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An investigation of the microbiota in uterine flush samples and endometrial biopsies from dairy cows during the first 7 weeks postpartum



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ABSTRACT

Metritis and endometritis commonly occur in dairy cows after calving. Although numerous studies have been performed to identify the causative pathogens, a complete overview has not been done. Metagenomic studies have analyzed the bacterial populations of uterine flush samples from postpartum (pp) dairy cows, but the microbiota in the uterine luminal fluid may differ from the microbiota of the endometrium itself, and important putative pathogens may have been overlooked. In the present study, we compared the microbiota of the uterine lumen and the endometrium of healthy, metritic, and endometritic cows. Samples were collected from 68 Holstein dairy cows at 1, 4, and 7 weeks pp, and the data were analyzed by deep sequencing of the V1 and V2 hypervariable regions of the 16S ribosomal RNA gene. The results showed that *Porphyromonadaceae*, *Fusobacteriaceae*, *Leptotrichiaceae*, and *Mycoplasmataceae* may be associated with uterine disease. The microbiota of the uterine flush samples and the endometrial biopsies were correlated, but the microbiota of the biopsies was more diverse. *Fusobacteriaceae* and *Leptotrichiaceae* were not observed in the biopsies at week 7, whereas they accounted for 20% and 13%, respectively, of the bacterial populations in the flush samples. The *Mycoplasmataceae* family was observed in much higher quantity in the flush samples than in the biopsies of the endometritis groups at weeks 4 and 7. Our findings support the observations of previous metagenomic studies and illustrate the importance of including endometrial biopsies to obtain more detailed knowledge of the pp uterine microbiota.

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1. Introduction

Apart from the postpartum (pp) period, the uterus is generally considered to be sterile [1,2]. During the first 10 to 14 days pp, an estimated 90% of all dairy cows have intra-uterine bacterial contamination [1]. Within 8 weeks pp, a sterile environment should be re-established in the healthy uterus through the process of involution [3]. However,

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bacterial infection may cause metritis in up to 20% of cows, or endometritis in 5% to 25% of cows, or develop into a subclinical infection within 4 to 8 weeks pp in 30% to 50% of cows [2,4]. Metritis is a profound inflammation of the uterine wall that usually occurs within 21 days pp but often within 10 days [5]. Endometritis and subclinical endometritis are inflammation in the inner lining of the uterus and occur at 21 days or more pp [5,6].

A variety of bacteria contaminate the bovine uterus shortly after calving, but the relationship between individual bacterial species or groups of bacteria and the development of uterine infection is poorly understood. The bacteria that contaminate the bovine uterus pp most likely originate from feces or the environment [7], which may explain why some phyla such as *Bacteroidetes*, *Fusobacteria*, *Firmicutes*, *Proteobacteria*, and *Tenericutes* have been observed in the pp uterus, regardless of the uterine health status [8]. These phyla are not considered part of a normal microbiota because the uterus is considered sterile between parturitions [1,2]. However, the hypothesis that the uterine environment is sterile between parturitions remains to be confirmed by the use of culture-independent methods.

In conventional culture studies, *Escherichia coli*, *Trueperella pyogenes*, *Prevotella* spp., and *Fusobacterium necrophorum* have commonly been detected in the uterus of cows with a pp uterine infection [5,9,10]. With the development of culture-independent methods that use next-generation sequencing to identify bacteria, it is possible to detect DNA from fastidious bacteria, which are difficult or impossible to isolate in conventional culture-based studies. Such metagenomic studies have shown an association between *Fusobacterium* spp., *Trueperella* spp., *Ureaplasma* spp., *Prevotella* spp., and *Bacteroides* spp. and uterine disease 35 days pp [11]. Endometritis has also been associated with *Bacteroidetes*, *Firmicutes*, and *Fusobacteria* [8]. Other bacteria, such as members of the phylum *Proteobacteria*, may be related to maintaining the uterine health of pp dairy cows [11]; furthermore, *Lactobacillus* spp. and *Propionibacter* spp. appear to be associated with good reproductive performance [11].

Profound insight into the diversity and composition of the microbiota of both the diseased and healthy uterus of dairy cows is a prerequisite for developing comprehensive treatments for these reproductive disorders. Although a number of potential causative microorganisms have been identified for bovine pp metritis and endometritis, the complete pathogenesis of these disorders is still unclear. Previous culture-independent studies of uterine flush samples have demonstrated that multiple bacterial species are associated with metritis and endometritis and that the bovine uterine microbiota is much more diverse than previously recognized on the basis of traditional cultivation [8,11–13]. Tissue-invasive bacteria, however, may not be predominant in the lumen but must adhere to and invade the endothelium to proliferate and cause disease. We therefore hypothesize that potentially pathogenic bacteria will be more prevalent in the endometrium than that in the lumen of the uterus. The purpose of this study was to identify metritis- and endometritis-related bacteria by performing in-depth analysis of the pp microbiota, including samples from both the uterine lumen and the endometrium of pp dairy cows. We applied next-

generation sequencing of the V1-V2 variable region of the 16S ribosomal RNA gene, which enables bacterial identification without cultivation [14,15].

2. Materials and methods

2.1. Animals

Samples were collected from 68 cows from a Danish dairy herd of approximately 1200 Holstein cows. The cows were kept in loose housing, fed ad libitum with total mixed rations, and milked twice daily with an average herd milk yield of 9700 kg energy corrected milk.

The cows included in the study calved between April and July 2012. Their parity ranged from 1 to 5. The herd used artificial breeding with semen from proven sires, and the herd was free of bovine diarrhoea virus infection and a number of other infections officially eradicated from Denmark [16].

