Veterinary Microbiology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

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

V.S. Machado, G. Oikonomou, M.L.S. Bicalho, W.A. Knauer, R. Gilbert, R.C. Bicalho *

Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853-6401, United States

ARTICLE INFO

Article history:
Received 17 February 2012
Received in revised form 24 April 2012
Accepted 25 April 2012

Keywords:
Arcanobacterium pyogenes
Dairy cows
Endometritis
Trace minerals
Reproduction
Fusobacterium necrophorum
Pyrosequencing

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.,

* Corresponding author. Tel.: +1 607 253 3140; fax: +1 607 253 3982.
E-mail address: rcb28@cornell.edu (R.C. Bicalho).

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http://dx.doi.org/10.1016/j.vetmic.2012.04.033

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.

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2.4. Barcoded pyrosequencing of the bacterial 16S rRNA genes

Amplicons were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) and combined in equimolar ratios into a single tube with a final concentration of 16 ng/μl. Pyrosequencing of the samples was carried at the Cornell University Life Sciences Core Laboratories Center using Roche 454 GS-FLX System Titanium Chemistry.

2.5. Bioinformatics

The obtained FASTA sequences file was uploaded in the Ribosomal Database Project (RDP) pipeline initial processor that trimmed the 16S primers, tag sorted the sequences, and filtered out additional sequences of low-quality. RDP Classifier was used to assign 16S rRNA gene sequences of each sample to the new phylogenetically consistent higher-order bacterial taxonomy (Wang et al., 2007).

2.6. Statistical analysis

Comparisons between the mean prevalence of bacteria genera in samples from metritic or non-metritic cows, cows that suffered or not from RP, cows that conceived or did not conceive, cows with different uterine lavage sample scores, and from TMS or control cows were made with the ANOVA function of JMP®PRO9. Comparisons between the percentage of metritic and non-metritic cows, cows that suffered or not from RP, cows that conceived or did not conceive, cows with different uterine lavage sample scores, and TMS or control cows that were positive for each different bacterial genus were made with the chi-square function of JMP®PRO9.

3. Results

Average prevalence of each different bacterial genus in samples from cows with different uterine lavage sample scores as well as the percentage of cows with different uterine lavage sample scores that were positive for each genus are presented in Table 1. *Bacteroides* spp., *Ureaplasma* spp., *Fusobacterium* spp., *Peptostreptococcus* spp., *Sneathia* spp., *Prevotella* spp. and *Trueperella* spp. prevalence was significantly ($P < 0.05$) higher in samples derived from cows that had a higher uterine lavage sample score.

Average prevalence of each different bacterial genus in samples from metritic or non-metritic cows as well as the percentage of cows that were positive for each genus is presented in Table 2. *Bacteroides* spp. and *Ureaplasma* spp. prevalence was significantly ($P < 0.05$) higher in samples derived from cows that suffered from metritis, while *Geobacillus* spp. prevalence was higher in samples derived from the non-metritic cows. *Ureaplasma* spp. was present in 65% of the metritic cows while it was present only in 40% of the non-metritic cows ($P < 0.05$).

Average prevalence of each different bacterial genus in samples from cows with RP and cows without RP as well as the percentage of cows that were positive for each genus are presented in Table 3. *Mycoplasma* spp. and *Ureaplasma* spp. prevalence was significantly ($P < 0.05$) higher in samples derived from cows with RP. The percent of animals positive to these genera was also significantly ($P < 0.05$) higher in cows that had RP.

Average prevalence of each different bacterial genus in samples from cows that were pregnant by 200 DIM and cows that were not pregnant by 200 DIM as well as the percentage of cows that were positive for each genus are presented in Table 4. *Bacteroides* spp., *Ureaplasma* spp., *Fusobacterium* spp., and *Trueperella* spp. prevalence was significantly ($P < 0.05$) higher in samples derived from cows that were not pregnant by 200 DIM.

Average prevalence of each different bacterial genus in samples from TMS and control cows as well as the percentage of cows that were positive for each genus are presented in Table 5. *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.

