80ºC until DNA extraction.

Isolation of genomic DNA was performed using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions for buccal swabs. To maximize bacterial DNA extraction, 5 µl of lysozyme (50 mg/mL, Thermal Fisher, Waltham, MA, USA) and 6 µL of mutanolysin (14.3 KU/mL, Sigma-Aldrich, Sait Louis, MO, USA) were mixed with 200 µL of the thawed fluid from the swab sample, and incubated at 37°C for 1 h before starting the QIAamp DNA Mini kit protocol. DNA concentration and purity were evaluated by optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at the wavelengths of 230, 260, and 280 nm, and the OD260/280 ratio of DNA ranged from 1.7 to 2.1.

16S rRNA gene amplification and sequencing

For each metagenomic DNA sample, amplification of the V4 hypervariable region of the bacterial/archaeal 16S rRNA gene was performed using the 515F and 806R primer set according to previously described methods and optimized for the Illumina MiSeq platform (19). PCR products were tagged with 280 different 12-bp error-correcting Golay barcodes (http://www.earthmicrobiome.org/) (20). PCR's were carried out in triplicate in 25 µl reactions using 12-300 ng of template DNA, 1X EconoTaq® Plus Green Master

Mix (Lucigen®, Middleton, WI) and 10 µM 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, 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, Rockland, DE) at wavelengths of 260 and 280 nm. Amplicons were standardized to the same concentration and pooled into three different 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 using the MiSeq Reporter proprietary metagenomics workflow based on the Greengenes database (21) as previously reported (6). 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; therefore, only classification up to genus level was attempted and presented (22). All the sequences were uploaded to the National Center for Biotechnology Information under Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) using two BioProjects; accession number PRJNA643565 with 165 samples and accession number PRJNA643556 with 280 samples.

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Total bacterial 16S rRNA gene copies quantification using qPCR

The total bacterial 16S rRNA gene copies quantification was performed using the Femto™ bacterial DNA quantification kit targeting the 16S rRNA gene (Zymo Research, El Cajon, CA, USA) following the manufacturer's protocol. Briefly, 18uL of the supplied master mix was added to each well with 2 µL of each sample or standards ranging from 2 fg to 20 ng of DNA. The cycling parameters were as indicated by the manufacture's protocol. Samples were run in duplicate. The amount of DNA in each sample was calculated based on the standard curve. Then, the 16S rRNA gene copy number was calculated using the following formula, provided by the company:

16S rRNA gene copy number = DNA in grams / grams to base pair constant x genome size

Where grams to base pair constant = 1.096×10^{-21} g and genome size = 5.5×10^{6} bp. Then, a logarithm to the base 10 conversion of the raw values was performed.

Effect of Chitosan microparticles on *Fusobacterium necrophorum* and *Bacteroides pyogenes in vitro*

Because the analysis of the uterine microbiome using 16S rRNA sequencing revealed that chitosan microparticles was ineffective against *Fusobacteria*, particularly *Fusobacterium,* we tested the effect of chitosan microparticles against *Fusobacterium necrophorum* (*F. necrophorum*) in vitro. We also used *Bacteroides pyogenes* (*B. pyogenes*), another highly abundant uterine pathogen (7), for comparison. *F. necrophorum* and *B. pyogenes* were inoculated into chopped meat broth (Anaerobe

Systems, Morgan Hill, CA) and incubated in anaerobic condition with GasPak Anaerobic System (BD, Franklin Lakes, NJ) at 37 ºC overnight. Then the culture was diluted to approximately 5 × 10^4 CFU/mL and incubated in chopped meat broth with 0, 0.1, 0.2, and 0.4% of chitosan microparticles respectively. After 24 h anaerobic incubation at 37 ºC, the bacterial cultures were serial diluted and plated on tryptic soy agar supplemented with 5% sheep blood to enumerate the bacteria. The assay was conducted in triplicate. Because ceftiofur is known to reach the minimum inhibitory concentrations in the endometrium and lochia of cows with metritis that are required to inhibit the growth of 90% of *F. necrophorum* and other uterine pathogens such as *Prevotella melaninogenica* and *Trueperella pyogenes* (0.125 µg/mL) (23, 24), ceftiofur was not tested.

Utilization of N-acetylglucosamine as a carbon source by *F. necrophorum* and *B. pyogenes*

Similar to what happens in the gastrointestinal tract, it is possible that the complex microflora of the uterus could hydrolyze chitosan microparticles into glucosamine and N-acetylglucosamine, which could then be available for bacterial growth (25). Lysozyme released after cell death or leukocyte degranulation may also hydrolyze chitosan microparticles into glucosamine and N-acetylglucosamine (26). Hence, we tested if *F. necrophorum* could use N-acetylglucosamine as a carbon source for growth using the AN MicroPlate (Biolog , Inc.) containing 95 carbon sources. We also used *B. pyogenes* for comparison. The AN Microplate does not include glucosamine as a carbon source.