2.2. Uterine sampling procedures

Samples were taken at pp week 1 (Days 4–12 pp), week 4 (Days 24–32 pp), and week 7 (Days 46–53 pp). To increase the group size of cows with a pp uterine infection, two cows with an overt uterine disease were included in the week 1 sampling, giving a total of 16 cows, and the remaining 52 cows were selected randomly by picking their ID numbers that were written on a folded piece of paper. Cows were not sampled after insemination, reducing the number of cows sampled at week 7. After washing the perineum with Lactacyd soap (Sanofi-Aventis, Paris, France) and drying with paper towels, a latex embryo flush catheter was inserted into the uterus; the uterine fluid was then aspirated. If no fluid was obtained by aspiration, 20 to 40 mL of sterile saline was flushed into the uterine lumen and then aspirated into a syringe. Approximately 1 mL of uterine fluid was added to prepared tubes containing 4 mL of RNAlater (Ambion, Austin, TX, USA) [17]. A smear of the uterine fluid was made on a microscope slide for cytology. The slides were stained with Hemacolor (Merck Millipore, Darmstadt, Germany) and mounted with Pertex (Histolab, Göteborg, Sweden). An endometrial biopsy was taken using a dividable biopsy instrument (Kruuse, Langeskov, Denmark). The biopsy instrument, protected by a plastic sleeve, was inserted into the cervix. The biopsy instrument was unsheathed in the cervix and advanced into the uterus, where the biopsy was taken. The biopsy was then enclosed within the instrument. The surface of the instrument was wiped with ethanol before opening the instrument to remove the biopsy. The biopsy was transferred into 2 mL of RNAlater with a sterile syringe. Samples in RNAlater were stored at room temperature for approximately 24 hours and then stored at -20°C .

2.3. Ethics statement

All animal procedures were approved by the Danish Animal Experiments Inspectorate under the Ministry of Justice (approval number 2011/561-90), and the animal experiments were conducted in strict accordance with their guidelines.

2.4. Classification

The cows at week 1 pp were grouped according to their uterine score and at weeks 4 and 7 by the ratio of neutrophils to endometrial cells observed on cytology. The uterine score was on the basis of vaginal exploration performed by the herd veterinarian, in accordance with the Danish scale for uterine scoring [18]. Briefly, scores of 0 to 4 characterized cows with odorless, mucoid uterine discharge in varying amounts, whereas scores of 5 to 9 characterized cows with smelly uterine discharge in increasing amounts. At the farm, the treatment threshold was a uterine score of 4. The cutoff was on the basis of this treatment threshold. Thus, cows were classified as “normal”, indicating no uterine disease, if they had a uterine score of 3 or less at week 1, and cows with a uterine score of 4 or higher at week 1 were considered to have metritis. At weeks 4 and 7, cows were classified as normal if they had neutrophil counts below 18% and 10%, respectively, as suggested by Kasimanickam et al. [19]. Cows were considered to have endometritis if they had a neutrophil count above 18% at week 4 or 10% at week 7.

2.5. DNA extraction from endometrial biopsies

After thawing, approximately 10 mg of endometrial tissue was placed in 300 μ L lysozyme buffer (20-mM Tris-HCl, 2-mM EDTA, 1.2% Triton X, and 5-mg Lysozyme per 100 mL). The samples were then incubated at 37 °C for 30 minutes. In total, 350- μ L lysis buffer from the Maxwell 16 LEV blood DNA kit (Promega, Madison, WI, USA) and a 5-mm stainless steel ball (QIAGEN, Hilden, Germany) were added before the tissue was bead-beaten at 20 Hz for 2 minutes in a TissueLyser II (QIAGEN). Next, 30- μ L Protease K was added, and the sample was incubated at 56 °C for 1 hour. DNA was extracted on a Maxwell 16 Research Instrument System using a Maxwell LEV Blood DNA Purification Kit (Promega). Afterward, the DNA concentration and quality was measured on a NanoDrop 1000 machine (Saveen Werner, Limhamn, Sweden).

2.6. DNA extraction from uterine flush samples

A volume of aspirated uterine fluid in RNAlater (Ambion) predicted to produce a pellet of approximately 10 mg (between 200 μ L and 1800 μ L) was measured, and PBS was added to a total volume of 1900 μ L. The samples were then centrifuged at 13,000 \times g at 4 °C for 30 minutes. The pellet was resuspended in 100- μ L lysozyme buffer (see above). DNA was extracted from the pellet in the same manner as DNA was extracted from the endometrial biopsies (see DNA extraction from endometrial biopsies).

2.7. Next-generation sequencing

16S ribosomal RNA polymerase chain reaction (PCR) was performed with primers targeting the V1 and V2 regions [20]. The forward primer sequence was: 5'-AGAGTTTGATCCTGGCTCAG-3', and the reverse primer sequence was 5'-CTGCTGCTYCCGTA-3'. Both the forward and reverse primers had a hexamer barcode on the 5' end.

A total of 51 primers with barcodes were used. The PCR mix consisted of 5 μ L of 10x PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 3 μ L of 25-mM dNTP, 2 μ L of 20-mM primers, 0.5 μ L of 5-U/ μ L Taq Gold polymerase (Applied Biosystems) and nuclease-free water to a volume of 50 μ L. The PCR amplification was performed as follows: an initial denaturation step at 94 °C for 3 minutes, then 30 cycles of 94 °C for 45 seconds, 57 °C for 45 seconds, and 72 °C for 90 seconds, followed by 10 minutes at 72 °C. A negative control for each primer set was included. The amplicons were analyzed with the Agilent DNA 1000 Reagents kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) for concentration and purity. Amplicons with a concentration below 5 ng/ μ L were discarded, resulting in 153 endometrial biopsies and 135 uterine flush samples. These were divided equimolarly into eight pools of up to 51 samples. Primer dimers and PCR reagents were removed using the MinElute PCR Purification Kit (QIAGEN). The samples were sent for Illumina MiSeq paired-end sequencing at the National High-throughput DNA Sequencing Center, University of Copenhagen, Denmark.

