Application of chitosan microparticles for treatment of metritis and *in vivo*
evaluation of broad spectrum antimicrobial activity in cow uteri

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Abstract

Uterine disease such as metritis is associated with multiple bacterial infections in the uteri after parturition. However, treatment of metritis is challenging due to considerably high antibiotic treatment failure rate with unknown reason. Recently, chitosan microparticles (CM) have been developed to exert broad spectrum antimicrobial activity against bacterial pathogens, including multi-drug resistant bacteria, without raising CM resistant mutants. In this study, we tested, using metagenomics analysis, if CM maintain strong antimicrobial activity against pathogenic bacteria such as *Fusobacteriaceae* and *Bacteroidaceae* in cow uteri and evaluated CM's potency as an alternative antimicrobial agent to cure metritis in cows. Here, we report that efficacy of CM treatment for metritis was comparable to the antibiotic ceftiofur, and CM greatly altered uterine microflora of sick animals to healthy uterine microflora. Among uterine bacteria, CM significantly decreased *Fusobacterium necrophorum*, which is known pathogenic bacteria within the uterus. Taken together, we observed the broad spectrum antimicrobial activity of CM *in vivo* with an animal model, and further evaluated treatment efficacy in cows with metritis, providing insights into promising use of CM as an alternative antimicrobial agent for controlling uterine disease.

Keywords: chitosan microparticles, uterine microflora, metagenomics, metritis, antimicrobial activity

Introduction

Uterine disease such as metritis is a major concern in the dairy industry [1]. Metritis, an acute inflammatory disease of multiple layers of the uterine lining with systemic implications, affects 20% to 40% of the postpartum dairy cows, with the incidence ranging from 8% to more than 40% in some farms. Metritis is characterized by the presence of fetid red-brownish uterine discharge and it has marked welfare, health, production, reproduction, and economic consequences [2-5]. Although intrauterine pathogenic *E. coli* (IUPEC) in the first week after parturition plays especially important roles in stimulating inflammation through lipopolysaccharides as well as supporting the growth of other bacteria, uterine disease is associated with multiple bacterial infections, including *Fusobacterium necrophorum*, *Arcanobacterium pyogenes* (renamed *Truuperella pyotenes*), *Prevotella melaninogenica*, and *Bacteroidetes* spp. [6-9]. In recent studies, metritis is shown to be highly associated with specific uterine microflora with abundance of *Bacteroidetes* [10] and *Fusobacteria* [11]. *Fusobacterium* spp. are known to produce several toxins including leukotoxin, endotoxin, and hemolysin [12], while *Bacteroides* spp. are thought to have mechanisms for antibiotic resistance and avoidance of phagocytosis [13]. Due to the fact that multiple pathogens are associated with this incidence, it is believed that antibiotic treatment against metritis is challenging and that may cause high rate of antibiotic treatment failure at 30% for this disease [14]. Therefore, it is an urgent need to develop effective treatment methods.

Although advanced antibiotic discovery programs including genomics, high-tech chemical approaches, and high-throughput screening methods have been applied to develop new antibiotics, the progress has slowed down considerably. Nano- and micro-materials have provided potential for treatment of diseases caused by antimicrobial resistant microorganisms.

68 Metallic nanoparticles (NPs), such as Ag, ZnO, TiO₂, Au, Cu, or Al, have been shown to kill
69 bacteria by damaging cellular components such as the cell wall and membrane, or inhibit enzyme
70 activity and DNA synthesis [15]. However, insufficient risk assessment for potential toxicity of
71 metallic NPs might have limited for clinical use [16].

72 Chitosan is a linear polysaccharide composed of randomly distributed β -(1-4)-linked D-
73 glucosamine and N-acetyl-D-glucosamine and is produced commercially by deacetylation of
74 chitin, which is the structural element in the exoskeleton of crustaceans, and cell walls of fungi
75 [17-23]. Due to its low toxicity, biocompatibility, and biodegradability, chitosan is widely used
76 in many areas including food, pharmaceutical, textile, agriculture, water treatment, and cosmetics
77 industries [24]. Japan and South Korea have approved chitosan for generally recognized as safe
78 (GRAS) status for the use of chitosan as a food additive since 1983 and 1995, respectively [25].
79 Of the interests, chitosan has an antimicrobial activity against bacteria, fungi, and yeasts [17-23].
80 Although the mechanisms of the antimicrobial activity are not clearly understood, it is widely
81 accepted that the bacterial membrane permeability is altered by interaction with positively
82 charged chitosan and negatively charged bacterial surface molecules, resulting in intracellular
83 component leakage that leads to cell death. However, due to the loss of positive charges on the
84 amino group of chitosan, the antimicrobial activity of chitosan is not effective in an environment
85 at neutral pH such as the uterus [19, 26, 27].

86 We have developed chitosan microparticles (CM), derived from chitosan by ionic cross-
87 linking, and reported that CM exert antimicrobial activity against various pathogens, including
88 clinically important antibiotic resistant pathogens with strong efficacy even at neutral pH where
89 chitosan lose antimicrobial activity [28, 29]. CM likely bind to the outer membrane protein
90 OmpA via hydrogen bonding and LPS via ionic interaction to kill bacteria [28]. In addition to the

broad spectrum antimicrobial activity, CM do not raise resistant mutants over a passage of 15 days and do not cause cross-resistance, which leads to multi-drug resistance and is a common problem for many other antibiotics [29]. Therefore, we hypothesized that CM may be a potential alternative antimicrobial agent that can be used to treat infectious disease caused by multi-drug resistant microorganisms and multiple causative agents including *E. coli*, *Bacteroides* spp., and *Fusobacterium* spp.. In this study, we tested if CM can exert strong broad spectrum antimicrobial activity in cow uteri and evaluated its potential use as an alternative antimicrobial agent to cure metritis in an animal model.