4. Discussion

In general, results presented here highlight the importance of known pathogens already associated in previous studies with uterine health and reproductive performance. Additionally, using pyrosequencing we were able to evaluate not only the presence or absence of microorganisms but the relative abundance of each bacterium in each sample and thus obtain more quantified results.

Fusobacterium necrophorum has been associated with acute uterine infections, mostly during the second week postpartum (Azawi, 2008; Bicalho et al., 2011; Santos et al., 2011); while *Trueperella pyogenes* has been associated with chronic uterine infections, later on the lactation period (Bicalho et al., 2011). We show here that RP and metritis were not risk factors for higher proportion of positive cows and higher average prevalence of *Fusobacterium* spp. and *Trueperella* spp. in the samples; these bacteria were found to be associated with uterine infection at 35 DIM though. It should be noted here that phylogenetic analysis performed (data not shown) showed that sequences representing *Fusobacterium* spp. and *Trueperella* spp. in our samples were closely affiliated to *Fusobacterium necrophorum* and *Trueperella pyogenes* respectively. In a previous study, Bicalho et al. (2011) evaluated, using PCR, the relationship between specific virulence factors of *Trueperella pyogenes*, *Escherichia coli* and *Fusobacterium necrophorum*, and incidence of metritis and endometritis; and found that at 35 DIM, only *Arcanobacterium pyogenes* was associated with endometritis.

Presence or absence in the uterine lumen of *Fusobacterium* spp. and *Trueperella* spp. at 35 DIM was not associated with reproductive performance; however, the average prevalence of these bacteria was associated with a decrease in reproductive performance. The lack of association between the presence of these bacteria and reproduction performance was also observed by Bicalho et al. (2011). We show here that it is the relative abundance of intrauterine *Fusobacterium* spp. and *Trueperella* spp. at 35 DIM and not just their presence or

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Table 1
Percentage of cows with different uterine lavage sample scores that were positive for each genus and average prevalence of each different bacterial genus in samples from cows with different uterine lavage sample scores. Endometritis was scored from 0 to 2 according with severity; 0 = no endometritis ($n = 80$), 1 = mild endometritis ($n = 12$), and 2 = severe endometritis ($n = 5$). Values in bold indicate statistically significant difference.

