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The microbiota of uterine biopsies, cytobrush and vaginal swabs at artificial insemination in Norwegian Red cows

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Abstract

The individual resistance or tolerance against uterine disease in dairy cattle might be related to variations in the uterine tract microbiota. The uterine tract microbiota in dairy cattle is a field of increasing interest. However, its specific taxonomy and functional aspects is under-explored, and information about the microbiota in the endometrium at artificial insemination (AI) is still missing. Although uterine bacteria are likely to be introduced via the vaginal route, it has also been suggested that pathogens can be transferred to the uterus via a hematogenous route. Thus, the microbiota in different layers of the uterine wall may differ. Norwegian Red (NR) is a high fertility breed that also has a high prevalence of subclinical endometritis (SCE), an inflammation of the uterus that has a negative

effect on dairy cattle fertility. However, in this breed the negative effect is only moderate, raising the question of whether this may be due to a favorable microbiota. In the present study we investigated the endometrial microbiota in NR at AI by biopsy and cytobrush samples, and comparing this to the vaginal microflora. The second objective was to describe potential differences at both distinct depths of the endometrium, in healthy vs SCE positive NR cows. We sampled 24 lactating and clinically healthy Norwegian red cows in their second heat or more after calving, presented for first AI. First, we obtained a vaginal swab and a cytobrush sample, in addition to a cytotape to investigate the animal's uterine health status with respect to SCE. Secondly, we acquired a biopsy sample from the uterine endometrium. Bacterial DNA from the 16S rRNA gene was extracted and sequenced with Illumina sequencing of the V3-V4 region. Alpha and beta diversity and taxonomic composition was investigated. Our results showed that the microbiota of endometrial biopsies was qualitatively different and more even than that of cytobrush and vaginal swab samples. The cytobrush samples and the vaginal swabs shared a similar taxonomic composition, suggesting that vaginal swabs may suffice to sample the surface-layer uterine microbiota at estrus. The current study gave a description of the microbiota in the healthy and SCE positive NR cows at AI. Our results are valuable as we continue to explore the mechanisms for high fertility in NR, and possible further improvements.

Keywords: Subclinical endometritis, microbiota, Norwegian Red, 16S, Uterine biopsy

Introduction

The optimization of dairy cattle fertility is crucial for production efficiency [1, 2] and to reduce emissions per unit of milk [3]. One factor with a major negative impact on fertility is early embryo death [4], which is affected by a suboptimal uterine environment [5]. Certain changes in the uterine microbiota cause uterine disease. For instance, there is an association between dysbiosis and the development of metritis and purulent vaginal discharge [6, 7]. The individual capacity of developing resistance or tolerance against uterine disease might also be related to variations in the uterine tract microbiota [8]. Modulations of the immune response have been suggested as a future perspective in the management of uterine disease [9]. Such advances require an expanded knowledge about variations in the uterine microbiota and associated outcomes. Hence, this is a field of increasing interest. The uterine microbiota is still under-explored in terms of specific taxonomy and functional aspects [8, 10]. Reasons for this might include the challenge in accessing the tissue in a sterile manner in living animals, and the expected low microbial mass. Our understanding of the uterine microbiota has changed with the introduction and development of 16S rRNA metagenomic sequencing. However, a challenge of microbiome studies from low-biomass sites such as the uterine tract is the introduction of contaminants, both during handling and from laboratory reagents. As part of current recommendations, a blank extraction control should be included [11]. Many studies have not included negative controls and might have erroneously appointed contaminants as microbiota present in body sites of low expected microbial biomass [12]. Some studies have shown contradicting results regarding body sites of expected low microbial mass, with one example being the question regarding a human placental microbiota [13, 14]. Hencet is highly relevant to continue the exploration of the reproductive tract microbiota in both healthy and diseased animals, along with refined methodological recommendations. Such data could help us to establish microbial biomarkers and dysbiosis indexes that could improve dairy cattle fertility [8].