Materials and Methods

Preparation of chitosan microparticles

A chitosan solution (0.25%) was prepared with a low molecular weight, 75-85% deacetylated chitosan (Sigma-Aldrich, St. Louis, MO) dissolved in acidic solutions containing 2% acetic acid (v/v) and 1% Tween 80 (v/v). For cross-linking the chitosan, 10% of sodium sulfate (w/v) was added to the chitosan solution until the solution became cloudy. The solution was then sonicated for 20 min. After sonication, the cross-linked chitosan (CM) was collected by centrifugation at $8,200 \times g$ for 10 min and washed with MiliQ water three times. The weight of CM was measured after freeze-drying.

In vitro antimicrobial activity of chitosan microparticles

Uterine fluid was collected from a dairy cow with clinical metritis and plated on CHROMagar *E. coli* (CHROMagar, Paris, France) to ensure there was no endogenous intrauterine pathogenic *E. coli* (IUPEC) in the uterine fluid. A single colony of an IUPEC strain [30] was inoculated in 5 ml of LB and incubated at 37°C with shaking at 200 rpm overnight. The next day, the culture was diluted 1:100 into fresh LB and incubated again to reach $OD_{600} = 1.0$. Approximately 5×10^4 CFU/ml of bacteria were inoculated into 2 mL of the uterine fluid containing different concentrations of CM (0%, 0.2%, 0.4%, 0.6%, and 0.8%) and incubated at 37°C for 24 h. Lastly, cultures were diluted and plated on CHROMagar *E. coli* to count CFU.

Animal management

All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC Protocol #: 201207405). Dairy cows used in this experiment

were housed in freestall barns equipped with fans and sprinklers that were activated when the ambient temperature exceeded 18.33°C. Barns were cleaned twice daily, and the freestalls were bedded with sand twice a week. Dairy cows were fed twice daily with a mixed ration formulated to meet or exceed the nutrient requirements of a lactating Holstein cow weighing 650 Kg and producing 45 Kg of 3.5% fat-corrected milk per day.

Treatment and sampling

Dairy cows with metritis (n= 30) were randomly assigned to three groups. One group received 8 g of CM dissolved in 10 ml of sterile water via intrauterine infusion (CM, n = 10). CM was set to the concentration of 0.2% (w/v) based on about 4 L of bovine uterus fluid because in the *in vitro* test, 0.2% CM showed the best antimicrobial activity to eliminate IUPEC in LB broth [28]. Another group received 2.2 mg/kg of ceftiofur hydrochloride (Excenel RTU sterile suspension, Zoetis, Madison, NJ) via intramuscular injection according to the U.S. Food and Drug Administration guidelines (Cef, n = 10). Treatments were conducted daily for five days from 0 to 4 days after diagnosis of metritis. As a control, the other group was not treated during the experiment (NT, n = 10). Two cows in CM and one in NT developed mastitis during the evaluation period and were treated with intramammary antibiotics, and hence were excluded from the trial. Therefore, the final sample size was 8, 10, and 9 for CM, Cef, and NT, respectively. Uterine swab samples were collected for 7 days from 0 to 6 days after diagnosis of metritis using the following procedure. Briefly, the perineum area of dairy cows was disinfected with 70% ethanol and a sterile pipette containing a sterile cotton swab (Har-Vet™ McCullough Double-Guarded Uterine Culture Swab, Spring Valley, WI) was introduced to the cranial vagina. To avoid vaginal contamination of the swab, the plastic sheath containing the pipette was directed into the cervix and then uterus. The plastic pipette was then protruded through the

plastic sheath, then the sterile swab within the plastic pipette was exposed and rolled against the uterine wall. The swab was pulled inside the pipette then the pipette pulled inside the plastic sheath and removed from the cow, avoiding contamination by vaginal fluid. Swabs were transferred to a 15 mL tube on ice and delivered to the laboratory within 4 h.

Rectal temperature and clinical cure rate

Rectal temperature, vaginal discharge score and abnormal clinical signs (including dehydration, anorexia, weakness, or severe depression) were examined by a veterinarian and recorded daily at the same time of day from day 0 to 11. The vaginal discharge scores were identified as described previously [14]. A cow with rectal temperature $< 39.5^{\circ}\text{C}$ and vaginal discharge score ≤ 3 was considered clinically cured. The cure rate of each treatment was recorded on day 6 and 11.

Bacterial culture

Swabs delivered from the dairy farm were directly suspended in 1 ml of 0.1% (w/v) peptone. To enumerate IUPEC, 200 μl of cell suspension was serially diluted up to 10^{-5} and plated on CHROMagar *E. coli* in duplicates. After incubation for 12 h at 37°C , blue colonies on CHROMagar *E. coli* were counted as IUPEC strains. For metagenomic sequencing, the rest of the cell suspension was frozen at -80°C .