Phylum	% of the cows tested positive				Average prevalence %			
	0	1	2	*P*-value	0	1	2	*P*-value
Firmicutes	100	92	100	0.13	**52.30**	**35.80**	**11.10**	<**0.01**
Genus								
Sporanaerobacter	**1**	**0**	**20**	**0.02**	<**0.01**	**0.00**	**0.05**	<**0.001**
Moryella	4	0	2	0.15	**0.01**	**0.00**	**0.10**	<**0.001**
Peptoniphilus	**18**	**16**	**100**	<**0.01**	0.05	0.04	0.20	0.10
Anaerococcus	20	16	40	0.52	0.07	0.10	0.30	0.08
Lactobacillus	41	50	20	0.49	0.40	0.20	<0.01	0.83
Anaerovibrio	37	50	40	0.71	**0.10**	**0.30**	**0.01**	**0.03**
Peptostreptococcus	26	25	60	0.25	**0.10**	**0.06**	**0.70**	<**0.01**
Clostridium	50	50	20	0.42	0.20	0.20	<0.01	0.40
Streptococcus	65	67	20	0.12	0.10	0.30	0.50	0.14
Helcococcus	**43**	**8**	**100**	<**0.01**	**0.30**	**0.01**	**0.40**	<**0.01**
Roseburia	57	50	20	0.25	0.08	0.20	0.30	0.10
Oscillibacter	78	75	40	0.14	1.31	1.00	0.01	0.14
Geobacillus	98	92	100	0.25	**13.00**	**9.30**	**0.40**	**0.02**
Staphylococcus	65	50	20	0.09	2.56	1.56	0.10	0.84
Bacteroidetes	100	100	100		**12.20**	**12.40**	**40.40**	<**0.01**
Genus								
Odoribacter	**5**	**0**	**40**	<**0.01**	0.01	0.00	0.03	0.79
Parabacteroides	27	33	0	0.18	0.04	0.06	0.00	0.48
Paludibacter	**47**	**42**	**0**	**0.04**	0.20	0.20	0.00	0.28
Prevotella	29	25	60	0.30	**0.10**	**0.03**	**1.00**	<**0.01**
Porphyromonas	47	33	60	0.53	0.30	0.80	1.00	0.05
Alistipes	67	75	20	0.07	0.90	0.80	<0.01	0.18
Bacteroides	36	33	80	0.13	**0.40**	**0.04**	**9.00**	< **0.01**
Proteobacteria	100	92	100	0.13	20.1	18.8	0.40	0.14
Genus								
Acidovorax	10	8	0	0.59	0.20	<0.01	0.00	0.75
Campylobacter	20	8	0	0.21	0.02	<0.01	0.00	0.30
Devosia	5	17	0	0.24	0.06	0.05	0.00	0.96
Escherichia/Shigella	46	41	20	0.48	0.30	0.10	0.01	0.29
Sphingopyxis	10	8	0	0.75	0.10	0.08	0.00	0.90
Proteus	54	58	20	0.31	0.80	0.20	<0.01	0.38
Acinetobacter	**84**	**67**	**20**	<**0.01**	0.60	0.20	0.01	0.12
Halomonas	87	83	60	0.23	1.00	0.30	0.03	0.05
Terenicutes	59	58	60	0.99	**7.00**	**23.20**	**24.30**	**0.02**
Genus								
Mycoplasma	14	25	0	0.37	0.70	4.90	0.00	0.21
Ureaplasma	45	50	60	0.78	**5.80**	**17.80**	**23.50**	**0.03**
Fusobacteria	73	58	100	0.11	**2.00**	**7.00**	**21.70**	<**0.01**
Genus								
Streptobacillus	16	8	0	0.49	0.20	0.02	0.00	0.61
Sneathia	30	41	40	0.67	**0.70**	**2.00**	**3.00**	**0.03**
Fusobacterium	**66**	**33**	**100**	**0.02**	**1.35**	**1.80**	**17.10**	<**0.01**
Actinobacteria	**100**	**92**	**80**	**0.02**	6.00	2.50	2.00	0.17
Genus								
Arcanobacterium	**21**	**25**	**80**	**0.01**	**0.10**	**0.07**	**2.0**	< **0.01**
Propionibacterium	**81**	**91**	**40**	**0.04**	0.10	0.40	0.60	0.08
Spirochaetes	39	20	0.00	0.07	0.17	0.12	0.00	0.47
Genus								
Treponema	**40**	**16**	**0.00**	**0.03**	0.10	0.08	0.00	0.40
Others	**61**	**42**	**0.00**	**0.01**	0.28	0.13	0.00	0.26
Genus								
Bacillariophyta	25	8	0.00	0.10	0.07	0.04	0.00	0.61
TM7_genera_incertae_sedis	32	25	0.00	0.28	0.07	0.03	0.00	0.49

absence in the uterus, which is associated with future reproductive performance.

RP is for long recognised as a risk factor for metritis and reduced reproductive efficiency (Benzaquen et al., 2007). However, in this study, RP did not seem to affect 35 DIM prevalence of bacteria that are traditionally correlated with endometritis and reproductive failure. The only significant effect that RP was found to have was on

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Mycoplasma spp. A higher proportion of cows that had RP were positive for *Mycoplasma* spp. while prevalence of *Mycoplasma* spp. was also higher in samples from cows that suffered from RP. Phylogenetic analysis (data not shown) of Mycoplasma spp. related OTU revealed its affiliation with *Mycoplasma bovigenitalium*.

Emphasis should be placed on the lack of association between *Escherichia/Shigella* spp. and uterine infection or

Table 2
Percentage of metritic ($n = 27$) and non-metritic ($n = 70$) cows that were positive for each different bacterial genus and average prevalence of each genus in samples from metritic or non-metritic cows. Values in bold indicate statistically significant difference.