The assay was carried out in triplicates according to the manufacturer's instructions. Briefly, *F. necrophorum* KG34 (GenBank accession no. SRX5402669) and *B. pyogenes* KG32 (GenBank accession no. SRS5092514) were grown on Wilkins-Chalgren agar medium (Sigma-Aldrich) and suspended in 14 ml of AN inoculating fluid (Biolog) to make bacterial suspension with a transmittance level of about 65%. A 100 µl of bacterial suspension was quickly plated into each well of the AN MicroPlate and incubated at 37°C in the GasPak EZ anaerobic pouch system (BD). Utilization of the carbon sources by *F. necrophorum* and *B. pyogenes* were indicated by tetrazolium violet forming blue color, which was read at 3, 6, 12, and 24 h post inoculation at OD590 using SmartSpec 3000 spectrophotometer (Bio-Rad). The well containing water in the Biolog AN MicroPlate was used as blank control and the actual OD for the carbon sources was obtained by subtracting the measured OD value from the time zero OD.

Data analysis

Swab samples resulted in 13,638,041 high-quality reads after filtering for size, quality, and the presence of chimeras. Four swab samples from three ceftiofur-treated cows did not pass the filtering criteria, and were excluded from the study.

Principle coordinates analysis (PCoA) of Bray-Curtis dissimilarity at the phylum and genus level was used to compare treatment groups and time points using PAST version 3.18. The effects of treatment, time, and the interaction between treatment and time on the uterine microbiome structure were evaluated with two-way PERMANOVA with 9999 permutations based on Bray-Curtis dissimilarity at the phylum level using PAST version 3.18. Linear discriminant analysis effect size (LEfSe) was used to

281 determine the phyla and genera most likely to explain differences between treatment
282 groups (27). A logarithm base 10 linear discriminant analysis (LDA) score cutoff of 3
283 was used. Alpha diversity at the genus level was compared among the groups using the
284 Chao1 richness index and the Shannon diversity index. Evenness was also evaluated.
285 Evenness was calculated by dividing the Shannon diversity index by the natural
286 logarithm of genus richness (i.e. number of genera in each sample). Continuous
287 outcomes such as relative or absolute abundances of bacteria, Chao1 richness index,
288 Shannon diversity index, and evenness were compared using ANOVA for repeated
289 measures using the MIXED procedure of SAS version 9.4 (SAS Institute, Cary, NC).
290 The ANOVA models included the effects of treatment, time, parity (multiparous vs.
291 primiparous) and the interaction between treatment and time. Parity was dropped from
292 the model if $P > 0.05$. Normality and homoscedasticity of residuals were assessed using
293 residual plots. For repeated measures, unstructured, autoregressive 1, and compound
294 symmetry covariance structures were tested, and the one with the smallest Akaike
295 information criterion was chosen. For all models, Tukey's honest significance test for
296 multiple comparisons was used. Significance was considered when $P \leq 0.05$.
297
298 **RESULTS**
299
300 **Effect of treatment on the uterine microbiome richness, diversity and evenness**
301 There was a main effect of treatment group on the Chao1 richness index ($P < 0.01$), the
302 Shannon diversity index ($P = 0.01$) and on evenness ($P < 0.01$). The Chao1 index was
303 greater ($P < 0.01$) for the non-metritic group than the metritic groups (Fig. 1A). On the

other hand, the Shannon index (Fig. 1B) and evenness (Fig. 1C) were lesser ($P \leq 0.05$) for the non-metritic group than the metritic groups. There was no difference ($P > 0.20$) among the metritic groups. There was also an effect of time on the Chao 1 index ($P = 0.01$), the Shannon index ($P = 0.04$) and on evenness ($P < 0.01$) (Fig. 1A, B, and C), but there was no interaction ($P > 0.45$) between treatment and time on the microbial community richness, diversity or evenness.

Effect of treatment on the uterine microbiome structure at the phylum level

Principle coordinates analysis (PCoA) paired with PERMANOVA based on the Bray-Curtis dissimilarity of relative abundance at the phylum level showed that there was an effect of treatment group ($P < 0.01$), time ($P < 0.01$) and an interaction between treatment and time ($P < 0.01$) on the structure of the uterine microbiome. The main effect of treatment showed that the uterine microbiomes of all metritic groups were different ($P < 0.01$) from the non-metritic group, and that the chitosan microparticles-treated group was different from the ceftiofur-treated ($P = 0.03$) and the metritic untreated group ($P = 0.04$), but there was no difference between the ceftiofur-treated and metritic untreated groups ($P = 0.34$) (Fig. 2A). Nonetheless, the interaction between treatment group and time showed that the uterine microbiomes of the metritic groups had different progression toward a healthy uterine microbiome overtime. On D0, the metritic groups were not different ($P > 0.30$) among themselves, but they were different ($P < 0.01$) from the non-metritic healthy group (Fig. 2B). The microbiomes of metritic cows were very homogeneous, whereas the microbiome of non-metritic cows was heterogeneous. On D3, all the metritic groups were different ($P < 0.01$) from the non-

metritic group, the chitosan microparticles-treated group was different from the ceftiofur-treated ($P < 0.01$) and the metritic untreated groups ($P = 0.03$), but there was no difference between the ceftiofur-treated and the metritic untreated groups ($P = 0.68$) (Fig. 2C). Both the ceftiofur-treated and the metritic untreated groups became more heterogeneous but the chitosan microparticles-treated group remained very homogeneous. On D6, the metritic groups were not different ($P > 0.13$) among themselves, but they were different ($P < 0.01$) from the non-metritic group, although the chitosan microparticles-treated group was still less heterogeneous than the ceftiofur-treated and metritic untreated groups (Fig. 2D). On D9, the metritic groups were not different ($P > 0.11$) among themselves, the chitosan microparticles-treated and the metritic untreated groups were still different ($P < 0.01$) from the non-metritic group, but the ceftiofur-treated group was not different ($P = 0.09$) from the non-metritic group (Fig. 2E). On D9, all groups became more heterogeneous but the chitosan microparticles-treated and metritic untreated groups lagged the ceftiofur-treated group. On D12, none of the groups were different ($P > 0.10$), although the chitosan microparticles-treated group was still less heterogeneous than the other groups. These results showed that the chitosan microparticles treatment delayed the progression towards a healthy uterine microbiome, whereas ceftiofur treatment hastened the progression of the uterine microbiome towards a healthy uterine microbiome.