2.8. Data analysis

The sequences were analyzed using BION software (<https://app.box.com/bion>). Paired sequences were trimmed with a 97% quality minimum in a 10 bp window; one mutation was allowed in the primers, and the minimum length of the reads was set to 260 bp after trimming. The reads were dereplicated, and reads with a chimera score above 30 were removed. The consensus sequences were mapped against the Ribosomal Database Project (RDP) database (<http://rdp.cme.msu.edu>) with a match minimum of 86%, and taxonomical classification was on the basis of best 1% of the similarities from the RDP database.

Each sample was normalized to 100,000 reads. Samples were excluded if the log₁₀-transformed number of read numbers in the samples was less than the overall average of reads in the experiment minus two standard deviations.

Rather than doing a statistical analysis on the average count of operational taxonomic units (OTUs) in each group, a two-tailed Fisher's exact test was applied because the group sizes were smaller than expected. Our application of the Fisher's exact test investigated the ratio of cows in which the number of observations of a given OTU exceeded 5% of the total number of normalized reads in the sample. For each taxonomic unit, the metritis and endometritis groups were tested against the normal group with a threshold value of 5% of the total normalized reads. The threshold, which was arbitrarily chosen on the basis of empirical data analysis, was estimated to be conservative. Spearman's rank test was performed on the average number of normalized reads per OTU to investigate the correlation between the biopsy and flush samples.

3. Results

A total of 20,128,922 paired-end reads in eight pools were obtained. After demultiplexing the reads according to barcodes and primers, 14,646,852 reads remained. The paired-end reads were joined, leaving 6,939,462 reads,

which were trimmed at the 5' and 3' ends of the sequences according to quality. Sequences with a quality below 97% or a trimmed length of less than 260 bp were discarded. This left 5,641,274 reads that were rarefied to 2,795,873 unique sequences. After the chimera filter, 2,575,771 reads over 288 samples were tested against the RDP database for taxonomic classification. Of these reads, 1,851,529 reads were matched to entries in the database, and 724,242 were not matched against any entries in the database. The rarefaction curves were not steep, but only a few of the curves plateaued (unpublished results). This indicates that a deeper sequencing would most likely have revealed more OTUs.

The obtained OTUs were identified to the family level, as most of the OTUs were unclassified below that taxonomic level. Operational taxonomic units that were unclassified at the family level were allocated to the nearest taxonomical level possible.

The 288 sequenced samples were classified according to the uterine health status of the cows from which they originated. A total of 162 samples were used for the analysis. The number of samples per group is shown in Table 1. In total, 126 samples were removed because they were derived from cows that had received treatment with antibiotics or because there were not enough cells on the cytology slide to make a uterine health classification. The samples from week 7 were excluded from the statistical analysis because of small group size. The cows generally did not stay in the same uterine health classification throughout the study. The cows with metritis at week 1 were treated with antibiotics, and therefore, the subsequent samples from those cows were discarded. The cows that developed endometritis did so at week 4, thereby shifting the uterine health category. Only two cows were in the healthy category at all three time points but that also reflects that few cows were left in the study by week 7.

3.1. Diversity

The normalized OTUs of the uterine flush samples and endometrial biopsies were correlated ($P < 0.01$). However, Shannon index scores, which took into account both the number of species found in each category and the evenness of their distribution, showed that the bacterial diversity

Table 1
Number of samples analyzed at each time point for both uterine flush samples and endometrial biopsies.

Sample type	Uterine status	Week 1	Week 4	Week 7
Flush	Normal	32	7	3
	Metritis	13	-	-
	Endometritis	-	19	6
Biopsy	Normal	31	8	4
	Metritis	11	-	-
	Endometritis	-	22	6

Samples taken at week 1 represent cases of metritis, whereas samples taken during weeks 4 and 7 originate from cows with endometritis. Samples were not analyzed if they were from cows that had received treatment with antibiotics or if where there were not enough cells on the cytology slide to make a uterine health classification. In addition, cows that had been inseminated were not sampled. This explains why the number of samples at weeks 4 and 7 is lower than week 1.

was higher in the endometrial biopsies compared with the uterine flush samples (Fig. 1).

3.2. Similar taxonomic composition between lumen and endometrium in cows with uterine disease at week 1 pp

The relative distribution of OTUs at week 1 pp is shown in Figure 2A. Table 2 is presented to easily discern the most abundant OTUs found per group. The uterine flush samples and the endometrial biopsies exhibited similar taxonomic compositions, with the families *Fusobacteriaceae*, *Porphyromonadaceae*, and *Streptococcaceae* constituting 59% of the flush samples microbiota and 62% of the biopsy microbiota.

When comparing the metritis group with normal cows, *Fusobacteriaceae* was found at a significantly higher frequency in endometrial biopsies ($P = 0.03$) and *Porphyromonadaceae* in the uterine flush samples ($P = 0.03$). An abundance of *Streptococcaceae* was observed in all sample types (Table 2). Fisher's exact test did not show any difference in frequency between the normal and the metritic cows with respect to the presence of *Streptococcaceae*.