Phylum	% of the cows tested positive			Average prevalence %		
	Non-metritis	Metritis	*P*-value	Non-metritis	Metritis	*P*-value
Firmicutes	98	100	0.44	**51.70**	**36.80**	<**0.01**
Genus						
Sporanaerobacter	1	4	0.42	<0.01	0.01	0.29
Moryella	**1**	**13**	<**0.01**	<0.01	0.04	0.05
Peptoniphilus	21	30	0.35	0.06	0.05	0.85
Anaerococcus	19	26	0.50	0.07	0.13	0.28
Lactobacillus	40	39	0.92	0.46	0.13	0.49
Anaerovibrio	39	43	0.69	0.15	0.06	0.164
Peptostreptococcus	28	35	0.56	0.11	0.24	0.14
Clostridium	46	52	0.62	0.16	0.28	0.27
Streptococcus	66	57	0.43	0.60	0.40	0.46
Helcococcus	42	52	0.39	0.34	0.48	0.59
Roseburia	55	52	0.80	0.42	0.41	0.93
Oscillibacter	76	83	0.52	1.20	1.40	0.49
Geobacillus	97	100	0.40	**13.70**	**8.03**	**0.04**
Staphylococcus	63	57	0.60	2.70	2.30	0.43
Bacteroidetes	100	100		12.70	18.90	0.07
Genus						
Odoribacter	4	13	0.15	<0.01	0.04	0.06
Parabacteroides	25	35	0.38	0.04	0.06	0.60
Paludibacter	42	52	0.39	0.15	0.25	0.09
Prevotella	31	35	0.76	0.13	0.34	0.27
Porphyromonas	43	61	0.14	0.60	1.50	0.22
Alistipes	66	70	0.73	0.80	0.90	0.68
Bacteroides	34	57	0.06	**0.34**	**2.42**	**0.03**
Proteobacteria	98	100	0.44	20.30	10.50	0.05
Genus						
Acidovorax	7	9	0.85	0.01	0.06	0.08
Campylobacter	15	26	0.23	0.02	0.03	0.32
Devosia	**1**	**17**	<**0.01**	<0.01	0.19	0.07
Escherichia/Shigella	49	30	0.12	0.30	0.12	0.16
Sphingopyxis	7	9	0.85	0.04	0.35	0.14
Proteus	54	57	0.82	0.71	0.27	0.24
Acinetobacter	79	70	0.35	0.49	0.54	0.79
Halomonas	84	87	0.70	0.80	0.80	0.83
Terenicutes	52	74	0.06	5.70	24.40	<0.01
Genus						
Mycoplasma	10	26	0.07	0.90	2.60	0.38
Ureaplasma	**40**	**65**	**0.04**	**4.50**	**20.70**	<**0.01**
Fusobacteria	75	74	0.95	3.40	5.10	0.53
Genus						
Streptobacillus	16	4	0.14	0.23	0.02	0.23
Sneathia	37	22	0.17	1.10	1.10	0.99
Fusobacterium	66	65	0.97	1.88	3.78	0.20
Actinobacteria	98	96	0.45	5.90	3.90	0.26
Genus						
Arcanobacterium	21	30	0.35	0.16	0.30	0.42
Propionibacterium	79	87	0.41	1.04	1.01	0.92
Spirochaetes	34	48	0.25	0.12	0.25	0.06
Genus						
Treponema	34	43	0.43	0.10	0.22	0.09
Others	60	39	0.09	0.24	0.09	0.06
Genus						
Bacillariophyta	25	17	0.43	0.08	0.03	0.25
TM7_genera_incertae_sedis	31	26	0.63	0.07	0.02	0.18

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reproductive performance that is reported here. *Escherichia coli* has been associated with uterine infections (Bicalho et al., 2010; Sheldon et al., 2010) and was reported to have an adverse effect on reproductive performance (Bicalho et al., 2011; Machado et al., 2012). However, recent studies have shown that presence of *E. coli* in the uterus at 35 DIM is not important for uterine disease and reproductive performance. It is the presence of *Escherichia coli* in the uterus during the first week after parturition that is related to uterine disease and may negatively affect reproduction

Table 3
Percentage of cows with retained placenta (RP, $n = 5$) and without RP ($n = 92$) that were positive for each different bacterial genus and average prevalence of each genus in samples from cows with RP and cows without RP. Values in bold indicate statistically significant difference.