DNA extraction and pyrosequencing

Cows that were positive for IUPEC at the day of calving were used for metagenomic sequencing. Samples ($n = 21$) collected at 0, 3, and 6 days after diagnosis of metritis from two cows in the CM group, three cows in the Cef group, and two cows in the NT group were thawed

on ice and spun at 14,000 rpm for 5 min to collect pellets. The pellets were re-suspended in 0.5 ml of TE buffer (10 mM Tris, 50 mM EDTA, pH 8) and incubated on ice for 5 min. To increase the extraction of nucleic acids from Gram-negative and Gram-positive bacteria, bead beating was conducted with an equal volume of 0.1-mm-diameter glass beads (Scientific Industries, Inc., Bohemia, NY) for 1 min at maximum speed using a BioSpec Mini-Bead Beater (Biospec Products Inc., Bartlesville, OK) and then cooled on ice for 1 min. This step was repeated three times. After bead-beating, cell lysates were collected by centrifuging at 12,000 rpm for 1 min. Genomic DNA was extracted from the cell lysates using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacture's protocol. Briefly, cell lysates were added with 20 µl of Proteinase K and then incubated at 56°C for 10 min. To remove RNA, 4 µl of RNase A (100 mg/ml) was added and incubated for 2 min at room temperature. Subsequently, 100 µl of AL Buffer was added to the samples and incubated at 70°C for 10 min, followed by adding 200 µl of 100% ethanol. The mixture from the previous step went through the QIAamp Mini spin column in a 2-ml tube, washed with 500 µl of AW1 and AW2 buffer, and eluted by 200 µl of AE buffer. The purity and concentration of genomic DNA were measured by a spectrophotometer (Nanodrop® 1000, Thermo Scientific, Waltham, MA) at wavelengths of 230, 260, and 280 nm.

The variable regions V1 through V3 of the 16S rRNA gene were amplified with primers 27F (5' AGRGTTTGATCMTGGCTCAG) and 519R (5' GTNTTACNGCGGCKGCTG) complemented with a linker primer sequence (5' AGRGTTTGATCMTGGCTCAG) and barcode sequence (5' AGACGAGT) using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA). PCR was carried out with an initial denaturing step at 94°C for 3 min, followed by 28 cycles of 94°C for 30 sec, 53°C for 40 sec, and 72°C for 1 min, and a final elongation step at 72°C for 5

min. PCR amplicons from different samples were mixed in equal concentrations, purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, Beverly, MA, USA), and sequenced on the Roche 454 FLX titanium instruments (Roche Applied Science, Indianapolis, IN) according to the manufacturer's guidelines.

Quality filtering and classification

After sequencing, reads were trimmed based upon quality scores 25 and binned into individual samples using a proprietary analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX). Short reads less than 200 bp, reads with ambiguous base calls, reads with homopolymer greater than 6 bp, and chimeras were discarded in samples. Operational taxonomic units (OTUs) were defined at 97% similarity after eliminating singleton sequences, and bacterial classification was conducted using BLASTn against a curated GreenGenes database [31]. Reads with > 97% identity to the reference sequence were designated at the species level, reads between 95% and 97% identity were designated at the genus level, reads between 90% and 95% identity were designated at the family level, reads between 85% and 90% identity were designated at the order level, reads between 80% and 85% identity were designated at the class level, and reads between 77% and 80% identity were designated at the phylum level [32-37]. The relative abundance of each gene that maps to the designated taxonomic classification was obtained by dividing the similarity hits for an individual gene by the total hits against **entire the database for all samples.**

Statistical analysis

The log of the number of IUPEC was used to minimize the correlation between the mean and variance of the data and was analyzed under the JMP Pro 11. ***In vitro* antimicrobial activity**

219 against IUPEC was evaluated at different concentrations of CM, where each of CM
220 concentrations was compared with 0% of CM using the Dunnett's test. *In vivo* antimicrobial
221 activity against IUPEC was analyzed by the generalized linear model which included the fixed
222 effects of treatments, days, and interaction between treatments and days. The number of IUPEC
223 are presented as means and standard error of the mean (SEM) in log transformation. The rectal
224 temperature was analyzed using the Mixed Procedure of SAS (Version 9.2 SAS Institute Inc.,
225 Cary, NC) and a statistical model that included treatment, days and the interaction. To observe
226 the overall structure of the uterine microflora in each group, the relative abundance of the
227 bacterial phyla, class, and order having greater than 1% was presented in stacked bars. Dunnett's
228 multiple comparisons was used to evaluate the relative abundance of the most abundant bacterial
229 phyla, class, and order at Day 3 and Day 6 against the Day 0 within each group. To observe the
230 dynamic change in abundance from the four most abundant families and the most abundant
231 species (*F. necrophorum*) in response to treatments, bacterial abundance at Day 0 was
232 normalized to 100%, and percentage change of bacterial abundance from 0 to 6 days after
233 diagnosis of metritis was calculated by the formula: Percentage change = (abundance at Day 6 –
234 abundance at Day 0) / abundance at Day 0 × 100%. A difference between the groups was
235 evaluated using Dunnett's multiple comparisons against the NT group at each time point. To
236 compare the susceptibility between pathogens and non-pathogens in response to CM and
237 ceftiofur, a percent to decimal of the pathogenic bacteria at the family level was examined in
238 each group at 0, 3, and 6 days after diagnosis of metritis. To visualize the relative abundance and
239 similarities among bacterial communities from each group at 0, 3, and 6 days after diagnosis of
240 metritis, a heat map and dendrogram were generated with the 15 most abundant species from all
241 samples based on Euclidean distance using the R software (<https://www.r-project.org/>). The

242 relative abundance of the 15 species was compared between Day 0 and Day 6 within each group
243 using the *t-test*. Principal coordinates analysis (PCoA) showed the relationships among bacterial
244 communities based on phylogenetic distance between OTUs. PCoA was generated by the
245 abundance weighted Unifrac metric (http://unifrac.colorado.edu/root?tool_id=pcoa). For all
246 statistical comparisons, differences with $P < 0.05$ were considered significant and differences
247 with $0.05 \leq P < 0.10$ were considered tendencies.
248