3.3. Differences in taxonomic composition between lumen and endometrium in cows with uterine disease at weeks 4 and 7 pp

None of the bacterial families that made up the major portion of the sequences in the metritis samples were among the three most abundant OTUs in the endometritis samples at week 4 (Fig. 2B). Furthermore, the uterine flush and endometrial biopsy samples displayed a more divergent pattern. In the uterine flush samples from endometritic cows, *Mycoplasmataceae* and *Leptotrichiaceae*, accounted for 60% of the OTUs at week 4. This observation was not mirrored in the endometrial biopsies at week 4, where *Ruminococcaceae*, *Mycoplasmataceae*, and *Bacteroidaceae* together constituted only 43% of the total OTUs (Table 2). Regardless of sample type, no OTUs were

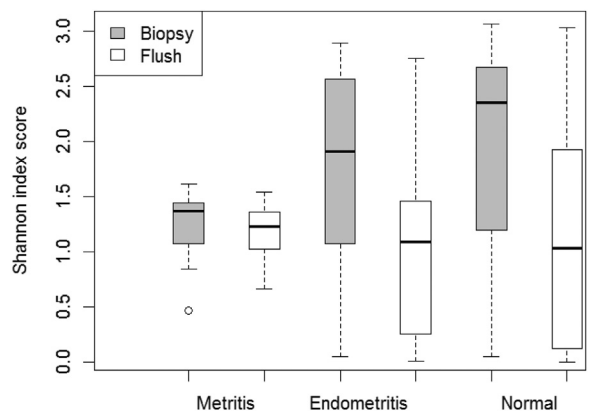


Fig. 1. Box plot of the distribution of Shannon index score for each sample in the metritis, endometritis, and normal groups. The lower part of the boxes represents the lower quartile, the upper part of the box represents the upper quartile, and the bar inside the box marks the median. The whiskers represent the minimal and the maximal values of the data, excluding outliers, which are shown as small circles.

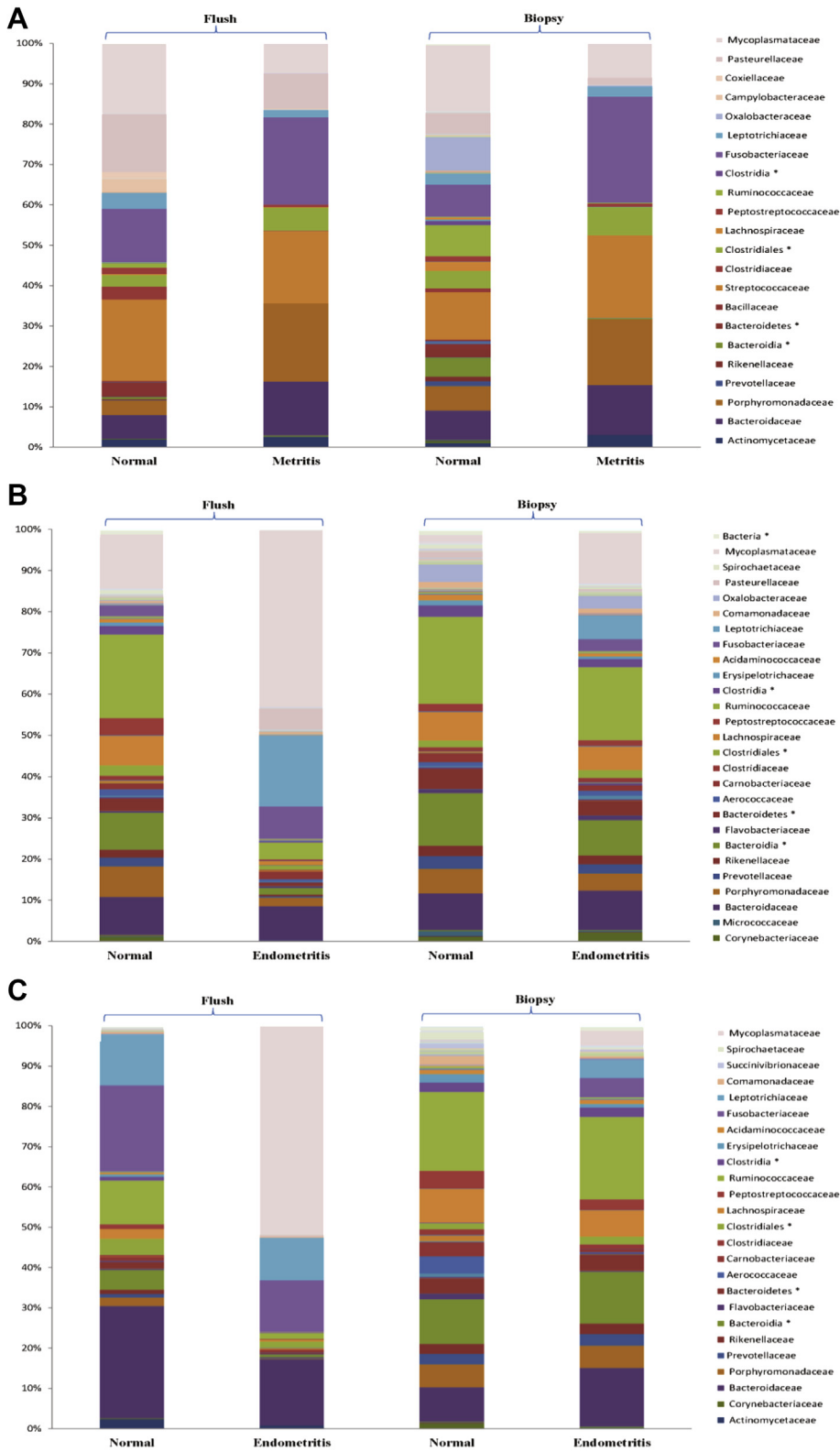


Fig. 2. Color-coded bar plot showing the relative uterine bacterial operational taxonomic unit (OTU) composition in uterine flush samples and endometrial biopsies from normal cows and cows with metritis and cows with endometritis. (A) Week 1 postpartum. (B) Week 4 postpartum. (C) Week 7 postpartum. The color code is given for families that constitute $\geq 1\%$ of the relative abundance of all the OTUs observed within each group. The OTUs are determined to the family level. OTUs that are unclassified at the family level are allocated to the nearest taxonomical level possible. These are indicated by *. The number of cows in each group is shown in Table 1.