Phylum	% of the cows tested positive			Average prevalence %		
	Non-RP	RP	*P*-value	Non-RP	RP	*P*-value
Firmicutes	99	100	0.76	48.10	43.60	0.72
Genus						
Sporanaerobacter	2	0	0.76	<0.01	0.00	0.76
Moryella	5	0	0.66	0.02	0.00	0.71
Peptoniphilus	23	25	0.94	0.06	0.02	0.54
Anaerococcus	22	0	0.29	0.08	0.00	0.49
Lactobacillus	39	50	0.68	0.38	0.28	0.91
Anaerovibrio	39	50	0.68	0.13	0.06	0.59
Peptostreptococcus	30	25	0.82	0.15	0.04	0.57
Clostridium	48	50	0.93	0.18	0.17	0.11
Streptococcus	63	75	0.62	0.62	0.15	0.44
Helcococcus	44	50	0.82	0.37	0.50	0.82
Roseburia	55	50	0.85	0.41	0.58	0.65
Oscillibacter	78	75	0.89	1.20	2.24	0.17
Geobacillus	98	100	0.76	1.30	5.90	0.36
Staphylococcus	62	50	0.64	2.30	0.17	0.71
Bacteroidetes	100	100		14.40	11.30	0.66
Genus						
Odoribacter	7	0	0.58	0.01	0.00	0.76
Parabacteroides	**26**	**75**	**0.03**	0.04	0.14	0.05
Paludibacter	44	50	0.82	0.17	0.21	0.73
Prevotella	33	25	0.75	0.19	0.04	0.70
Porphyromonas	48	50	0.93	0.88	0.11	0.63
Alistipes	66	75	0.72	0.83	1.02	0.72
Bacteroides	39	50	0.68	0.91	0.10	0.70
Proteobacteria	99	100	0.76	18.50	3.80	0.17
Genus						
Acidovorax	8	0	0.55	0.02	0.00	0.72
Campylobacter	16	50	0.08	0.02	0.02	0.83
Devosia	5	25	0.08	0.05	0.02	0.87
Escherichia/Shigella	46	0	0.07	0.27	0.00	0.34
Sphingopyxis	8	0	0.55	0.12	0.00	0.78
Proteus	52	100	0.06	0.62	0.11	0.51
Acinetobacter	77	75	0.94	0.10	0.45	0.42
Halomonas	85	75	0.59	0.86	0.15	0.23
Terenicutes	**56**	**100**	**0.03**	**9.20**	**37.90**	**0.02**
Genus						
Mycoplasma	**13**	**50**	**0.04**	**0.81**	**3.78**	**<0.01**
Ureaplasma	45	75	0.24	8.10	23.10	0.16
Fusobacteria	77	25	0.03	4.00	1.10	0.61
Genus						
Streptobacillus	14	0	0.42	0.18	0.0	0.61
Sneathia	35	0	0.15	1.20	0.0	0.73
Fusobacterium	67	25	0.08	2.40	1.10	0.67
Actinobacteria	98	100	0.67	5.60	2.00	0.36
Genus						
Arcanobacterium	22	50	0.20	0.20	0.07	0.70
Propionibacterium	80	100	0.32	1.10	0.54	0.44
Spirochaetes	36	75	0.12	0.15	0.22	0.65
Genus						
Treponema	36	50	0.57	0.13	0.15	0.87
Others	56	25	0.22	0.21	0.01	0.24
Genus						
Bacillariophyta	24	0	0.26	0.07	0.00	0.44
TM7_genera_incertae_sedis	30	25	0.82	0.06	0.01	0.51

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in dairy cows (Bicalho et al., 2010, 2011; Machado et al., 2012).