Results

In vitro and in vivo antimicrobial activity of CM

We generated chitosan microparticles (CM) as described previously; the diameter of the prepared CM was about 600 nm with a spherical shape which we analyzed by scanning electron microscopy [28]. We first evaluated the antimicrobial activity of CM *in vitro* using uterine fluid from a dairy cow with metritis to optimize the treatment dose before applying CM to an animal model. After 24 h incubation in the uterine fluid, 0.2% of CM decreased the concentration of intrauterine pathogenic *E. coli* (IUPEC) to less than 6 logs (4-log reduction compared to control), while 0.6% and 0.8% of CM could reduce IUPEC to less than 3 logs (Fig. 1A). When compared to 0% of CM, the log number of IUPEC was significantly lower ($P < 0.01$) in CM concentrations from 0.2% to 0.8%. The result showed that 0.2% was able to inhibit the growth of the pathogen in the uterine fluid significantly. Therefore, we decided to use 0.2% of CM to infuse into uteri of cows for further *in vivo* evaluation.

Because IUPEC is considered an important pathogen during early infection of the uterus, the antimicrobial activity of CM against IUPEC could be used as a critical indicator to evaluate if CM may treat metritis in cows. Since not all dairy cows with retained placenta were positive for IUPEC, we first screened IUPEC positive cows on day 0 to determine which animals would be selected for IUPEC counting during CM infusion into uteri. Three cows in the CM group, six cows in the antibiotic ceftiofur group, and two cows in the no-treatment (NT) group were positive for IUPEC. IUPEC was greatly decreased during CM treatment from Day 0 to Day 4 and completely removed in the uteri of CM-treated cows on Day 5 (Fig. 1B). This result indicates that CM have *in vivo* antimicrobial activity against IUPEC. Although there was no significant difference among groups at each time points, we found a significant effect in both treatments ($P = 0.01$) and days ($P < 0.01$), indicating that IUPEC decreased differentially as a

function of treatments and days. However, no significant difference was observed for the interaction between treatments and days ($P = 0.08$).

Clinical cure rate and rectal temperature

To investigate if CM can be used to treat metritis, the treatment efficacy of CM was compared with the antibiotic ceftiofur on rectal temperature reduction and clinical cure rate. For cure rate, on day 6 more than 20% of cows were cured in CM treatment whereas only 10% were cured with ceftiofur, indicating CM was slightly better than the antibiotic (Fig. 2A). However, on day 11, both CM and ceftiofur treatments showed similar efficacy above 60%. In addition, since the rectal temperature is used as a critical animal health indicator, it was measured for 12 days during treatment (Fig. 2B). A marginal difference ($P = 0.06$) between the CM and Cef groups was found at day 2, but no statistical difference was observed ($P = 0.34$) during treatment.

Diversity of uterine microflora

To investigate the overall effect of CM treatment against multiple pathogens in the uteri, we performed metagenomic analysis to evaluate microflora changes during treatment which allowed us to evaluate both aerobic and anaerobic bacteria. Uteri swab samples were collected in cows at 0, 3, and 6 days after diagnosis of metritis from each group. PCR amplified variable regions V1 through V3 of 16S rRNA genes, resulting in an average of 12,087.24 reads per sample and 1,889 OTUs from all samples. Rarefaction curves started to reach a plateau in all of the samples, suggesting that the number of sequences identified is sufficient to describe uterine microflora (data not shown). The Chao1 for species richness predicted 2,152 OTUs and the Shannon index for species diversity predicted an average of 4.21 in all samples. There was no significant difference in the number of reads, number of OTUs, and Chao1 among three groups,

but the Shannon index was significantly higher ($P < 0.05$) in the CM group than in the Cef group at Day 6. The number of reads, OTUs, Chao1, and Shannon index of individual samples are presented in Table 1.

Responses of bacterial phylotypes to CM, ceftiofur and no-treatment

Taxonomic analysis of the metagenomic analysis of the 16S rRNA genes revealed eight phyla through all samples (Fig. 3A), in which Bacteroidetes (51.61%), Fusobacteria (26.23%), Firmicutes (18.78 %), Proteobacteria (1.62%), and Actinobacteria (1.26%) were the major phyla, having an abundance greater than 1%; Tenericutes, Spirochaetes, and Planctomycetes were detected but were less frequent than the major phyla. CM treatment significantly decreased Bacteroidetes at Day 6 ($P = 0.03$) when compared to Day 0, but highly increased Firmicutes at Day 3 ($P = 0.04$) and Day 6 ($P = 0.02$). Fusobacteria seemed to be decreased by CM or ceftiofur treatment, but no significant difference was found among Day 0, 3, and 6 in the CM ($P > 0.13$) and Cef ($P > 0.11$) groups. On the other hand, in untreated cows, Fusobacteria and Firmicutes increased and Bacteroidetes decreased slightly at Day 6, with no significant difference when compared to Day 0. Bacterial class and order having greater than 1% of abundance are presented in Fig. 3B and 3C. Bacteroidia (51.58%) and Bacteroidales (51.45%) were the most abundant class and order in the phylum Bacteroidetes; Fusobacteria (26.23%) and Fusobacteriales (26.19%) were the most abundant class and order in the phylum Fusobacteria; Clostridia (18.23%) and Clostridiales (18.59%) were the most abundant class and order in the phylum Firmicutes. The responses of major bacteria in the class and order level to CM, ceftiofur, and no-treatment agreed with those in the phylum level (Fig. 3A).