Table 2

The three most abundant operational taxonomic units (OTUs) observed in the uterine flush samples and the endometrial biopsies (measured in percentages within groups).

Sample type	OTU	Flush	Biopsy
Week 1			
Normal	<i>Streptococcaceae</i>	20%	12%
	<i>Mycoplasmataceae</i>	17%	16%
	<i>Pasteurellaceae</i>	14%	5% ^b
	<i>Oxalobacteraceae</i>	0% ^b	8%
	<i>Fusobacteriaceae</i>	22%	26%
Metritis	<i>Porphyromonadaceae</i>	19%	16%
	<i>Streptococcaceae</i>	18%	20%
Week 4			
Normal	<i>Ruminococcaceae</i>	20%	21%
	<i>Mycoplasmataceae</i>	13%	2% ^b
	<i>Bacteroidia</i> ^a	9% ^b	13%
	<i>Bacteroidaceae</i>	9%	9%
Endometritis	<i>Mycoplasmataceae</i>	43%	12%
	<i>Leptotrichiaceae</i>	17%	6% ^b
	<i>Ruminococcaceae</i>	4% ^b	18%
	<i>Bacteroidaceae</i>	8%	10%
Week 7			
Normal	<i>Bacteroidaceae</i>	28%	9%
	<i>Fusobacteriaceae</i>	21%	0% ^b
	<i>Ruminococcaceae</i>	11% ^b	20%
	<i>Leptotrichiaceae</i>	13%	0% ^b
	<i>Bacteroidia</i> ^a	5% ^b	11%
Endometritis	<i>Mycoplasmataceae</i>	52%	4% ^b
	<i>Ruminococcaceae</i>	2% ^b	20%
	<i>Bacteroidaceae</i>	16%	15%
	<i>Fusobacteriaceae</i>	13%	5% ^b
	<i>Bacteroidia</i> ^a	1% ^b	13%

Operational taxonomic units marked with a superscript alphabet (a) were unclassified at the family level and are given at the class level.

Operational taxonomic units marked with a superscript character (b) were not among the three most abundant in the group but are displayed to make comparisons between sample types more straightforward.

observed at a significantly higher frequency in cows with endometritis compared with normal cows.

The relative distribution of OTUs at week 7 is shown in Figure 2C. At this time point, *Mycoplasmataceae* still dominated the uterine flush samples of cows with endometritis, accounting for 52% of the found OTUs (Table 2). *Fusobacteriaceae* OTUs were observed at a relatively high abundance in uterine flush samples from both normal cows and endometritic cows at week 7. In the endometrial biopsies, *Fusobacteriaceae* only accounted for 5% of the OTUs observed in the endometritic cows and appeared to be absent from the endometrial biopsies of normal cows.

Ruminococcaceae, *Bacteroidaceae*, and an unclassified family that belonged to class *Bacteroidia* were the three most abundant families in the endometrial biopsies from cows with endometritis at week 7 as well as in endometrial biopsies from normal cows at weeks 4 and 7 (Table 2). Such a consistent pattern of microbial composition was not observed in the uterine flush samples. The microbial population of the endometrial biopsies appeared to be more stable over time (and independent of uterine score) than the microbial population of the uterine flush samples.

3.4. Cows with no apparent uterine disease

Differences were also observed between uterine flush samples and endometrial biopsies from the healthy cows.

In the uterine flush samples, *Mycoplasmataceae*, *Leptotrichiaceae*, and *Bacteroidaceae* were the most abundant families, although some variations between time points were observed. In the endometrial biopsies, *Ruminococcaceae* and *Bacteroidaceae*, and an unclassified family that belonged to class *Bacteroidia* were the most abundant taxa at weeks 4 and 7 pp.

Ruminococcaceae was present at a significantly higher frequency in endometrial biopsies from normal cows than in cows with metritis ($P = 0.003$) at week 1 and in uterine flush samples from normal cows compared with endometritic cows at week 4 ($P = 0.0005$). *Ruminococcaceae*, however, was also observed in high abundance in endometrial biopsies from cows with endometritis at weeks 4 and 7.

Lachnospiraceae ($P = 0.002$) and *Porphyromonadaceae* ($P = 0.03$) were observed at a significantly higher frequency in uterine flush samples from normal cows at week 4 compared with endometritic cows. In the endometrial biopsies, *Porphyromonadaceae* and an unclassified family that belonged to phylum *Bacteroidetes* were also found at a significantly higher frequency in normal cows compared with endometritic cows.

Bacteroidaceae was generally abundant in samples from both normal cows and cows with endometritis. *Fusobacteriaceae* and *Leptotrichiaceae* were observed in relatively high quantities in the uterine flush samples but not in the endometrial biopsies at week 7.

4. Discussion

Until now, the cultivation-independent studies on bacterial diversity in the pp bovine uterus have analyzed uterine flush samples [8,11–13]. Consequently, potential tissue-invading bacteria that are mainly present in the uterine wall may have been underestimated or even overlooked. In the present investigation, both uterine flush samples and endometrial biopsies were sampled three times over a period of 7 weeks from 68 pp dairy cows. This approach allowed us to compare over time the microbial composition and relative bacterial abundance in the uterus of healthy cows and cows with pp uterine infection and to investigate whether the microbiota found in the uterine lumen corresponded to the bacterial population of the endometrium.