The LEfSe showed that differences in phyla relative abundance most likely to explain the differences between the metritic groups and the non-metritic group on D0 were greater relative abundance of *Bacteroidetes* and *Fusobacteria* in metritic cows and greater relative abundance of *Proteobacteria* and *Tenericutes* in non-metritic cows (Fig.

3A). On D3, the differences in phyla relative abundance most likely to explain the differences between the chitosan microparticles-treated and ceftiofur-treated groups were greater relative abundance of *Fusobacteria* in the chitosan microparticles-treated group and greater relative abundance of *Firmicutes* in the ceftiofur-treated group (Fig. 3B). On D3, the differences in phyla relative abundance most likely to explain the differences between the chitosan microparticles-treated and the metritic untreated group were greater relative abundance of *Fusobacteria* in the chitosan microparticles-treated group and greater relative abundance of *Verrucomicrobia* in the metritic untreated group (Fig. 3C). In order to develop a more intuitive understanding of the microbiome composition and dynamics, we also presented stacked bars for each day showing the average phylum relative abundances for each treatment (Fig. S1) and the phylum-level compositions of all D0 and D12 samples by treatment (Fig. S2).

Effect of treatment on the uterine microbiome structure at the genus level

The results for PCoA paired with PERMANOVA based on the Bray-Curtis dissimilarity of relative abundance at the genus level mostly agree with the results at the phylum level, showing that chitosan microparticles treatment delayed the progression towards a healthy uterine microbiome, whereas ceftiofur treatment hastened the progression of the uterine microbiome towards a healthy uterine microbiome (Fig. S3). The LEfSe results at the genus level mostly agree with results at the phylum level, with metritic cows having greater relative abundance of *Fusobacterium, Bacteroides,* and *Porphyromonas* than non-metritic cows on D0, and chitosan microparticles treatment

having greater relative abundance of *Fusobacterium* than ceftiofur treatment and untreated controls on D3 (Fig. S4).

Effect of treatment on total bacterial 16S rRNA gene counts

There was an effect of treatment group ($P < 0.01$), time ($P < 0.01$) and an interaction between treatment and time ($P = 0.01$) on total bacterial 16S rRNA gene counts in the uterus. The main effect of treatment showed that total bacterial 16S rRNA gene counts of all metritic groups were different ($P < 0.01$) from the non-metritic group, but there was no difference ($P \geq 0.06$) among the metritic groups (Fig. 4). Nonetheless, the interaction between treatment group and time showed that the total bacterial 16S rRNA gene counts of the metritic groups had different progression toward a healthy uterine microbiome overtime. On D0, total bacterial 16S rRNA gene counts were not different ($P > 0.20$) among the metritic groups, but they were all greater ($P < 0.01$) than the non-metritic group (Fig. 4). On D3, total bacterial 16S rRNA gene counts were not different ($P > 0.13$) among treatment groups (Fig. 4). On D6, the chitosan microparticles treatment had greater ($P \leq 0.05$) total bacterial 16S rRNA gene counts than the other groups, which were not different ($P > 0.60$) among themselves (Fig. 4). On D9, the chitosan microparticles treatment had greater ($P \leq 0.05$) total bacterial 16S rRNA gene counts than the untreated control and non-metritic groups, and not different ($P = 0.08$) from the ceftiofur treatment, whereas the ceftiofur treatment, untreated control, and non-metritic groups were not different ($P > 0.06$) among themselves (Fig. 6). On D12, there was no difference ($P > 0.35$) among the chitosan microparticles treatment, ceftiofur treatment and untreated control groups, but the chitosan microparticles treatment and

untreated control groups had greater ($P \leq 0.03$) total bacterial 16S rRNA gene counts than the non-metritic group, whereas there was no difference (P = 0.16) in total bacterial 16S rRNA gene counts between the ceftiofur treatment and the non-metritic group. These results showed that chitosan microparticles treatment delayed the progression of the uterine microbiome towards a healthy uterine microbiome.

Effect of Chitosan microparticles on *F. necrophorum* and *B. pyogenes in vitro*

Chitosan microparticles was able to inhibit the growth or to eliminate both *F. necrophorum* and *B. pyogenes* depending on the dose used (Fig. 5). For *F. necrophorum* (Fig. 5A), compared with control at 24 h, chitosan microparticles resulted in lesser ($P < 0.01$) bacterial concentrations at 0.1, 0.2 and 0.4%, although bacterial concentration at 0.1% chitosan microparticles increased ($P = 0.004$) from 0 to 24h, and bacterial concentration at 0.2% chitosan microparticles was unchanged ($P = 0.41$) from 0 to 24h. Bacterial concentration of *F. necrophorum* at 0.4% chitosan microparticles was greatly reduced ($P < 0.001$) from 0 to 24h. For *B. pyogenes* (Fig. 7B), compared with control at 24 h, chitosan microparticles resulted in lesser ($P < 0.001$) bacterial concentrations at 0.2 and 0.4%, but not at 0.1 ($P = 0.64$). Bacterial concentration at 0.1% chitosan microparticles increased ($P < 0.001$) from 0 to 24h, and was reduced ($P < 0.001$) at 0.2 and 0.4%. In summary, chitosan microparticles inhibited *F. necrophorum* growth at all concentrations, but was only able to eliminate *F. necrophorum* at a dose of 0.4% chitosan microparticles. In the case of *B. pyogenes*, chitosan microparticles was able to eliminate *B. pyogenes* at a dose of 0.2 and 0.4% chitosan microparticles, although a dose response was not observed.