To investigate the uterine microbiota, it is common to use flush samples, swabs, or cytobrush samples. One previous study investigated the microbiota of endometrial biopsies, arguing that the deeper layers of the endometrium might possess a different microbiota than the uterine lumen. They speculated that the findings could reveal more invasive bacteria with associations to different diseases or disorders [15]. It has also been suggested that pathogens can be transferred to the uterus via a hematogenous route from the gut [16], and those bacteria might be more abundant in the deeper cell layers of the uterus that can be reached by a biopsy sample.

There are a vast number of studies investigating the microbiota related to metritis, endometritis, or purulent vaginal discharge [7]. Subclinical endometritis (SCE) is another condition that affects fertility in dairy cattle that has been explored during the last 20 years [17]. The condition consists of a persistent presence of polymorphonuclear cells in the post-partum endometrium, exceeding a naturally occurring first line of defense [18]. According to the definition, SCE occurs when there are no symptoms of clinical disease, when the cytological changes occur at a pre-defined level (elevated PMN; referred to as cytological endometritis (CYTO)), and when fertility is reduced [19]. Only two studies have investigated the microbiota related to this condition [20, 21]. Both studies concluded that SCE is not associated with changes in the uterine microbiota. Hence, the current hypothesis states that SCE is mainly affected by uterine immune regulation [20]. However, these studies only investigated the superficial endometrial microbiota at set timepoints post-partum. One previous study investigated the cultivable aerobic bacteria in the uterus at artificial insemination (AI) in healthy cows and cows with mild endometritis defined by vaginal mucus with flecks of pus [22]. However, information about the complete bacterial microbiota at AI is still missing, both concerning the superficial layers compared to deeper layers of the endometrium, as well as potential associations with SCE at this point of the production cycle.

93 Norwegian Red (NR) is the main dairy cattle breed in Norway. Female fertility has been included in the
94 total merit index in Norway since 1972 [23]. As a result, Norwegian Red cows are notable for their good
95 reproductive performance. The breed has a reported pregnancy incidence of 62.9% and calving rate of
96 56.3%, both to first AI [24], and a more recently reported 56-d nonreturn rate (NRR) of 72.9% [25].
97 Recently, we found that NR had a high prevalence of SCE at AI with only a moderate effect on fertility
98 compared to Holstein [26]. One study showed an indication of this condition having a heritable
99 component in NR [27], but the majority of the variation was not explained by this factor. Hence, it is not
100 known if the relatively positive outcome from SCE in NR is due to a beneficial immune regulation, certain
101 traits of the microbiota, other factors, or combinations of such. One step towards a better
102 understanding of this question, was to investigate the uterine microbiota at AI. The main objective of
103 the current study was to investigate the endometrial microbiota in NR at AI, by comparing the deep
104 layer to the superficial layer of the endometrium and considering associations to the vaginal microflora.
105 The second objective was to describe potential differences at these distinct depths of the endometrium,
106 in healthy vs SCE positive NR cows.

Materials and Methods

Experimental design and study population

The present observational cross-section study was conducted at The Animal Production Experimental Centre, NMBU in Ås, Norway, from October 2017 to March 2018. Ethical approval was provided by the Norwegian Food Safety authority with approval ID 11732. The study unit was lactating NR cows in their second heat or more after calving, presented for first AI. The reproductive tract samples were collected from 24 cows on different days according to their natural heat, as detected by activity monitors and visual inspection. Before sampling, all animals were clinically examined by one of three veterinarians. The following parameters were controlled: General appearance, desire to feed, mucous membrane color, rectal temperature, heart rate, respiration rate, and udder appearance. To evaluate the vaginal mucus characteristics, a mucus sample was collected using a Metricheck (Simcro, Hamilton, New Zealand). After washing the vulva and perineum with lukewarm water and chlorhexidine digluconate (Hibiscrub, Mölnlycke Health Care AB, Göteborg, Sweden) the device was advanced to the level of the cervix and withdrawn. The evaluation was conducted according to a scale from 0 to 3 [28]. Only healthy animals with vaginal mucus score 0 (clear or translucent mucus) were included in the study. Body condition scoring at the sampling day was registered by DeLaval Delpro (DeLaval, Ski, Norway) on a scale from 1-5 with 0.1 intervals, which was based on the NR body condition scoring system [29].