Similar to previous culture-independent studies, [8,11] most of the sequence reads greater than 1.8 M were affiliated with the phyla *Bacteroidetes* (*Bacteroidaceae* and unclassified families that belong to class *Bacteroidia*), *Fusobacteria* (*Fusobacteriaceae* and *Leptotrichiaceae*), *Firmicutes* (*Ruminococcaceae* and *Streptococcaceae*), and *Tenericutes* (*Mycoplasmataceae*) regardless of the uterine health status of the cow. The differences in the phylogenetic composition and bacterial abundance of both normal and diseased cows were tracked over time (Fig. 2).

The Shannon index, which measures species diversity in a population by taking into account both the number of species present and the relative proportion of each observed species, was calculated for both uterine flush samples and biopsies. The differences in the Shannon indexes showed that the biopsies had a more diverse and

evenly distributed microbial population than that observed in the uterine flush samples. To test the hypothesis that tissue-invasive species are more likely be identified from endometrial biopsies than from uterine flush samples, we compared the microbiota of the two specimen types. According to Spearman's rank test, the two sample types were correlated. Despite this correlation, considerable differences were observed among the most abundant bacterial families of the endometrial biopsies and uterine flush samples at weeks 4 and 7 pp. At week 1 pp, a higher bacterial diversity in the endometrial biopsies was the biggest difference between the sample types. The most surprising finding was the apparent absence of *Fusobacteriaceae* and *Leptotrichiaceae* in the biopsies at week 7, whereas they accounted for approximately 20% and 13%, respectively, of the OTUs observed in the flush samples (Table 1). The *Mycoplasmataceae* family was also found in much higher numbers in the uterine flush samples than that in the endometrial biopsies. The difference was striking for the endometritis group at weeks 4 and 7, where this family accounted for 43% and 52% of the OTUs in flush samples but only 12% and 4% of the endometrial biopsies, respectively (Table 1). Variation in processing may have caused some differences between sample types. Histologic and bacteriological examinations of endometrial biopsies have been studied by Bonnet et al. [21–23], and although it is debated whether taking biopsies has an adverse effect on fertility [24,25], histologic examination of endometrial biopsies is a sensitive method for diagnosing uterine disease [21–24]. The differences we observed for the bacterial families indicate that the uterine flush samples were more easily skewed than the endometrial biopsies. It would be interesting to compare these results to the results of a cytobrush sampling, as these might differ from both the flush samples and the biopsies. A surprisingly rich bacterial flora was observed in the endometrium, and pinpointing invasive species was therefore not an easy task. Fluorescence *in situ* hybridization or similar methods are needed to draw more solid conclusions regarding the tissue distribution and invasiveness of the bacteria found in the biopsies.

A relatively clear pattern emerged from the bacterial composition of the metritis group. Operational taxonomic units from the *Fusobacteriaceae* and *Porphyromonadaceae* families were more abundant and more frequent in cows with metritis than those in normal cows at week 1 pp. It is therefore likely that bacteria from these families play an important role in the development of metritis in dairy cattle during the first week after calving. *Fusobacterium necrophorum* and *Porphyromonas levii*, which are members of the *Fusobacteriaceae* and *Porphyromonadaceae* families, respectively, have been identified in culture-independent studies of bovine pp diseases. *F. necrophorum* has previously been isolated from cases of bovine metritis [7] and is a well-known pathogen that causes profound necrotizing inflammation in cattle [26]. *P. levii* has previously been associated with metritis [13] and bovine necrotic vulvovaginitis [27].

By contrast, bacteria from the *Porphyromonadaceae* family occurred more frequently in normal cows than in cows with endometritis from week 4 uterine flush samples and endometrial biopsies. The role of bacteria from

Porphyromonadaceae in the development of pp uterine infections in dairy cows needs further investigation.

Escherichia coli and *T. pyogenes* are considered important bacteria in the development of metritis and endometritis. The presence of *E. coli* in the uterus at an early time point after parturition has been linked with the development of endometritis in culture-based studies [28–30], often followed by *T. pyogenes* and Gram-negative bacteria [28,30]. *Trueperella pyogenes* was observed in the uterus of cows with uterine disease by culture-independent methods by Machado et al. [11], and virulence factors from *E. coli* and *T. pyogenes* were found in cows with uterine disease by Bicalho et al. [7]. Furthermore, pp uterine disease has been induced by infusion of *T. pyogenes* [31,32], and Lipopolysaccharide from *E. coli* has been used to mimic uterine infection *in vitro* [29,33]. However, in other culture-independent studies neither *E. coli* nor *T. pyogenes* was found in significant numbers [8,12]. This is in accordance with the present study, where fewer than 1% of the sequence reads could be assigned to *Enterobacteriaceae* (the family that includes *E. coli*) or *Actinomycetaceae* (the family that includes *T. pyogenes*) at any of the investigated time points. The present data therefore do not support the hypothesis that *E. coli* and *T. pyogenes* are important etiologic factors for the development of metritis or endometritis in dairy cattle, if judged by the number of OTUs found. The fact that we did observe a few *Enterococcaceae* OTUs and that a spiking experiment with *E. coli* ruled out the possibility that the DNA-extraction method eliminated the *E. coli* DNA (unpublished results), demonstrates that the method used does not discriminate against *E. coli*. The same spiking experiment was, however, not performed for *T. pyogenes*. The scarcity of *E. coli* and *T. pyogenes* in the pp uterus should be kept in mind when evaluating antimicrobial treatment of pp uterine diseases. However, *E. coli* and *T. pyogenes* may produce potent toxins that influence the course of pp uterine infections despite their low numbers. Finally, the role of bacteria from the seemingly prevalent *Mycoplasmataceae* and *Porphyromonadaceae* families should be further investigated.