Utilization of N-acetylglucosamine as a carbon source by *F. necrophorum* and *B. pyogenes*

The carbon source utilization assay showed that *F. necrophorum* and *B. pyogenes* did not preferentially utilize the N-acetylglucosamine as a carbon sources (Table S1). N-acetylglucosamine was utilized only minimally by *F. necrophorum* and not at all by *B. pyogenes*. Use of N-acetylglucosamine by *F. necrophorum* and *B. pyogenes* ranked 55th and 79th out of 95 carbon sources, respectively. The preferred carbon sources for *F. necrophorum* were L-glutamine, pyruvic acid methyl ester, L-alanyl-L-histidine, pyruvic acid, and β-hydroxybutyric acid (BHBA). The preferred carbon sources for *B. pyogenes* were urocanic acid, L-malic acid, D-galacturonic acid, L-glutamine, and fumaric acid. In summary, *F. necrophorum* did not preferentially utilize N-acetylglucosamine as a carbon source, and *B. pyogenes* was not able to utilize N-acetylglucosamine as a carbon source.

DISCUSSION

Previous studies showed that intrauterine infusion of chitosan microparticles was as effective as ceftiofur in reducing *E. coli* counts (12) and the relative abundance of *F. necrophorum*, and appeared to be even more effective than ceftiofur in reducing the relative abundance of Bacteroidetes (13); therefore, chitosan microparticles seemed to be a promising alternative to traditional antibiotics. Therefore, in this study we assessed the effectiveness of chitosan microparticles treatment in returning the uterine

microbiome of metritic cows to a healthy state compared with ceftiofur treatment and untreated controls.

First, we did not observe any difference among the metritic groups in microbial richness, diversity or evenness. However, PCoA and total bacterial count showed that, in contrast to our preliminary findings (12, 13), treatment with chitosan microparticles was less effective than treatment with ceftiofur in returning the uterine microbiome of metritic cows to a healthy state. In fact, untreated cows showed a faster recovery than chitosan microparticles-treated cows, which showed that chitosan microparticles hindered the progression of the uterine microbiome towards a healthy state. This could be clearly observed in the PCoA at the phylum and genus level on D3 and D6, and the total bacterial counts on D6 and D9. The LEfSe showed that differences between chitosan microparticles-treated and ceftiofur-treated and between chitosan microparticles-treated and untreated controls on D3 were mainly due to greater relative abundance of *Fusobacteria*, particularly *Fusobacterium* in chitosan microparticles-treated compared with ceftiofur-treated and untreated controls. *Fusobacterium* from the phylum *Fusobacteria* is one of the major pathogens associated with metritis in dairy cows (16); therefore, our data indicates that chitosan microparticles treatment altered the uterine microbiome in favor of *Fusobacterium.*

The negative effects of chitosan microparticles treatment on the uterine microbiome of metritic cows were unexpected because chitosan is categorized as GRAS (generally recognized as safe) for general use in foods by the United Stated Food and Drug Administration. Because chitosan is safe in humans, we expected it to be safe in animals. Nonetheless, in our larger study, we observed that chitosan

463 microparticles treatment resulted in increased culling (i.e. sold or died) from the herd in
464 the first 60 days postpartum (17). When we looked at the reasons for culling, chitosan
465 microparticles treatment resulted in a greater proportion of cows being culled because
466 of pelvic inflammation and peritonitis. Others have also reported increase inflammation
467 after infusion of chitosan hydrogel in the mammary gland of cows (28). Therefore, the
468 negative effects on the microbiome observed herein may have been a result of
469 exacerbated inflammation after chitosan microparticles administration. It is not clear why
470 the discrepancies between this study and our previous study evaluating the effect of
471 chitosan microparticles on the uterine microbiome (13), but it may be a result of having
472 a very small sample size in our earlier study (two to three cows per group), which could
473 have led to spurious observations. Another difference was the dose administered, which
474 was increased three fold in this study compared with our previous study. Hence, our
475 finding of delayed progression of the uterine microbiome towards a healthy uterine
476 microbiome, marked by a greater relative abundance of *Fusobacteria*, particularly
477 *Fusobacterium*, corroborates the finding of poorer recovery after chitosan microparticles
478 treatment. Some possible confounders that were not investigated in this study is the
479 effect of chitosan microparticles on viruses, protozoa, and fungi, and the interaction
480 between chitosan microparticles and mucin, which could have affected its overall effect
481 on the uterine microbiome. Therefore, these factors should be investigated in further
482 studies.