Additionally, higher average prevalence of *Ureaplasma* spp. was associated with reproductive failure and greater mucus score. Phylogenetic analysis (data not shown) showed that *Ureaplasma* spp. in this study was probably *Ureaplasma diversum*. *Ureaplasmas* were firstly isolated from the bovine reproductive tract by Taylor-Robinson et al. (1967), and *Ureaplasma diversum* has been associated with granular vulvitis, endometritis

Table 4
Percentage of cows that were pregnant ($n = 67$) and cows that were not pregnant ($n = 30$) by 200 DIM that were positive for each different bacterial genus and average prevalence of each genus in samples from cows with different pregnancy status. Values in bold indicate statistically significant difference.

Phylum	% of the cows tested positive			Average prevalence %		
	Non-pregnant	Pregnant	*P*-value	Non-pregnant	Pregnant	*P*-value
Firmicutes	100	98	0.40	44.00	49.60	0.31
Genus						
Sporanaerobacter	4	2	0.53	0.01	<0.01	0.38
Moryella	4	5	0.82	0.02	0.01	0.55
Peptoniphilus	30	21	0.35	0.05	0.06	0.65
Anaerococcus	**7**	**27**	**0.04**	0.05	0.09	0.46
Lactobacillus	**18**	**49**	**<0.01**	0.03	0.53	0.27
Anaerovibrio	37	41	0.71	0.11	0.14	0.61
Peptostreptococcus	22	33	0.29	0.17	0.13	0.67
Clostridium	41	51	0.38	0.11	0.23	0.15
Streptococcus	48	70	0.05	0.25	0.74	0.07
Helcococcus	44	44	1.00	0.76	0.21	0.02
Roseburia	44	59	0.21	0.33	0.46	0.45
Oscillibacter	74	79	0.58	1.10	1.30	0.57
Geobacillus	96	98	0.53	**8.43**	**13.90**	**0.04**
Staphylococcus	**44**	**68**	**0.03**	5.20	0.90	0.09
Bacteroidetes	100	100		15.60	13.70	0.57
Genus						
Parabacteroides	18	32	0.19	0.03	0.05	0.18
Paludibacter	30	51	0.06	0.12	0.20	0.18
Prevotella	26	35	0.40	0.33	0.12	0.26
Porphyromonas	41	51	0.38	1.66	0.50	0.10
Alistipes	59	70	0.33	0.61	0.94	0.17
Bacteroides	44	38	0.57	**2.14**	**0.33**	**<0.05**
Proteobacteria	100	98	0.40	11.20	20.60	0.049
Genus						
Acidovorax	4	9	0.34	<0.01	0.03	0.34
Campylobacter	11	21	0.28	<0.01	0.02	0.07
Devosia	0	8	0.13	0.00	0.07	0.48
Escherichia/Shigella	33	49	0.16	0.07	0.33	0.03
Sphingopyxis	0	11	0.07	0.00	0.17	0.39
Proteus	41	60	0.09	0.13	0.80	0.06
Acinetobacter	67	81	0.14	0.26	0.60	0.10
Halomonas	78	87	0.25	0.69	0.88	0.47
Terenicutes	67	54	0.26	**18.44**	**7.13**	**0.04**
Genus						
Mycoplasma	11	16	0.56	2.20	0.96	0.51
Ureaplasma	56	43	0.27	**15.30**	**6.00**	**0.048**
Fusobacteria	78	73	0.63	6.40	2.80	0.15
Genus						
Streptobacillus	22	9	0.10	0.31	0.12	0.24
Sneathia	33	33	1.00	0.89	1.25	0.81
Fusobacterium	67	65	0.88	**4.80**	**1.30**	**0.01**
Actinobacteria	96	98	0.55	4.80	5.70	0.61
Genus						
Arcanobacterium	22	24	0.87	**0.44**	**0.08**	**0.03**
Propionibacterium	70	86	0.09	**0.53**	**1.2**	**0.02**
Spirochaetes	26	43	0.12	0.07	0.19	0.09
Genus						
Treponema	26	41	0.17	0.06	0.16	0.094
Others	**30**	**65**	**<0.01**	**0.05**	**0.27**	**<0.01**
Genus						
Bacillariophyta	11	29	0.07	**0.01**	**0.09**	**<0.05**
TM7_genera_incertae_sedis	**11**	**38**	**0.01**	**0.01**	**0.08**	**0.04**

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Table 5
Percentage of control ($n = 50$) and trace mineral supplemented (TMS, $n = 47$) cows that were positive for each different bacterial genus and average prevalence of each genus in samples from control and TMS cows. Values in bold indicate statistically significant difference.