To observe the dynamic change of uteri bacteria in response to CM, ceftiofur, and no-treatment, we analyzed the percentage change of the relative abundance in the four most

abundant families (Fig. 4A). *Porphyromonadaceae* (29.01%), *Fusobacteriaceae* (26.21%), *Bacteroidaceae* (20.20%), and *Clostridiaceae* (12.33%) were the four most abundant families in all samples, accounting for 87.75% of uterine microflora. In response to CM, *Fusobacteriaceae* and *Bacteroidaceae* decreased by 72.45% and 62.56% respectively, whereas *Porphyromonadaceae* and *Clostridiaceae* increased by 17.93% and 136.67%, respectively, at Day 6. In response to ceftiofur, *Fusobacteriaceae* only decreased by 54.85%. In multiple comparisons among groups using Dunnett's test, a significant difference ($P = 0.04$) between the CM and NT groups was observed in *Fusobacteriaceae* at Day 6, and a marginal difference ($P = 0.05$) was observed between the Cef and NT groups in *Fusobacteriaceae* at Day 6. Because *Fusobacteriaceae* and *Bacteroidaceae* belonging to a pathogenic group are more influenced by CM than by ceftiofur, CM seem to be more effective in controlling uterine pathogens than ceftiofur.

To further evaluate the susceptibility of uterine pathogens to CM and ceftiofur, we examined the proportion of pathogenic bacteria at the family level in each group from Day 0 to Day 6 (Fig. 4B). According to a previous study [6], *Fusobacteriaceae*, *Bacteroidaceae*, *Prevotellaceae*, *Staphylococcaceae*, *Streptococcaceae*, *Actinomycetaceae*, and *Pseudomonadaceae* were believed to be pathogens or potential pathogens associated with uterine disease in dairy cows. Therefore, the proportion of seven pathogenic families was examined as a percent to decimal (Fig. 4B). At Day 0, before cows received treatments, pathogenic bacteria were more abundant in all samples than non-pathogenic bacteria as they showed higher proportions of pathogenic bacteria above 0.5. The high proportion of pathogenic bacteria continued in untreated cows up to 6 days after diagnosis of metritis (from 0.65 to 0.57). However, the proportion of pathogenic bacteria dropped to 0.21 at Days 3 and Day 6 in response

to CM, indicating that the proportion of pathogenic bacteria decreased, while the proportion of non-pathogenic bacteria increased after CM treatment. This suggests that pathogenic bacteria are more susceptible to CM than non-pathogenic bacteria. Similarly, the proportion of pathogenic bacteria in cows treated with ceftiofur decreased from 0.65 to 0.40 up to 6 days after diagnosis of metritis. Although ceftiofur was also effective in reducing pathogenic bacteria in the uterus, it was not as effective as CM in reducing pathogenic bacteria. Finally, we investigated the response of *F. necrophorum* to CM, ceftiofur, and no-treatment (Fig. 4C) because *F. necrophorum* is a well-known pathogen causing uterine disease such as metritis and endometritis in dairy cows [6, 11, 38]. In response to CM, *F. necrophorum* decreased by an average of 68.31% and 45.51% by CM and ceftiofur, respectively, at Day 6; one cow from the ceftiofur group was excluded in the species level analysis of DNA sequence data because reads were classified into *Fusobacterium* spp.. Nevertheless, the abundance of *Fusobacterium* spp. was also decreased from 37.22% to 3.35% by ceftiofur treatment up to Day 6. In contrary to cows treated with either CM or ceftiofur, *F. necrophorum* was increased by 7.33% in untreated cows at Day 6 when compared to Day 0. According to the multiple comparisons among groups, abundance of *F. necrophorum* in the CM group was significantly lower ($P = 0.03$) than that in the NT group at Day 6, whereas there was no difference ($P = 0.30$) between the Cef and NT groups. A comparison between the CM and Cef groups at Day 3 and Day 6 showed no significant difference ($P > 0.52$).

CM alter uterine microflora

To visualize the bacterial abundance and similarity among treatment groups, we generated a heat map with a dendrogram using the 15 most abundant species having $\geq 1\%$ of abundance (Fig. 5A). *F. necrophorum* (23.43%) was the most abundant species in all samples, followed by *Porphyromonas levii* (12.95%), *Bacteroides heparinolyticus* (12.15%), and

368 *Porphyromonas endodontalis* (9.34%). *F. necrophorum* was similarly abundant in three groups
 369 at Day 0. However, *F. necrophorum* decreased in treated cows and, was significantly ($P = 0.03$)
 370 lower in the CM group at Day 6 when compared to the NT group at Day 6 as shown in Fig. 4C.
 371 The abundance of *P. levii* decreased from 32.02% to 8.13% by CM treatment, while it increased
 372 in the Cef and NT groups. The abundance of *B. heparinolyticus* decreased in all groups (29.60%
 373 to 0.60% in the CM group, 18.85% to 15.31% in the Cef group, and 14.76% to 3.33% in the NT
 374 group). On the other hand, the abundance of *P. endodontalis* increased after CM treatment,
 375 which indicates that *P. endodontalis* is less influenced by CM. The relative abundance of 15
 376 species at Day 0 and Day 6 is described in Table 2. The dendrogram showed three clusters at the
 377 distance of about 40: one cluster contained three nodes (NT-0, NT-3, and NT-6), one cluster
 378 contained four nodes (Cef-0, Cef-3, Cef-6, and CM-0), and another cluster contained two nodes
 379 (CM-3 and CM-6). This indicates that bacterial communities from the Cef and NT groups were
 380 similar within the groups, regardless of treatment and days. However, cows treated with CM
 381 shifted bacterial communities at Days 3 and 6, which were distant from the bacterial community
 382 at Day 0. To investigate relationships among bacterial communities based on the phylogenetic
 383 distance between OTUs, Principal coordinates analysis (PCoA) was performed using the
 384 abundance weighted Unifrac metric (Fig. 5B). The first principal component was the main axis
 385 with 90.17% of the variability in the data. The second principal component explained only 7.2%
 386 of the variation and thus it rarely contributed to the ordination. In PCoA, bacterial communities
 387 at Day 0 from the CM, Cef, and NT groups were similarly located by the first axis, indicating
 388 that uterine microflora were similar in all cows at Day 0 before cows received treatments.
 389 However, bacterial communities from cows treated with CM at Days 3 and 6 (CM-3 and CM-6)
 390 were remotely placed from the bacterial community from untreated cows at Day 0 (CM-0).