We did not detect any OTUs that occurred more frequently in cows with endometritis than those in normal cows, but a larger sample size would be necessary to increase statistical power. Furthermore, observing the absolute amount of bacteria in all the samples would enable a comparison of OTU counts corrected for individual bacterial load. This could give more insight into possible pathogens at weeks 4 and 7. In the present study, the bacterial load was not measured, and all samples were normalized equally. As mentioned above, the most notable differences at weeks 4 and 7 were the increasing discrepancies between the most abundant OTUs observed in the uterine flush samples and the endometrial biopsies. The prevalence of uterine disease at weeks 4 and 7 was higher than anticipated, even including the extra cows with a high uterine score that were sampled. At week 7, this can be explained partly by the normal cows being excluded when they were inseminated.

Endometrial biopsies from the healthy cows were characterized by a high diversity and a stable microbiota, most notably *Ruminococcaceae*, *Bacteroidaceae*, and unclassified families that belong to class *Bacteroidia*. The bacterial

population observed in the uterine flush samples was less stable and mainly dominated by the *Mycoplasmataceae*, *Leptotrichiaceae*, and *Bacteroidaceae* families.

The normal pp cows had a very diverse uterine microbiota. We did not find any OTU that was consistently associated with a healthy bovine pp uterus without also being observed in samples from cows with endometritis or metritis. For example, OTUs mapping to *Ruminococcaceae*, *Lachnospiraceae*, *Porphyromonadaceae*, and unclassified families that belong to phylum *Bacteroidetes* were significantly associated with normal cows in different sample types and at different time points. However, all these families were also found either in high frequency or in high abundance in samples from cows with metritis or endometritis. Likewise, although *Ruminococcaceae*, *Bacteroidaceae*, and unclassified families that belong to class *Bacteroidia* were found in high abundance in the endometrial biopsies from normal cows at weeks 4 and 7, these families were also present in abundance in samples from cows with endometritis at week 7. The diversity observed in the normal cows, coupled with several frequently occurring families that are also found in high quantities in bovine fecal matter [34], most likely reflects the fecal and environmental contamination of the uterus. This could be viewed as a part of a temporary core microbiota; a microbiota that will most likely disappear when the pp uterus undergoes involution, during which bacterial contamination is cleared [3]. If the hypothesis that the uterine environment is sterile between parturitions was challenged by future studies using culture-independent methods, the possibility of a normal uterine microbiota should be explored.

This study did not include a comparison between the culture-independent method and the traditional culture-based method of identifying the bacteria in the pp uterus, although such a comparison would be interesting. In addition, a sampling of feces and the stable environment would have been interesting, as it would provide information regarding the contamination of both the uterus and the samples. In pigs, the fecal microbiota is very similar to the vaginal microbiota [35] and thus possibly to the uterine pp microbiota.

Clinically, the results of the present study suggest that there are fewer *E coli* and *T pyogenes* in the pp uterus than expected. Perhaps other species that cannot be cultured traditionally will be detected as more important pathogens. This may alter the strategy for the antimicrobial treatment of pp uterine diseases.

4.1. Conclusions

The authors found that the microbial composition in the uterus pp is complex. There was a clear association between metritis at week 1 pp and *Fusobacteriaceae* and *Porphyromonadaceae*. However, at weeks 4 and 7, none of the OTUs that were observed frequently in samples from cows with endometritis had an unequivocal association with the disease. Additional studies with larger sample sizes are needed to completely understand the role of these bacteria in pp uterine disease. While comparing the endometrial biopsies and the uterine flush samples, we found that although they were correlated, there were

discrepancies between their most abundant bacterial families, and the microbial population found in the biopsies had a higher diversity and was more stable over time.

5. Nucleotide sequences

The data discussed in this publication have been deposited in the NCBI Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/Traces/sra/>) and are accessible through accession number: SRP052611.

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References

- [1] Sheldon IM, Dobson H. Postpartum uterine health in cattle. *Anim Reprod Sci* 2004;82:83:295–306.
- [2] Dubuc J. Postpartum uterine diseases: prevalence, impacts, and treatments. *WCDS Adv Dairy Technol* 2011;23:255–67.
- [3] Földi J, Kulcsár M, Pécsi A, Huyghe B, de Sa C, Lohuis JACM, et al. Bacterial complications of postpartum uterine involution in cattle. *Anim Reprod Sci* 2006;96:265–81.
- [4] LeBlanc SJ. Reproductive tract inflammatory disease in postpartum dairy cows. *Animal* 2014;8(Suppl 1):54–63.
- [5] Sheldon IM, Lewis GS, LeBlanc S, Gilbert RO. Defining postpartum uterine disease in cattle. *Theriogenology* 2006;65:1516–30.
- [6] Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. Definitions and diagnosis of postpartum endometritis in dairy cows. *J Dairy Sci* 2010;93:5225–33.
- [7] Bicalho MLS, Machado VS, Oikonomou G, Gilbert RO, Bicalho RC. Association between virulence factors of *Escherichia coli*, *Fusobacterium necrophorum*, and *Arcanobacterium pyogenes* and uterine diseases of dairy cows. *Vet Microbiol* 2011;157:125–31.
- [8] Santos TMA, Bicalho RC. Diversity and succession of bacterial communities in the uterine fluid of postpartum metritic, endometritic and healthy dairy cows. *PLoS One* 2012;7:e53048.
- [9] Königsson K, Gustafsson H, Gunnarsson A, Kindahl H. Clinical and bacteriological aspects on the use of oxytetracycline and flunixin in primiparous cows with induced retained placenta and post-partal endometritis. *Reprod Domest Anim* 2001;36:247–56.
- [10] Williams EJ, Fischer DP, Pfeiffer DU, England GCW, Noakes DE, Dobson H, et al. Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. *Theriogenology* 2005;63:102–17.
- [11] Machado VS, Oikonomou G, Bicalho MLS, Knauer WA, Gilbert R, Bicalho RC. Investigation of postpartum dairy cows' uterine microbial diversity using metagenomic pyrosequencing of the 16S rRNA gene. *Vet Microbiol* 2012;159:460–9.
- [12] Peng Y, Wang Y, Hang S, Zhu W. Microbial diversity in uterus of healthy and metritic postpartum Holstein dairy cows. *Folia Microbiol* 2013;58:593–600.
- [13] Santos TMA, Gilbert RO, Bicalho RC. Metagenomic analysis of the uterine bacterial microbiota in healthy and metritic postpartum dairy cows. *J Dairy Sci* 2011;94:291–302.
- [14] Cho I, Blaser M. The human microbiome: at the interface of health and disease. *Nat Rev Genet* 2012;13:260–70.
- [15] Jarvie T. Next generation sequencing technologies. *Drug Discov Today Technol* 2005;2:255–60.
- [16] FVST, Ministry of Food Agriculture and Fisheries, Danish Veterinary and Food Administration. Animal health in Denmark 2012. Glostrup: Danish Veterinary and Food Administration; 2013.
- [17] Vlčková K, Mrázek J, Kopečný J, Petrzelková KJ. Evaluation of different storage methods to characterize the fecal bacterial