483 Because the efficacy of chitosan microparticles against *Fusobacterium* had not
484 been previously tested *in vitro,* we tested the efficacy of chitosan microparticles against
485 *F. necrophorum*, the main *Fusobacterium* species (16). We observed that although

chitosan microparticles inhibited *F. necrophorum* growth at all concentrations, chitosan microparticles was only able to significantly reduce *F. necrophorum* at a dose of 0.4%. Herein, we aimed for a concentration of 0.6% chitosan microparticles in the uterus because, although chitosan microparticles was effective at eliminating *E. coli* at a concentration of 0.2% chitosan microparticles *in vitro* (12), a dose of 0.6% chitosan microparticles was required *ex vivo* using uterine fluid from cows with metritis (18). Therefore, it is possible that a dose higher than 0.6% chitosan microparticles would be needed to significantly reduce *F. necrophorum in vivo*. In the case of *B. pyogenes*, chitosan microparticles was able to reduce *B. pyogenes* concentrations at a dose of 0.2%; therefore, a dose of 0.6% may have been able to inhibit its growth *in vivo*.

Because of the potential for conversion of chitosan microparticles into glucosamine and N-acetylglucosamine in the uterus, we evaluated if *F. necrophorum* could utilize N-acetylglucosamine as a carbon source; however, we observed that N-acetylglucosamine was not a preferred carbon source for *F. necrophorum*, although a minor utilization was observed. Similar to our findings, utilization of N-acetylglucosamine by a *F. necrophorum* strain isolated from sheep was not observed unless iron was restricted (29). Iron restriction is not expected to happen given the extensive tissue damage and hemolysis in the case of metritis. N-acetylglucosamine was not utilized by *B. pyogenes.* The Biolog AN MicroPlate does not include glucosamine or as a carbon source. However, because of the similarities and congruent metabolism of these amino sugars, it is unlikely that glucosamine could be utilized as a carbon source. Nonetheless, slower growth rate of *E. coli* on glucosamine compared with N-acetylglucosamine has been reported (30); therefore, further investigation is needed to

completely rule out the utilization of glucosamine by *F. necrophorum*. Finally, although *E. coli* can utilize glucosamine and N-acetylglucosamine as a carbon source, we did not observed any shift in the uterine microbiome towards greater abundance of *Escherichia* in the chitosan microparticles group; therefore, conversion of chitosan microparticles into glucosamine or N-acetylglucosamine or utilization of glucosamine or N-acetylglucosamine may be a minor contributor to for bacterial growth *in vivo*.

An interesting finding was the predilection of *F. necrophorum* for BHBA as an energy source. Cows undergo a period of negative energy balance around calving, which leads to lipid mobilization and increased production of BHBA in the liver, which end up in the blood circulation (31), hence can reach the uterus because of vascular degeneration postpartum (32). More specifically, cows that develop metritis have higher BHBA concentrations than healthy cows (33), which could be the reason for greater abundance of *Fusobacterium* in cows that develop metritis. The preference of *F. necrophorum* for L-glutamine and pyruvic acid seems to be of lesser relevance for its rapid growth in the uterus during early lactation because glutamine and pyruvic acid are actually decreased in early lactation because of mammary gland uptake for milk production (34, 35). Therefore, energy metabolites and their availability in the uterus may regulate uterine pathogen growth, and deserve further research.

We also observed that ceftiofur treatment hastened the progression of the uterine microbiome of metritic cows towards a healthy uterine microbiome. Using PCoA we observed that the ceftiofur treatment group was not different from the non-metritic group by D9, and was the closest to the non-metritic group by D12. We also observed that the ceftiofur treatment group was the only group in which the total bacterial count was not

532 different from the non-metritic group by D12. This finding corroborate the finding of
533 increased cure rate for the ceftiofur treatment compared with the chitosan microparticles
534 treatment and untreated controls by D12 (17). Interestingly, although an advantage for
535 the ceftiofur treatment in returning the uterine microbiome to heath could be observed,
536 the differences between ceftiofur and untreated were not as marked as one would
537 expect. This finding is intriguing, given the clear advantage observed in cure rates for
538 ceftiofur treatment compared with untreated controls in our companion study (17), and
539 in previous studies (9, 10). Nonetheless, our findings herein, agree with our previous
540 findings, where we showed only minor differences in the microbiome of ceftiofur-treated
541 and untreated cows (14). Therefore, it seems that even minor differences in the uterine
542 microbiome are associated with significant changes in cure rates.

543 Lastly, as it has been shown before (6, 16, 36-38), metritic cows had a greater
544 relative abundance of *Bacteroidetes*, particularly *Bacteroides* and *Porphyromonas* and
545 *Fusobacteria*, particularly *Fusobacterium*, whereas healthy cows had a greater relative
546 abundance of Proteobacteria and Tenericutes on D0. Similar to previous findings (6, 16,
547 39), we also showed that metritic cows had decreased richness (i.e. Chao1) on D0. On
548 the other hand, contrary to what we observed previously (16, 39), metritic cows were
549 shown to have increased diversity (i.e. Shannon), which was a result of having
550 increased evenness of the uterine microbiota compared with healthy cows. Others have
551 also shown no difference in richness (OTU number), diversity (Shannon) or evenness
552 (38), or even increased alpha diversity (i.e. measure not specified) in metritic cows
553 compared with healthy cows (37). It was clear from the PCoA that the uterine
554 microbiome of metritic cows on D0 was very homogeneous, whereas the uterine

555 microbiome of healthy cows was heterogeneous; therefore, our interpretation of
556 decreased richness but increased evenness in metritic cows is that the uterine
557 microbiome of metritic cows is consolidated into fewer genera with more similar relative
558 abundance than that of healthy cows.

