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Investigation of postpartum dairy cows' uterine microbial diversity using metagenomic pyrosequencing of the 16S rRNA gene

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ABSTRACT

The objective of this study was the use of metagenomic pyrosequencing of the 16S rRNA gene for the investigation of postpartum dairy cows' uterine bacterial diversity. The effect of subcutaneous supplementation of a trace mineral supplement containing Zn, Mn, Se, and Cu (Multimin North America, Inc., Fort Collins, CO) at 230 days of gestation and 260 days of gestation on dairy cows' uterine microbiota was also evaluated. Uterine lavage samples were collected at 35 DIM and were visually scored for the presence of purulent or mucopurulent secretion. The same samples were also used for the acquisition of bacterial DNA. The 16S rRNA genes were individually amplified from each sample. Pyrosequencing of the samples was carried at the Cornell University Life Sciences Core Laboratories Center using Roche 454 GS-FLX System Titanium Chemistry. The Ribosomal Database Project online tools were used for the analysis of the obtained sequences library. *Bacteroides* spp., *Ureaplasma* spp., *Fusobacterium* spp., *Peptostreptococcus* spp., *Sneathia* spp., *Prevotella* spp. and *Arcanobacterium* spp. prevalence was significantly ($P < 0.05$) higher in samples derived from cows that had a higher uterine lavage sample score. *Bacteroides* spp., *Ureaplasma* spp., *Fusobacterium* spp., and *Arcanobacterium* spp. prevalence was significantly ($P < 0.05$) higher in samples derived from cows that were not pregnant by 200 DIM. *Anaerococcus* spp., *Peptostreptococcus* spp., *Parabacteroides* spp., and *Propionibacterium* spp. prevalence was significantly ($P < 0.05$) lower in samples derived from cows that were trace mineral supplemented.

1. Introduction

Postpartum uterine diseases are important for both animal welfare and economic reasons, contributing to cow discomfort, elimination from the herd and impaired reproductive performance. Although presence of *Escherichia coli* and *Truperella pyogenes* has been more commonly associated with uterine inflammation and impaired reproductive performance (Bicalho et al., 2011), other pathogenic bacteria, such as *Fusobacterium necrophorum, Bacteroides* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Prevotella melaninogenicus* and *Streptococcus* spp. have also been associated with uterine diseases (Williams et al., 2005; Azawi, 2008; Santos et al., 2011).

Metagenomics refers to culture-independent studies of the collective set of genomes of mixed microbial communities. Barcoded pyrosequencing on the Genome Sequencer FLX/454 Life Sciences platform enable a dramatic increase in throughput via parallel in-depth analysis of many samples with limited sample processing and lower costs (Meyer et al., 2008); such an approach has not yet been used for the investigation of dairy cows' uterine microbial diversity.

Trace minerals play an important role in dairy cows' immune function, fertility and growth (Underwood and Suttle, 1999). Some positive effects of injectable trace minerals supplementation on cows' reproductive traits have already been shown (Harrison et al., 1984; Sales et al.,

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2011). However, the effect of systemic trace minerals supplementation on uterine microbiota remains unknown.

Therefore, the aim of this study was the use of metagenomic pyrosequencing of the 16S rRNA gene for the investigation of the uterine bacterial diversity and the evaluation of the effect of subcutaneous supplementation of a trace mineral supplement.

2. Materials and methods

2.1. Animals, treatment, case definitions and sample collection

Ninety seven primiparous Holstein cows kept in one dairy farm located near Ithaca, New York, were enrolled from September 16 of 2010 until June 30 of 2011. All cows were offered a total mixed ration (TMR) consisting of approximately 55% forage (corn silage, haylage, and wheat straw) and 45% concentrate (corn meal, soybean meal, canola, cotton seed, and citrus pulp) on a dry matter basis of the diet. The diets were formulated to meet or exceed the NRC nutrients requirements for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% fat corrected milk (FCM).