To evaluate the milk progesterone level and hence confirm the heat status, a milk sample was collected from each animal, by hand from one teat. A Broad Spectrum MicroTabs tablet was added (D&F Control Systems Inc., Dublin, USA), and the samples were stored frozen at −20°C before laboratory analysis. The progesterone concentration was measured using an enzyme immunoassay [30], modified by a second antibody coating technique. The specificity of the monoclonal antibody for this method was described previously: The intraassay coefficient of variation was <10%, while the inter-assay coefficient of variation

was 9.2% and 5.3%, at milk progesterone concentrations of 1.48 and 19.66 ng/mL, respectively [31]. In the present study, a progesterone concentration of >3.0 ng/mL was considered to indicate that the cow was not in estrus.

Uterine sampling and diagnostic method for cytological endometritis

Sampling from the uterus of each animal was performed together by two veterinarians who had also practiced the procedure together on organs from slaughterhouses. The sampling was performed in two phases. In the first step, we obtained a vaginal swab from the vagina and a cytobrush sample from the endometrium. Here, we also used an ordinary paper tape glued to the top of the inseminator (further referred to as cytotape) to collect cells from the endometrium with the ragged side of the tape. This step investigated the animal's uterine health status with respect to SCE. The second step consisted of acquiring a biopsy sample from the uterine endometrium. A dual-purpose instrument for the collection of a cytotape sample and a cytobrush sample was prepared in the laboratory, according to the method developed by Pascottini et al. [32]. A cytobrush (535010, Jan F.Andersen A/S, Jevnaker, Norway) was attached to the stylet of a sterile stainless steel insemination gun. Cytotape was glued around the top of the same gun, and the device was covered with a disposable plastic tube (Sheath protector tubes, Continental plastic, Delavan, USA). Before sampling, the vulva and the perineum were cleaned with lukewarm water and chlorhexidine digluconate (Hibiscrub, Mölnlycke Health Care AB, Göteborg, Sweden) and dried with paper towels. Avoiding any contact with the external genitalia, a sterile cotton swab was used to collect bacteria from the vagina, and further deposited into a sterile Eppendorf tube. The dual-purpose instrument was then inserted into the vaginal canal and advanced through the cervix to the uterine body. The top of the protective tube was penetrated, uncovering the insemination gun with the cytotape. First, the cytotape was rolled against the endometrium to collect cells, giving it a slight pressure with a finger from the rectum. Secondly, the cytobrush was released into the uterine

153 body and rotated towards the uterine wall in the same manner. The cytobrush was then retracted into
154 the insemination gun, and the insemination gun was again pulled back into the protective plastic tube.
155 After that, the tube was carefully drawn back out of the reproductive canal.
156 The sampling device was transported to the laboratory. To avoid contamination, the plastic tube was
157 dried off with paper and cut off at the top using sterile scissors, and the sample was taken out on the
158 clean, cut, end. The cytobrush sample was directly transferred to a sterile Eppendorf tube, and instantly
159 frozen in liquid nitrogen. It was further stored in a freezer at -80° Celsius. The same freezing conditions
160 were used for the vaginal swabs. The cytotape was rolled against a glass slide and air dried, followed by
161 a fixation and staining using Dip Quick Stain (Jorvet, J0322A1, A2, A3 Jorgensen Laboratories, Loveland,
162 USA). After this, the sample was evaluated by the two veterinarians at 400X magnification in a bright
163 field microscope, counting in total 300 representative PMN and epithelial cells in several fields, and
164 calculating the proportion of PMN. A threshold of 5% PMN was used to diagnose SCE [33].