559 To conclude, despite promising preliminary results, chitosan microparticles
560 treatment slowed the progression of the uterine microbiome towards a healthy uterine
561 microbiome; thus, failing as an alternative to traditional antibiotics. Therefore, our study
562 indicates with great degree of confidence that chitosan microparticles, as tested herein,
563 are detrimental to uterine health. As expected, but not previously reported, ceftiofur
564 treatment hastened the uterine microbiome progression towards a healthy uterine
565 microbiome. Therefore, we provide data on the expected shift in the uterine microbiome
566 when an ineffective and an effective treatment are administered. Finally, given the
567 continuous rise in antibiotic resistance, further research is needed to develop effective
568 alternative therapies to traditional antibiotics.

569

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712

713 **Figure 1.** Chao1 richness (A) index, Shannon diversity index (B), and evenness (C) of
714 the uterine microbiota at enrollment (D0), D3, D6, D9, and D12. CM (n = 21) =
715 intrauterine infusion of 24 g of chitosan microparticles at the time of diagnosis (D0), D2
716 and D4; CEF (n = 25) = subcutaneous injection of ceftiofur at D0 and D3; UNT (n = 23)

= no intrauterine or subcutaneous treatment; NME (n = 20) = non-metritic healthy cows matched with metritic cows based on days postpartum at metritis diagnosis. * Indicates significant ($P \leq 0.05$) differences between each group of metritic cows and the NME group. $^{+}$ Indicates significant ($P \leq 0.05$) differences between CEF and NME in A and between CM and NME in C.

Figure 2. Assessment of the overall effect of treatment group on the uterine microbiome (A), and its progression from enrollment (D0; B) to D3 (C), D6 (D), D9 (E), and D12 (F) using principal coordinate analysis of Bray-Curtis dissimilarity of relative abundance at the phylum level. CM (n = 21) = intrauterine infusion of 24 g of chitosan microparticles at the time of diagnosis (D0), D2 and D4; CEF (n = 25) = subcutaneous injection of ceftiofur at D0 and D3; UNT (n = 23) = no intrauterine or subcutaneous treatment; NME (n = 20) = non-metritic healthy cows matched with metritic cows based on days postpartum at metritis diagnosis. a, b, c, d letters indicate significant ($P \leq 0.05$) differences between treatment groups.

Figure 3. Linear discriminant analysis effect size (LEfSe) was used to determine the phyla most likely to explain differences between metritic cows and healthy cows (A), CM and CEF (B), and CM and UNT (C) three days after enrollment. A logarithm base 10 linear discriminant analysis (LDA) score cutoff of 3 was used. CM (n = 21) = intrauterine infusion of 24 g of chitosan microparticles at the time of diagnosis (D0), D2 and D4; CEF (n = 25) = subcutaneous injection of ceftiofur at D0 and D3; UNT (n = 23) = no

intrauterine or subcutaneous treatment; NME (n = 20) = non-metritic healthy cows matched with metritic cows based on days postpartum at metritis diagnosis.

Figure 4. Total uterine bacterial 16S rRNA gene copy number (Log_{10}) at enrollment (D0), D3, D6, D9, and D12. CM (n = 21) = intrauterine infusion of 24 g of chitosan microparticles at the time of diagnosis (D0), D2 and D4; CEF (n = 25) = subcutaneous injection of ceftiofur at D0 and D3; UNT (n = 23) = no intrauterine or subcutaneous treatment; NME (n = 20) = non-metritic healthy cows matched with metritic cows based on days postpartum at metritis diagnosis. * Indicates significant ($P \leq 0.05$) differences between each group of metritic cows and the NME group. $^{+}$ Indicates significant ($P \leq 0.05$) differences between CM and all the other groups. ‡ Indicates significant ($P \leq 0.05$) differences between CM and UNT, and between CM and NME. † Indicates significant ($P \leq 0.05$) differences between CM and NME and between UNT and NME.

Figure 5. *In vitro* Bacterial count (Log_{10}) in chopped meat broth (Anaerobe Systems, Morgan Hill, CA) containing 0.0%, 0.1%, 0.2%, and 0.4% chitosan microparticles (CM) at 0 and 24h in culture. a, b, c, d letters indicate significant ($P \leq 0.05$) differences between chitosan microparticles concentrations at 24h. * Indicates significant ($P \leq 0.05$) differences between 0 and 24h for each CM concentration.