Pregnant heifers were randomly allocated into one of two treatments; trace mineral supplemented (TMS) or control. Randomization was completed in Excel (Microsoft, Redmond, WA) using the random number function and imported into the farms' Dairy Comp 305® program. Cows that were randomly assigned to the treatment group received 2 injections of trace minerals (Multimin North America, Inc., Fort Collins, CO) at approximately 230 days of gestation and 260 days of gestation; each injection contained 300 mg of zinc oxide, 50 mg of manganese carbonate, 25 mg of sodium selenite, and 75 mg of copper carbonate. Control cows were not injected with a negative placebo.

Signs of uterine inflammation were evaluated at 35 ± 3 DIM by visual inspection of a uterine lavage sample for the presence of purulent secretion as described by Machado et al. (2011). For the acquisition of a uterine lavage sample the cows were restrained, the perineum area was cleansed and disinfected with 70% ethanol, and a plastic infusion pipette was introduced into the cranial vagina and manipulated through the cervix into the uterus. A total of 20 ml of sterile saline solution was infused into the uterus and agitated gently, and a sample of the fluid was aspirated. The volume of recovered fluid ranged from 5 to 15 ml. All of the samples were visually scored by one investigator, who assessed the presence of a purulent or mucopurulent secretion in the uterine lavage sample. The score ranged from 0 to 2, with 0 indicating absence of a purulent or mucopurulent secretion in the lavage sample, 1 indicating a bloody but not purulent sample, and 2 the presence of pus in the lavage sample. The obtained uterine lavage sample was also used for the acquisition of uterine bacterial DNA.

Retained placenta (RP) was defined as a condition where cows failed to release their fetal membranes within 24 h of calving (Kelton et al., 1998). Metritis was diagnosed and treated by properly trained farm personnel that followed a specific diagnostic protocol designed by the staff of the Ambulatory and Production Medicine Clinic, Cornell University. Data regarding reproductive performance during the subsequent lactation were extracted from the farm's DairyComp 305® database (Valley Agricultural Software, Tulare, CA). Cows were right censored if not diagnosed as being pregnant before culling, death, or the end of the data collection period, which was at 200 DIM. This project proposal was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (# 2011-0111).

2.2. DNA extraction

Isolation of microbial genomic DNA was performed by using a QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions. Some modifications, such as the addition of 400 μg of lysozyme and incubation for 12 h at 56 °C, were included to maximize bacterial DNA extraction. The DNA concentration and purity were evaluated by optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 230, 260 and 280 nm.

2.3. PCR amplification of the V1-2 region of bacterial 16S rRNA genes

The 16S rRNA genes were individually amplified from each sample using a composite pair of primers containing unique 10-base barcode, which was used to tag the PCR products from respective samples. The forward primer used was 5′-**CGTATCGCCTCCCTCGCGCCATCAG**NNNNNNNNNNTC*AGAGTTTGATCCTGGCTCAG*-3′: the bold sequence is the GS FLX Titanium Primer A, and the italicized sequence is the universal broadly conserved bacterial primer 27F. The reversed primer used was 5′-**CTATGCGCCTTGCCAGCCCGCTCAG**NNNNNNNNNNCA*TGCTGCCTCCCGTAGGAGT*-3′: the bold sequence is the GS FLX Titanium Primer B, and the italicized sequence is the broad-range bacterial primer 338R. The sequence NNNNNNNNNN, which is identical in the forward and reverse primer of each pair, designates the unique 10-base barcode used to tag each PCR product. A two-base linker sequence (underlined) was inserted between the barcode and the template-specific sequence to help diminish any effect the composite primer might have on the efficiency of the amplifications. PCR were carried out in triplicates 20-μl reactions containing 0.3 μM forward and reverse primers, approximately 50 ng of template DNA and 10 μl HotStar Taq Plus Mix kit (Qiagen). A modified touchdown thermal cycling was used for amplification and consisted of initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing (starting at 68 °C and subsequently decreased by 2 °C/2 cycles until it reached 58 °C, temperature at which the 20 remaining cycles were performed) for 30 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 7 min. Replicate amplicons were pooled, purified with the QIAquick PCR Purification Kit (Qiagen), and visualized by electrophoresis using 1.2% (w/v) agarose gels stained with 0.5 μg/ml ethidium bromide before sequencing. Blank controls, in which no DNA was added to the reaction, were performed similarly and, since these failed to produce visible PCR products, they were not analyzed further.