165 After the SCE diagnosis was set, the second phase of sampling was initiated. Again, the vulva and
166 perineum were washed and dried in the same manner. A sterile biopsy forceps (Kruuse biopsy
167 instrument, 141700 Kruuse, Norway) was covered with a sanitary sleeve (340842 Kruuse, Drøbak,
168 Norway) and introduced into the reproductive tract without touching the external genitalia. The forceps
169 were advanced into the uterine body and the sanitary sleeve was penetrated at the top by pulling it
170 back. With the pressure from a finger on the rectal side, the forceps were pressed against the
171 endometrium to cut off the biopsy. The forceps were then withdrawn from the reproductive tract. Any
172 tissue or mucus on the outside of the forceps was cut off with a sterile surgical blade, and the closed
173 instrument was cleaned of with a paper towel drenched in 70% ethanol. The biopsy was transferred to a
174 sterile Eppendorf tube using a new sterile surgical blade, and instantly frozen in liquid nitrogen, then
175 transferred to a freezer holding -80° Celsius.

After sampling, each animal was artificially inseminated with cryopreserved semen. Information about the pregnancy status at 56 days of gestation was retrieved from the breeding company Geno SA.

DNA extraction, qPCR and sequencing

Thawing and DNA extraction from biopsy, vaginal swabs and cytobrush samples was performed in 13 rounds with 4-5 samples at a time, using the QIAamp Cador mini Pathogen kit (QIAGEN, Hilden, Germany), according to the manufacturer's recommendations. As a negative control for the extraction process, DNA was extracted from DEPC water, and the resulting material was sequenced in the same manner as the rest of the samples. Each extraction round contained one negative extraction control. The negative controls from three different rounds were pooled before sequencing. The biopsies had a weight of 12-25 mg per sample. For efficient lysis of tissue, biopsies were pretreated enzymatically using protocol T2 as described by the manufacturer. In this step, ATL mixed with Proteinase K was added, followed by vortexing and an overnight incubation at 56°C. The negative controls were also subjected to this pretreatment. For further lysis of bacteria, biopsy samples, negative controls, cytobrush and swab samples were subjected to the pretreatment B1, before continuing with the remaining extraction procedure. Nanodrop (Nanodrop 1000, Thermo Fisher Scientific, Waltham, USA) was used to assess DNA quality using the 260/280 and 260/230 ratios, and a Qubit fluorometer with the dsDNA HS Assay kit (0,1-120 ng/ul, Thermo Fisher Scientific, Waltham, USA) was used to assess DNA concentration.

The bacterial count in the different sample types was estimated using qPCR quantification and compared to a dilution series of a standard with known 16S rRNA copy number. For this estimate, a subgroup of 30 samples (the first 30 samples that we extracted DNA from) were individually subjected to this analysis (20 biopsies, 5 cytobrush and 5 vaginal swabs). Copies of the 16S rRNA gene were quantified using a previously described primer set (forward primer: 5'-TCCTACGGGAGGCAGCAGT-3'; reverse primer: 5'-GGACTACCAGGGTATCTAATCCTGTT-3') [34] in a total reaction volume of 20 µl on a

Mx3005p Real-Time PCR System (Agilent Technologies, Santa Clara, USA). Each reaction contained: SYBR GreenER qPCR Supermix Universal Kit (Invitrogen, Waltham, USA), 0.2 µM of each primer, 50 nM ROX dye and 2 µl of template DNA. The cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C, and dissociation for 1 min at 95°C, 30 sec at 55°C and 30 sec at 95°C.

To explore the bacterial microbiota, the V3-V4 hypervariable regions of the bacterial 16S gene were targeted using the 341F/785R primer pair [35]. In addition to the biopsies, vaginal swabs and cytobrush samples, pooled negative extraction controls, negative control (sterile water) and positive control (ZymoBIOMICS Microbial Community DNA Standard II (Zymo Research, Irvine, USA)) were included. Amplification of the 16S V3-V4 region was performed based on the 2-step PCR procedure described in the Illumina application note (https://support.Illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf, 05.01.2023). 32+8 cycles were used. Library size was checked using an Agilent Tape station 4200 with High Sensitivity reagents (Agilent, Santa Clara, USA). Sequencing libraries of expected size (~630 bp) were pooled and size selected on a gel. Sequencing was performed on an Illumina MiSeq, using V3 reagents with 2 x 300 bp reads. 30 % PhiX control library was added to the 16S libraries, and cluster density was reduced to 80% of regular levels. Base calling was performed using Real Time Analysis Software (RTA) version 1.18.54, followed by bclfastq v2.18.0.12 to demultiplex the raw data and produce fastq files.