Figure 1

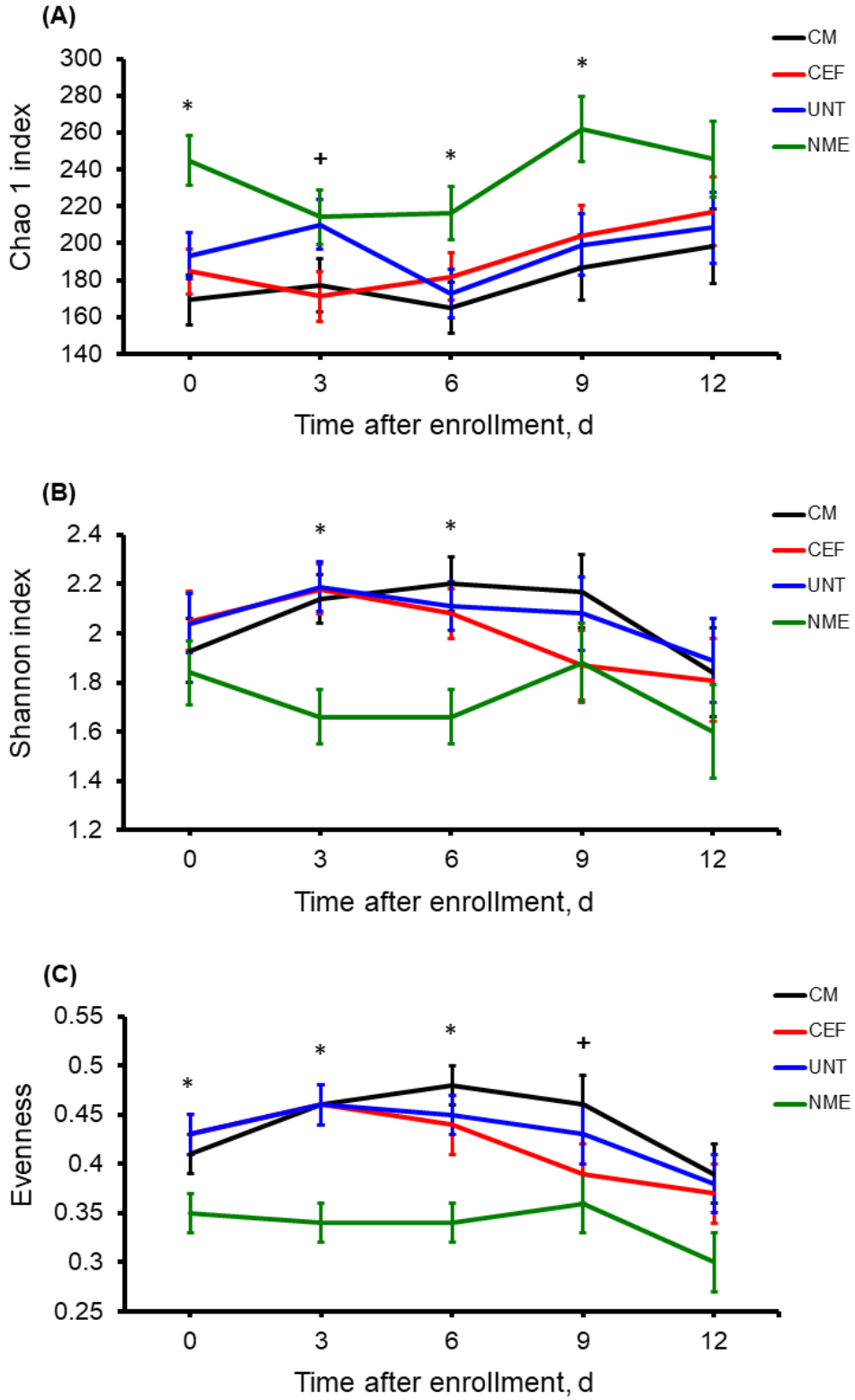

Figure 2

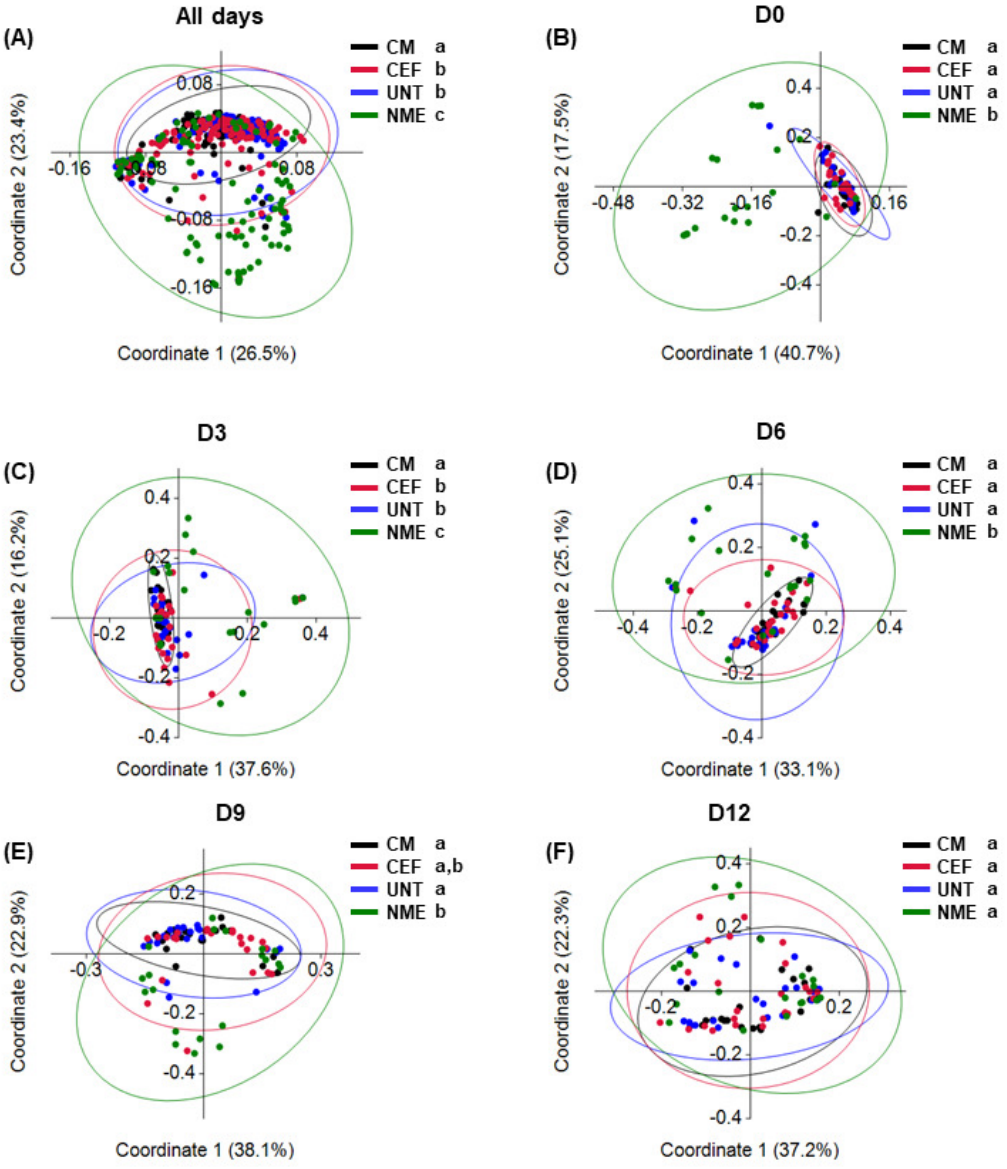