Phylum	% of the cows tested positive			Average prevalence %		
	Control	Treatment	*P*-value	Control	Treatment	*P*-value
Firmicutes	98	100	0.25	50.80	45.30	0.25
Genus						
Sporanaerobacter	4	0	0.17	0.01	0.00	0.18
Moryella	**8**	**0**	**<0.05**	0.03	0.00	0.10
Peptoniphilus	30	15	0.08	0.06	0.04	0.54
Anaerococcus	**36**	**4**	**<0.01**	**0.10**	**0.01**	**<0.01**
Lactobacillus	**52**	**30**	**0.03**	0.60	0.07	0.13
Anaerovibrio	44	34	0.31	0.20	0.08	0.13
Peptostreptococcus	**54**	**0**	**<0.01**	**0.20**	**0.00**	**<0.01**
Clostridium	52	45	0.47	0.20	0.20	0.36
Streptococcus	70	55	0.13	0.60	0.60	0.89
Helcococcus	**54**	**30**	**0.02**	0.30	0.40	0.92
Roseburia	62	46	0.13	0.50	0.20	0.07
Oscillibacter	84	68	0.07	1.50	0.90	0.07
Bacteroidetes	100	100		16.20	11.10	0.07
Genus						
Odoribacter	6	6	0.94	0.02	0.01	0.53
Parabacteroides	32	21	0.23	**0.06**	**0.02**	**0.02**
Paludibacter	46	43	0.73	0.20	0.10	0.53
Prevotella	38	21	0.07	0.30	0.04	0.09
Porphyromonas	**68**	**23**	**<0.01**	1.10	0.50	0.35
Alistipes	70	62	0.39	0.80	0.80	0.85
Bacteroides	**62**	**13**	**<0.01**	0.60	1.00	0.62
Proteobacteria	98	100	0.25	**13.90**	**24.30**	**<0.02**
Genus						
Acidovorax	10	9	0.8	0.01	0.03	0.55
Campylobacter	24	11	0.08	0.03	0.01	0.10
Devosia	8	4	0.44	0.09	0.01	0.39
Escherichia/Shigella	44	45	0.95	0.30	0.30	0.96
Sphingopyxis	12	6	0.34	0.20	0.02	0.21
Proteus	48	57	0.35	0.50	0.80	0.38
Acinetobacter	74	83	0.28	0.40	0.60	0.13
Halomonas	80	92	0.11	0.60	1.00	0.06
Terenicutes	54	66	0.23	8.70	11.10	0.61
Genus						
Mycoplasma	10	19	0.2	1.10	1.10	0.92
Ureaplasma	38	55	0.09	7.30	9.20	0.65
Geobacillus	96	100	0.17	12.00	13.00	0.42
Staphylococcus	74	47	0.01	3.80	0.80	0.16
Fusobacteria	**84**	**62**	**0.01**	4.00	3.30	0.73
Genus						
Streptobacillus	18	11	0.30	0.10	0.20	0.34
Sneathia	36	28	0.38	1.50	0.60	0.49
Fusobacterium	**78**	**49**	**<0.01**	0.80	0.90	1.00
Actinobacteria	96	100	0.10	6.00	4.70	0.40
Genus						
Arcanobacterium	**36**	**13**	**<0.01**	0.20	0.20	0.99
Propionibacterium	**90**	**70**	**0.01**	**1.50**	**0.50**	**<0.01**
Spirochaetes	40	32	0.41	0.20	0.10	0.14
Genus						
Treponema	38	32	0.53	0.20	0.10	0.21
Others	48	62	0.17	**0.10**	**0.30**	**<0.03**
Genus						
Bacillariophyta	20	23	0.68	0.06	0.07	0.70
TM7_genera_incertae_sedis	22	38	0.08	**0.02**	**0.10**	**0.02**

(Doig et al., 1980) and reproductive failure (Kreplin et al., 1987). A possible mechanism of action of *Ureaplasma diversum* might be related to its ability to disturb prostaglandin production by endometrial cells (Kim et al., 1994). Average prevalence of *Prevotella* spp. was also associated with mucus score, while the average prevalence of *Bacteroides* spp. was associated with reproductive failure. Both these genera have already been associated with postpartum uterine infection (Williams et al., 2005; Azawi, 2008).