Statistical analyses

The raw sequences were deposited in the SRA archive (NCBI) with bioproject ID PRJNA841790. Bioinformatic analyses to obtain taxonomy and diversity data were performed using the QIIME2 pipeline version 2021.8 [36]. The DADA2 plugin [37] was applied for filtering, denoising and chimera removal.

The demultiplexed sequences were trimmed at 18 base pairs at the 5' end for all reads, and at 300 and 255 base pairs at the 3' end for forward and reverse reads, respectively. This decision was based on a Phred score with lowest median of 28 and lowest value of 18 in two base pair positions. The resulting high-quality sequences were clustered to amplicon sequence variants (ASVs).

The bacterial taxonomic analyses were performed using a Naïve Bayes classifier trained on SILVA reference sequences version 138.1 [38] that was preprocessed using the rescript plugin [39]. The classifier was further adapted to the investigated region of 16S using the q2-feature-classifier [40]. Once the taxonomy file was generated, the sequences were filtered for mitochondria, chloroplasts, and archaea. Additionally, all features that did not reach a classification of at least phylum level were removed as we suspected them to be a result of host-specific DNA (q2-taxa plugin) [36]. The resulting features were used in downstream analyses. For the generation of the taxa barplot figure in QIIME2, the features were filtered to retain only those that appeared in at least two samples and at least at a frequency of 4000. This was done for the purpose of visual clarity to retain the most highly represented features.

For phylogenetic diversity analyses, a phylogenetic fasttree [41] was generated by aligning the ASVs with MAFFT [42], which integrated the mask method [43]. Further, the alpha rarefaction plot was generated to find the optimal rarefaction depth and investigate whether the sequencing was deep enough.

To study the alpha diversity, we used Chao1 [44], Pielou's Evenness [45] and Shannon metrics [46] in the QIIME2 pipeline at a rarefaction depth of 27.500 at which all samples were included. Pairwise comparisons between sample types and SCE status were calculated using the Kruskal-Wallis analysis of variance after multiple testing correction with Benjamini/Hochberg (non-negative) FDR adjustment. Bray-Curtis measure of dissimilarity [47] and Weighted unifrac [48] in the QIIME2 pipeline were used as estimates for beta diversity. The latter, but not the first, takes phylogeny into consideration. Based on

the beta diversity, the differences between sample types and the SCE status were calculated using PERMANOVA. An adjusted p-value (q-value) of < 0.05 was considered significant.

To investigate which taxa that best could explain the differences between the groups, we used Linear discriminant analysis effect size (LDA-LEfSe) [49] through the online Galaxy tool (https://huttenhower.sph.harvard.edu/galaxy/ 14.11.2022). The class was sample type, and the subclass was SCE status. A significance level of 0.05 was used for factorial Kruskal-Wallis test among classes and the pairwise Wilcoxon test between subclasses, and the effect size threshold was set to 3.0. The strategy for multi-class analysis was one-against-all. We included the negative extraction controls in the analyses in order to eliminate potential contaminating taxa from the comparison.

The low prevalence of SCE in the present study marks a limitation in the statistical analysis of how the microbiota is affected by SCE, and this part of the study should be considered as descriptive.

Results

Descriptive statistics

Twenty-four animals were included in the study, of which three cows were positive for subclinical endometritis. Supplementary Table 1 provides an overview of the descriptive statistics. Out of all included animals, only one was considered to have been sampled and inseminated outside of heat, based on milk progesterone levels. The median DIM was 53 (41 - 67) with one outlier sampled at day 170 after parturition. The body condition score ranged between 3.1 and 4.1 with a median of 3.8. At 56 days after AI, 10 out of the 24 sampled animals did not enter a new estrus with a subsequent insemination, resulting in a NRR of 41.7%.

The sequencing provided a median of 113098 (12970 – 214853) raw sequences per sample. After data cleaning, which included denoising, chimera removal and taxonomic filtering, a median of 60537 (9011-91454) high quality sequences per sample were kept for further analyses. The alpha rarefaction plot confirmed that the sequencing depth was sufficient to describe the bacterial microbiota, as it leveled out for all sample types.