391 Consistent with the dendrogram, PCoA also indicates that uterine microflora is greatly altered in
392 response to CM ($P < 0.05$). On the other hand, the shift of uterine microflora caused by ceftiofur
393 was less when compared to CM.
394

Discussion

Here, we report that CM, derived from chitosan by ionic cross-linking, were effective to inhibit IUPEC both *in vitro* and *in vivo*. CM was as effective as the antibiotic ceftiofur in metritis treatment in high-risk cows. In addition, *F. necrophorum* (23.43%) was the most abundant species in all dairy cows with uterine disease, followed by *P. levii* (12.95%), *B. heparinolytica* (12.15%), and *P. endodontalis* (9.34%). *F. necrophorum* and *A. pyogenes*, which are etiological agents for uterine disease, were reduced by CM treatment, whereas non-pathogenic *P. endodontalis* was increased.

Metritis is a uterine disease characterized by an abnormally enlarged uterus and a fetid, red-brown uterine discharge within 21 days after calving [39]. Metritis is predicted to cost \$650 million annually based on 20% incidence of 8.5 million dairy cows in the US due to reduced milk yield, prolonged conception, increased culling and treatment cost [40, 41]. Ceftiofur, a third-generation cephalosporin, is commonly used to treat metritis. However, the efficacy of ceftiofur is dose-dependent and the cure rate of administration of the widely used concentration, 2.2 mg of ceftiofur equivalents/kg, were about 77% [14]. The reason of the 23% treatment failure is not well understood. Although it is not clear why high antibiotic treatment failure rate is reported with metritis in cows, it is reasonable to hypothesize that antimicrobial resistance is associated with this phenomenon since antibiotics have been used to prevent or treat uterine disease in cows [14, 42-45]. Third-generation cephalosporins, such as Ceftiofur hydrochloride (Excenel[®], Zoetis) and ceftiofur crystalline-free acid (Excede[®], Zoetis) and oxytetracycline Liqueamycin[®] LA-200[®] (Zoetis) are approved to treat metritis in cows. However, cephalosporins are widely used due to the long withdrawal time for milk (4 days) and meat (28 days) for Liqueamycin. Cephalosporin resistant microorganisms have steadily increased over the last

decade in cows [46], and ceftiofur resistance is widely spread because beta-lactamase gene, *bla* CMY-2, is encoded on plasmids and easily transferred to neighboring pathogens in animals [47, 48]. The prevalence of ceftiofur resistance in dairy cows with metritis is about 30 % among dairy cows (unpublished data, Ma et. al.), implying that the antibiotic treatment failure with ceftiofur is probably associated with antibiotic resistance.

Uterine infection is a health concern in women because it leads to puerperal endometritis. The estimates of initial visits to physicians for pelvic inflammatory disease was 88,000 visits in 2013 [49]. The prevalence of puerperal endometritis is highly influenced by the type of delivery, which ranged from 2-5% in vaginal deliveries up to 20-25% in the cesarean deliveries [50]. Similar to bacteria found in cattle with metritis, aerobic and anaerobic Gram-negative bacteria such as *E. coli* and *Bacteroides* spp. and Gram-positive bacteria such as *Streptococcus* spp. were commonly isolated in the amniotic fluid following cesarean section [51] and the endometrium [52]; therefore, we believe that there is great potential for using CM as an alternative human medicine.

According to the Centers for Disease Control and Prevention's report, approximately 23,000 people were killed in 2013 by antimicrobial resistant microorganisms in the US [53, 54]. Unfortunately, the number of people killed by antibiotic resistant microorganisms are expected to increase in the future due to insufficient methods of alternative therapies for infectious diseases caused by multi-drug resistant microorganisms. Nano- and micro-particles have been sought as potential alternatives due to diverse mechanisms to kill multi-drug resistant microorganisms, although they have not been extensively applied in human and animal clinics yet [55]. In this study, CM showed strong antimicrobial activity against IUPEC both *in vivo* and *in vitro*, indicating CM is a promising candidate to be used as an alternative of ceftiofur (Fig. 1).

In addition, in the analysis of cure rate and rectal temperature measurements of dairy cows with metritis, CM was as effective as ceftiofur (Fig. 2). These data indicate that CM remain effective *in vivo* animal models and are valuable to be further developed to treat bacterial infectious disease. It is beneficial to use CM as an alternative antimicrobial agent of ceftiofur to treat metritis. CM, like cationic antimicrobial peptides, have lower mutation rates due to their mechanism of breaking the bacterial cell membrane [29], while many antibiotics increase the mutation rate significantly [56]. In addition, continuous exposure of bacteria to sublethal concentrations of CM did not raise resistance for 15 days or cause cross-resistance [29]. One study showed that a lactose chitosan derivative reduced the concentration of *Staphylococcus aureus* at different cell ages (mid-exponential, late-exponential and late-stationary phase) by more than 3 logs [57]. Likewise, CM have significant antimicrobial activity against bacterial pathogens not only in log phase but also in stationary phase, which gives CM more potential than many other antibiotics as they can only target growing bacteria. Furthermore, chitosan is considered GRAS in many countries [25]. In an *in vitro* ruminal digestibility assay, CM did not disrupt the homeostasis and normal function of rumen, indicating CM may not show adverse side effects [29]. Therefore, CM are applicable and beneficial as a treatment option for metritis and other bacterial infectious disease.