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Percentage of cows positive for *Staphylococcus* spp. was significantly higher for cows that were pregnant by 200 DIM. However, average prevalence was higher in cows that were not pregnant by 200 DIM ($P = 0.09$). *Staphylococcus* spp. have been previously reported as being detrimental for uterine health (Paisley et al., 1986). Other bacteria were beneficial to uterine health or reproductive performance. Percentage of cows positive for *Lactobacillus* spp. was higher in cows that were pregnant by 200 DIM; *Lactobacillus* spp. are usually benign, and have been previously isolated from dairy and meat cows' vaginal vault (Rodriguez et al., 2011) and from buffaloes' uteri (Azawi et al., 2008). Azawi et al. (2008) suggested that presence of *Lactobacillus* spp. in the buffaloes' uterus is beneficial for uterine health. Additionally, *Propionibacter* spp. was positively associated with reproductive performance. This bacterium has never been isolated from bovine uteri before. However, there are studies showing beneficial effects of feeding *Propionobacteria* on milk production (Stein et al., 2006) and reproduction (Lehloenya et al., 2008).

Pyrosequencing of the 16S rRNA gene also allowed the detection of some pathogenic bacteria that, to the best of our knowledge, have never been detected or associated with uterine health so far. For instance, bacteria from the genus *Odoribacter*, described as an inhabitant of the human intestine that have the potential to become an opportunistic pathogen (Goker et al., 2011), and has been isolated from surgically removed appendices (Hardham et al., 2008) and peritoneal pus (Labbe et al., 1977) were associated with uterine disease and poor reproductive performance. In addition, *Peptoniphilus* spp. was also associated with uterine disease; bacteria from this genus have been reported as intramammary infections (Bexiga et al., 2011) or human clinical infections pathogens (Citron et al., 2011). Moreover, bacteria from the genus *Helcococcus*, an emerging pathogen related with bovine valvular endocarditis (Kutzer et al., 2008), were reported here to be associated with uterine infection.

Systemic trace mineral supplementation significantly decreased the proportion of positive cows for some genera that were associated with uterine infection or reproductive failure, such as *Helcococcus* spp., *Bacteroides* spp., *Fusobacterium* spp., and *Trueperella* spp. It is possible that transition cows may have increased trace minerals needs that a diet formulated to meet the current NRC recommendations for Holstein cows may not satisfy, especially since dietary mineral supplements may not be absorbed properly due to interactions with other nutrients at the ruminal level (Underwood and Suttle, 1999) or due to modifications in the rumen, while antagonists located in drinking water may also have a negative effect on the efficiency of trace minerals absorption from the digestive tract (Spears, 2003). Therefore, systemic trace mineral supplemented cows might have experienced immunessupression caused by the trace mineral deficiencies in a lesser extent (Shankar and Prasad, 1998), and this immune response increase might have been sufficient to reduce the presence in the uterine lumen of bacteria that can be detrimental to uterine health and reproductive performance. (Harrison et al., 1984) reported that selenium supplementation reduced metritis incidence in dairy cows.

5. Conclusion

In conclusion, metagenomic pyrosequencing of the 16S rRNA genes came to confirm the importance of known pathogens associated with uterine health and reproductive performance, such as *Fusobacterium* spp., *Trueperella* spp., *Ureaplasma* spp., *Prevotella* spp. and *Bacteroides* spp. Additional information regarding other pathogens potentially associated with uterine health and reproductive performance, such as *Odoribacter* spp., *Peptoniphillus* spp. and *Helcococcus* spp was revealed. Finally, systemic trace mineral supplementation had an impact on the microbiota profile of dairy cows; decreasing the incidence of some genera associated with uterine infection or reproductive failure.

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