Quantification by qPCR

Supplementary figure 1 shows the number of genome copies estimated by qPCR for a subgroup of 30 samples. Results showed that the bacterial load was highest in the vagina (13.4 - 6126.3 genome copies/µl, median 706), and lower in the cytobrush (1.3 - 16.1 genome copies/µl, median 7.5) and biopsy samples (1.4 - 340.9 genome copies/µl, median 18.6). One cytobrush sample had a very high number of gene copies compared to the others (78048.1 copies/ µl). Using Grubb's test (https://www.graphpad.com/quickcalcs/grubbs1/ 06.01.2023) this sample was detected as an outlier ($p < 0.05$) and was not included in the figure.

Alpha and beta diversity analysis

Figure 1 shows the alpha diversity measurement for each sample type. The Chao1 measurement showed no difference in richness between the different sample types, while both Pileou's evenness and Shannon showed a difference between biopsy and each of the two other sample types ($q < 0.0008$ for both). Supplementary Table 2 presents the outcome from Kruskal-Wallis calculations for each pairwise comparison. No difference in alpha diversity was seen between SCE positive and negative individuals.

The beta diversity is visualized in PCoA plots (Figure 2). For Bray Curtis, the three axes explained 43.1 % of the total differences between the samples, while for Weighted unifrac the corresponding number was 74.1%. For both measurements, there was a clear clustering of the biopsy samples compared to the other sample types, which were more scattered in general in all dimensions. The three biopsy samples belonging to SCE positive animals were clustered together, but that cluster did not differ visually from the other biopsy samples. The PERMANOVA calculations for the pairwise comparison (Supplementary Table 3), revealed a difference between biopsy and the other two sample types ($q = 0.0015$). There was no difference between cytobrush and vaginal swab based on the Weighted unifrac diversity measurement. In contrast, cytobrush vs vaginal swab showed a difference with the Bray-Curtis dissimilarity measurement. For the SCE-status, there was no difference in beta diversity for either of the measurements. There was no clustering of samples based on the lab extraction round or pregnancy outcome.

The negative extraction controls had a low total richness, and a high evenness meaning that the microbiota consisted of an even mix of few taxa, see Supplementary Figure 2. For the beta diversity, these samples were distributed together with the biopsy samples, but also clustered at one end.

Taxonomy composition and differential abundance analysis

In total, 319 bacterial genera were identified. To simplify visualization and interpretation, the dataset was filtered to show only those ASVs appearing in at least two samples and at a frequency of 4000 highlighting the 29 most abundant bacterial genera. Their relative abundances in the different samples are shown in Figure 3. From this outcome, the dominant represented phyla were Proteobacteria, Firmicutes, Actinobacteriota and Bacteroidota. The differential abundance analysis of the complete dataset (Figure 4) showed that Bacilli had a higher abundance in the vaginal swabs and the cytobrush samples, compared to the biopsy samples. In the biopsy samples, Clostridia, Bacteroidia and Bacteroidota were among the enriched taxa.

The bacterial genus with the highest overall abundance in all samples combined was *Streptococcus*, which was present in 16 out of 18 cytobrush samples and all vaginal samples. In 11 out of these samples, *Streptococcus* represented more than half of the relative abundance, and up to 99.5%. It was barely detected in the biopsy samples (< 0.8%). The differential abundance analysis confirmed that Streptococcus was enriched in the cytobrush and vaginal swab samples compared to the biopsy samples. Escherichia-Shigella had a similar pattern with highest abundance in the vaginal swabs. Mycoplasma was also more abundant in the cytobrush samples (identified in 3 samples) and the vaginal swabs (identified in 3 samples).

In the 23 biopsies, we found a high abundance of *Oscillospiraceae UCG-005* in 17 samples (relative abundance 0.6 - 44.7%) and *Bacteroidetes_vadinHA17* in 15 samples (0.3 - 17.8%). Other taxa with high relative abundance in the biopsies were genera *Ruminococcus*, *Bacteroides*, *Alysiella* and four different genera of the family Lachnospiraceae. The mentioned taxa were not present in the negative extraction controls, and barely in the cytobrush and vaginal swab samples.