Metagenomic analysis is a powerful tool to understand microbial communities. Many studies have examined microbiota in the feces and rumen of cattle to evaluate animal health, growth performance, and feed efficiency [34, 58]. Other studies have investigated uterine microbiota in uterine fluids to evaluate uterine disease and reproduction performance [38, 59]. We used metagenomic analysis to evaluate the effects of CM on alteration of uterine bacteria. The sequencing technique allowed us to understand overall microbial communities by being able

to detect anaerobic and fastidious bacteria simultaneously. In our study, the abundance of the uterine pathogenic bacteria (*P. levii*, *B. heparinolytica*, and *F. necrophorum*) decreased greatly in CM treatment whereas the relative abundance of *P. endodontalis* increased (Fig. 4 and Table 2), showing that CM restore uteri shifting to a healthy microflora. A similar alteration was observed with ceftiofur treatment, but the reduction of relative abundance of pathogens was not as effective as with CM treatment (Table 2). Santos *et al.* revealed five major bacteria phylums in uteri, including *Gammaproteobacteria*, *Firmicutes*, *Fusobacteria*, *Bacteroidetes*, and *Tenericutes*, in which *Fusobacteria* was more dominant in cows with metritis, which is consistent with our data, whereas phylum *Gammaproteobacteria* has higher prevalence in healthy cows [11]. Consistent with our data, Peng *et al.* described that in metritic cows, more abundance of *Bacteroidetes*, *Peptostreptococcus*, and *Fusobacterium* were observed on day 10 [60]. These reports suggest that the abundance of uterine bacteria is a critical indicator for health evaluation.

Conclusions

In summary, we generated CM from chitosan and infused them into the uteri of dairy cows to control bacterial pathogens. We confirmed a broad spectrum of antimicrobial activity of CM against pathogens causing metritis in cows. CM shifted uterine microflora by reducing pathogenic bacteria, including *F. necrophorum* and *A. pyogenes*. The antimicrobial activity of CM was positively associated with treatment of dairy cows with uterine disease. Taken together, engineered CM provide great promises as an alternative antimicrobial agent to control pathogens in animals to enhance animal and public health.

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Figure 1

Figure 2

Figure 3

694 **Figure 4**
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Figure 5

Figure Captions

Figure 1. CM have *in vitro* and *in vivo* antimicrobial activity against intrauterine pathogenic *E. coli* (IUPEC). (A) Antimicrobial activity of CM against inoculated IUPEC in the uterine fluid from dairy cows with metritis after 24 h incubation. Data are means $\pm$ SEM of three independent experiments and an asterisk indicates a significant difference when compared to 0% of CM ($P < 0.01$, Dunnett's test). (B) Dairy cows were treated for 6 days with chitosan microparticles (CM, $n = 8$), antibiotic ceftiofur (Cef, $n = 10$), or left without treatment (NT, $n = 9$). IUPEC from uteri swabs collected daily for seven days in each group was enumerated on CHROMagar. Each point indicates the mean values ($\log$) $\pm$ SEM. There is a significant effect of both treatments ($P = 0.02$) and days ($P < 0.01$) by the generalized linear model.

Figure 2. Cure rate and rectal temperature of cows treated with CM or antibiotic ceftiofur. (A) Cure rate (%) of cows treated with CM or ceftiofur on Day 6 and 11. (B) Rectal temperature ($^{\circ}\text{C}$) change of cows treated with CM, ceftiofur, or without treatment. Each point represents the mean $\pm$ SEM and an asterisk indicates a tendency between the CM and Cef groups at the given time point ($P = 0.06$).

Figure 3. The relative abundance of the uterine bacteria in response to chitosan microparticles (CM), ceftiofur (Cef), and no-treatment (NT) at the phylum (A), class (B), and order (C) levels. Relative abundance of bacteria having greater than 1% is presented in the stacked bars showing the mean percentage of reads assigned to the respective taxonomic group based on 16S rRNA sequences. Numbers 0, 3, and 6 indicate the days after diagnosis of metritis. Dunnett's multiple

comparison was used to evaluate a change in bacterial abundance compared with Day 0 within each group. An asterisk represents a significant difference at $P < 0.05$.

Figure 4. Pathogenic bacteria are more susceptible to CM than non-pathogenic bacteria. (A) The percentage change of the four most abundant bacterial families in response to chitosan microparticles (CM), ceftiofur (Cef), and no-treatment (NT) from 0 to 6 days after diagnosis of metritis. Dunnett's multiple comparison was used to compare treatment groups against the NT group at each time point. An asterisk inside the box indicates a significant difference ($P < 0.05$) and a cross inside the box indicates a tendency towards significance († , $0.05 \leq P < 0.10$), when compared to the NT group at Day 6. (B) Proportion of pathogenic bacteria to non-pathogenic bacteria at the family level. *Fusobacteriaceae*, *Bacteroidaceae*, *Prevotellaceae*, *Staphylococcaceae*, *Streptococcaceae*, *Actinomycetaceae*, and *Pseudomonadaceae* were assigned to a pathogenic group, and the rest were assigned to a non-pathogenic group. A percent to decimal of the pathogenic bacteria is presented in each group at 0 ($\square$), 3 (Δ), and 6 ($\circ$) days after diagnosis of metritis. (C) Percent change of *Fusobacterium necrophorum* in response to chitosan microparticles (CM), ceftiofur (Cef), and no-treatment (NT) from 0 to 6 days after diagnosis of metritis. *F. necrophorum* is an important pathogen causing uterine disease such as metritis and endometritis in dairy cows. Each point represents the mean percentage (%) $\pm$ SEM. An asterisk indicates a significant difference between the CM and NT groups at Day 6.