Figure 3

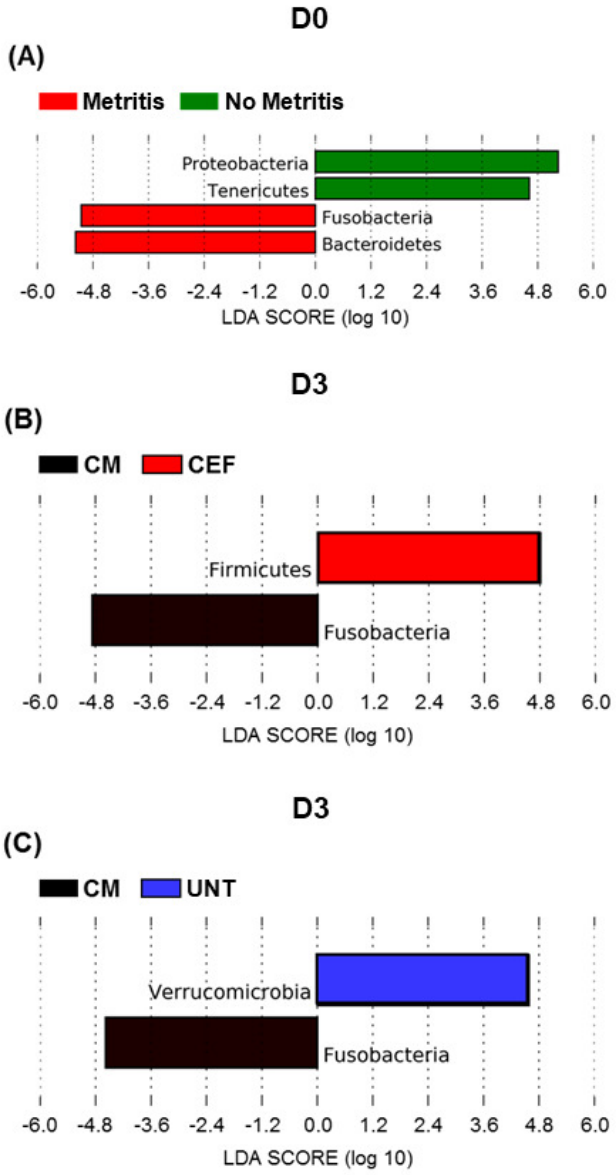

Figure 4

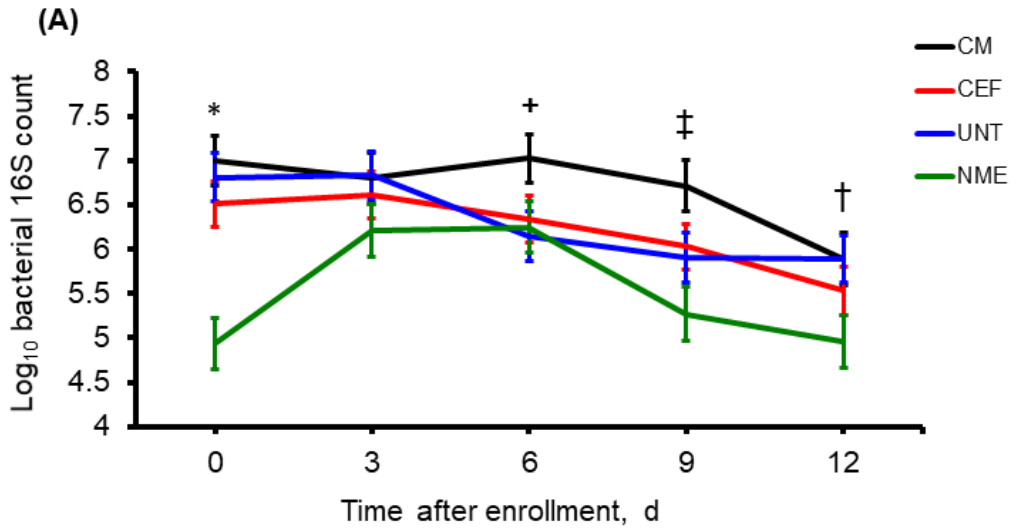

Figure 5