322 The negative extraction controls showed a variety of taxa that were also present in the other sample
323 types. They were dominated by genera *Massilia*, *Burkholderia*, *Polaromonas*, and *Flavobacterium*. The
324 first three were also present in high abundance in the other sample types. *Massilia* was the second
325 highest represented genus in all samples combined. It was present in all negative extraction controls
326 (relative abundance 32.1 - 58.0%), in all biopsies (1.7 - 89.5%), 15 out of 18 cytobrush samples (0.2 -
327 72.4%), and 4 out of 13 vaginal swab samples (3.9 - 46.6%). *Burkholderia* and *Polaromonas* were the
328 fifth and sixth most abundant genera in general, and had a similar pattern to *Massilia* with respect to
329 appearance in the different sample types. *Flavobacterium* represented up to 15.5% of the relative
330 abundance in the negative extraction controls, but it was barely present in the other sample types (<
331 2.1%).

332

Discussion

Ecologic diversity and bacterial load: sample type

The present study investigated the microbiota of the reproductive tract in NR cows at AI, using three different sample types. We demonstrated that the microbiota of endometrial biopsies is qualitatively different, and more even than that of cytobrush and vaginal swab samples. There were more inter-individual differences in the microbiota of the cytobrush and vaginal samples, than in the biopsy samples. We also found that the microbiota from cytobrush samples and vaginal swabs had no significant difference in alpha or beta diversity and a similar taxonomic composition. This result suggest that the vagina and uterus share a common microbiota, at least when the cow is presented for AI at natural heat. Following this, one may also question the necessity of taking a cytobrush sample instead of a vaginal swab in future investigations of uterine microbiota and its correlation to different disorders and diseases, at least in periods where the anatomical restriction between vagina and uterus is weak, such as in estrus [8]. The microbiota of a biopsy sample, however, could reveal important information that is not captured through the other two sample types. There is some evidence suggesting the transmission of pathogens from the gut to the uterus via the hematogenous route [16]. Possibly, deeper layers have a higher load of bacteria descended from this route while the superficial endometrium and vagina are more likely to be affected by extrinsic and ascending pathways. Nevertheless, the theory about the hematogenous route has also been condemned along with a critical attitude towards the putative existence of a microbiome within the healthy uterine environment [50]. Another explanation of a differing microbiota between the biopsy and cytobrush samples, could be that the findings in the biopsies represents a persistent colonization. After parturition, the caruncular regions of the endometrium are exposed to the uterine lumen. A remodeling and regeneration of the endometrium results in a re-epithelialization at these sites, and the process takes about 30 days [18]. It might be that

356 the bacteria that invaded the tissue after calving and before restoration of the epithelium, are present in
357 the biopsies, while the cytobrush represent bacteria that colonized the endometrium at a later point,
358 without the capacity to cross the epithelial barrier. It is important to remember that the microbial
359 biomass in the biopsy samples and the other two sample types may differ. Our results from the biopsies
360 have to be interpreted with caution, as earlier literature shows that when the starting microbial mass is
361 low, the proportion of contaminant bacterial DNA increases [51].

362 One previous study compared the uterine microbiota using biopsies and flush samples at week 1, 4 and
363 7 post-partum. In concordance with our study, they concluded that the microbiota of the biopsy
364 samples was more diverse. However, their conclusion was based on the Shannon index which takes both
365 richness and evenness into account, while our results showed a higher evenness but not a higher
366 richness in the biopsy samples. Further, they hypothesized that the richness of the uterine microbiota
367 would decrease as the cow got closer to completing the involution process [15]. The present study
368 implies that the uterine biopsies still have a rich microbiota far after the involution process and at the
369 normal timepoint for AI in NR. Moreover, the number of days between calving and sampling (41-170)
370 did not seem to influence the microbial composition in the present study. This result is interesting from
371 a practical point of view, as NR cows are commonly inseminated from day 42 after calving, which is the
372 recommendation from the NR breeding association Geno SA [52].