Figure 5. CM alter uterine microflora. (A) A heat map shows mean relative abundance of the 15 species in bacterial communities from the CM (chitosan microparticles), Cef (ceftiofur), and NT (no-treatment) groups at 0, 3, and 6 days after diagnosis of metritis. Columns represent each

751 bacterial community and rows represent the 15 most abundant species having greater than 1%.
752 The color of each cell indicates the relative abundance of bacterial species. The dendrogram at
753 the top of the heat map was generated based on Euclidean distance. (B) Principal coordinates
754 analysis (PCoA) showing the CM, Cef, and NT groups at 0, 3, and 6 days after diagnosis of
755 metritis using the weighted UniFrac distance. The percent of variation in the first and second
756 principal coordinates is indicated in the axis labels. Red circles represent the CM group, blue
757 squares represent the Cef group, and green triangles represent the NT group. Numbers 0, 3, and 6
758 in the symbol indicate the days after diagnosis of metritis.

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761 **Table 1.** Diversity indices of uterine microflora in dairy cows

Sample #	Treatment [†]	Day	No. of reads	No. of OTUs*	Shannon	Chao1
SJ1	Ceftiofur	0	22,730	711	4.34	899.88
SJ2	Ceftiofur	0	21,550	168	3.12	190.56
SJ3	Ceftiofur	0	15,988	480	4.00	573.08
SJ4	Ceftiofur	3	17,951	769	4.85	927.04
SJ5	Ceftiofur	3	2,854	153	3.56	204.36
SJ6	Ceftiofur	3	3,059	254	4.06	325.32
SJ7	Ceftiofur	6	3,495	303	4.16	413.68
SJ8	Ceftiofur	6	3,249	217	4.02	277.07
SJ9	Ceftiofur	6	12,590	451	4.12	569.03
SJ10	CM	0	13,499	458	4.04	559.67
SJ11	CM	0	5,953	320	3.82	475.04
SJ12	CM	3	12,935	592	4.82	721.73
SJ13	CM	3	10,218	451	4.61	575.76
SJ14	CM	6	14,647	622	4.95	746.26
SJ15	CM	6	4,626	318	4.63	472.90
SJ16	NT	0	8,226	333	4.03	403.31
SJ17	NT	0	15,950	557	4.39	679.12
SJ18	NT	3	23,741	461	3.74	512.14
SJ19	NT	3	16,362	664	4.52	860.84
SJ20	NT	6	8,811	316	4.03	374.86
SJ21	NT	6	15,398	687	4.70	823.95

762 [†]CM: chitosan microparticles; NT: no-treatment. *Sequences with >97% identity were assigned to operational
763 taxonomic units (OTUs).
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766 **Table 2.** The relative abundance of the 15 most abundant microbial species. A comparison was
767 made within each group between before (Day 0) and after (Day 6) treatment. Asterisks indicate
768 significant difference ($P < 0.05$).

Group	Pooled	Cef		CM		NT	
	Mean %, n = 7	Mean %, n = 3		Mean %, n = 2		Mean %, n = 2	
Bacterial species		Before	After	Before	After	Before	After
<i>Fusobacterium necrophorum</i>	23.43	26.78	16.54	15.28	4.18	36.28	39.10
<i>Porphyromonas levii</i>	12.95	4.69	24.93	32.02	8.13	0.20	9.27
<i>Bacteroides heparinolyticus</i>	12.15	18.85	15.31	29.60	0.60	14.76	3.33
<i>Porphyromonas endodontalis</i>	9.34	5.83	4.16	0.16	28.45	0.00	0.00
<i>Bacteroides denticanum</i>	4.47	1.40	0.20	0.32*	11.71*	7.30	4.94
<i>Porphyromonas somerae</i>	4.07	10.15	1.79	0.00	0.01	10.11	7.25
<i>Helcococcus ovis</i>	3.69	4.66	3.35	1.89	1.39	3.55	4.16
<i>Bacteroides pyogenes</i>	2.36	0.80	3.90	4.78	0.63	0.61	0.27
<i>Peptoniphilus asaccharolyticus</i>	2.31	1.88	1.82	3.40	2.80	1.36	3.20
<i>Fusobacterium spp.</i>	1.74	12.41	1.12	0.07	0.00	0.32	0.18
<i>Parvimonas micra</i>	1.63	0.04	1.53	0.89	0.06	3.57	4.55
<i>Prevotella spp.</i>	1.47	0.64	0.85	1.47	1.46	1.55	2.50
<i>Peptostreptococcus anaerobius</i>	1.30	0.33	1.86	0.76	1.97	0.22	1.31
<i>Sporanaerobacter acetigenes</i>	1.22	0.00	2.76	0.00	4.50	0.00	0.00
<i>Clostridiisalibacter bacterium</i>	1.13	0.41	0.75	0.20	3.40	0.00	0.43

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● / Pathogenic bacteria ● Chitosan microparticles (CM)