- communities of captive western lowland gorillas (*Gorilla gorilla gorilla*). *J Microbiol Methods* 2012;91:45–51.
- [18] Elkjær K, Ancker M-L, Gustafsson H, Friggens NC, Waldmann A, Mølbak L, et al. Uterine bacterial flora in postpartum Danish Holstein dairy cows determined using DNA-based fingerprinting: correlation to uterine condition and calving management. *Anim Reprod Sci* 2013;138:39–48.
- [19] Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS, et al. Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. *Theriogenology* 2004;62:9–23.
- [20] Wilmotte A, Auwera Van der G, Wachter De R. Structure of the 16 S ribosomal RNA of the thermophilic cyanobacterium *Chlorogloeopsis HTF* (“*Mastigocladus laminosus* HTF”) strain PCC7518, and phylogenetic analysis. *FEBS Lett* 1993;317:96–100.
- [21] Bonnet BN, Miller RB, Etherington WG, Martin SW, Johnson WH. Endometrial biopsy in Holstein-Friesian dairy cows. I. Technique, histological criteria and results. *Can J Vet Res* 1991;55:155–61.
- [22] Bonnet BN, Miller RB, Martin SW, Etherington WG, Buckrell BC. Endometrial biopsy in Holstein-Friesian dairy cows. II. Correlations between histological criteria. *Can J Vet Res* 1991;55:162–7.
- [23] Bonnet BN, Martin SW, Gannon VP, Miller RB, Etherington WG. Endometrial biopsy in Holstein-Friesian dairy cows. III. Bacteriological analysis and correlations with histological findings. *Can J Vet Res* 1991;55:168–73.
- [24] Bonnett BN, Martin SW, Meek AH. Associations of clinical findings, bacteriological and histological results of endometrial biopsy with reproductive performance of postpartum dairy cows. *Prev Vet Med* 1993;15:205–20.
- [25] Chapwanya A, Meade KG, Narciandi F, Stanley P, Mee JF, Doherty ML, et al. Endometrial biopsy: a valuable clinical and research tool in bovine reproduction. *Theriogenology* 2010;73:988–94.
- [26] Nagaraja TG, Narayanan SK, Stewart GC, Chengappa MM. *Fusobacterium necrophorum* infections in animals: pathogenesis and pathogenic mechanisms. *Anaerobe* 2005;11:239–46.
- [27] Elad D, Friedgut O, Alpert N, Stram Y, Lahav D, Tiomkin D, et al. Bovine necrotic vulvovaginitis associated with *Porphyromonas levii*. *Emerg Infect Dis* 2004;10:505–7.
- [28] Dohmen M, Joop K, Sturk A, Bols P, Lohuis J. Relationship between intra-uterine bacterial contamination, endotoxin levels and the development of endometritis in postpartum cows with dystocia or retained placenta. *Theriogenology* 2000;54:1019–32.
- [29] Sheldon IM, Rycroft AN, Dogan B, Craven M, Bromfield JJ, Chandler A, et al. Specific strains of *Escherichia coli* are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. *PLoS One* 2010;5:e9192.
- [30] Williams EJ, Fischer DP, Noakes DE, England GCW, Rycroft A, Dobson H, et al. The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. *Theriogenology* 2007;68:549–59.
- [31] Amos MR, Healey GD, Goldstone RJ, Mahan SM, Düvel A, Schubert H-J, et al. Differential endometrial cell sensitivity to a cholesterol-dependent cytolysin links *Trueperella pyogenes* to uterine disease in cattle. *Biol Reprod* 2014;90:1–13.
- [32] Ayliffe T, Noakes D. Effects of exogenous oestrogen and experimentally induced endometritis on absorption of sodium benzylpenicillin from the cow's uterus. *Vet Rec* 1982;110:96–8.
- [33] Herath S, Lilly ST, Fischer DP, Williams EJ, Dobson H, Bryant CE, et al. Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F_{2α} to prostaglandin E₂ in bovine endometrium. *Endocrinology* 2009;150:1912–20.
- [34] Dowd SE, Callaway TR, Wolcott RD, Sun Y, McKeegan T, Hagevoort RG, et al. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiol* 2008;8:125.
- [35] Lorenzen E, Kudirkiene E, Gutman N, Grossi AB, Agerholm JS, Erneholm K, et al. The vaginal microbiome is stable in prepubertal and sexually mature Ellegaard Göttingen Minipigs throughout an estrous cycle. *Vet Res* 2015;46:1–13.