373 Quantification by qPCR of a subgroup of samples showed that the median bacterial load was much
374 higher in the vaginal swabs compared to cytobrush and biopsy. This is not surprising and concords with
375 results from the female genital tract by Chen et al. [53]. In that study, both the endometrium and the
376 vagina were sampled with sterile swabs. Our results also support our hypothesis that the contamination
377 from the vagina to the uterine samples during the sampling process in field in the current study was low.
378 The bacterial load was similar and of low biomass in the biopsies and the cytobrush samples. The one

379 earlier study that investigated bovine uterine biopsies by 16S analysis did not perform any quantification
380 [15], and neither did the two studies that investigated the microbiota in healthy cows and cows with SCE
381 [20, 21].

382 ## Ecologic diversity: SCE diagnosis

383 Along with collecting three different sample types from the reproductive tract, the individuals in the
384 present study were investigated for SCE. This design was initially set up to calculate the differences in
385 microbiota related to this condition in NR, but it also provided important knowledge of the uterine
386 status in our study population in general. Instead of using the term SCE, CYTO has been suggested as a
387 more appropriate alternative when referring to cytological changes in the endometrium alone.
388 However, the two expressions are not used in a consequent manner in literature. We chose to use SCE
389 in the present article, as it is more commonly used and better coordinates with the two earlier studies of
390 the microbiota related to PMN in the uterus. Interestingly, very few individuals were positive for SCE in
391 the investigated herd, compared to the earlier presented prevalence level of 28% [26]. The prevalence
392 discrepancy confirms that herd factors, management and seasonal variability affect the occurrence of
393 SCE [26, 54, 55]. The three biopsies from SCE positive animals formed a cluster in the beta diversity
394 PCoA visualization which indicates that there are similarities between them, although this cluster did not
395 seem to separate from the remaining biopsy samples. Our results coincide with studies from Wang et al.
396 [21] and Pascottini et al. [20], who both concluded that there was no difference between SCE positive
397 and healthy cows at 30 and 10/21/35 days postpartum, respectively. These two studies used uterine
398 flush samples or cytobrush samples, but neither used biopsies. The microbiota from the deeper layers of
399 the uterus might have a correlation with SCE even if the superficial bacterial population does not show
400 such a correlation. The mechanism for the presence of PMN in the endometrium may well be more
401 affected by invasive bacteria or bacteria present in the deeper layers of the endometrium than the

superficial layers. Likewise, our study pointed to differences between the different sample types. By all means, studying uterine biopsies could influence the current understanding of SCE as a condition affected mainly by the immune regulation of the animal [9, 18]. However, while a biopsy might provide important information, the sampling is less feasible to perform on at high throughput. Furthermore, taking a biopsy from the uterus at AI might itself affect fertility, which is relevant for routine diagnostics or in studies where downstream fertility is a response variable. In the present study, fertility was most likely affected by the biopsy sampling. Few sampled animals (41.7%) maintained their pregnancy at 56 days after AI, comparing our results to the average non-return-rate in NR of 72.9% [25]. Recently, Ramirez-Garzon et al [56] published a review on the effect of endometrial sampling procedures on the subsequent pregnancy rate in cattle. They concluded that endometrial biopsy does not have a negative effect on fertility. However, their paper did not include evaluations of biopsies taken the same day as performing the AI, which was the method used in the current study.

Taxonomic composition

The most highly represented phyla in the present study were Proteobacteria, Firmicutes, Actinobacteriota and Bacteroidota. Our results concord with earlier studies of the bovine reproductive tract using next generation sequencing in healthy cows or cows with SCE [15, 21, 57]. Interestingly, all the mentioned studies also found a high abundance of Fusobacteriota which was not detected at all in the present study. Fusobacteriota is associated with the development of metritis [7, 57, 58] and purulent vaginal discharge [6]. NR has a uniquely low occurrence of metritis and endometritis based on a low registered treatment rate in the Norwegian Dairy Herd Recording System of 1.3 treatments per 100 cow-years [59]. Likewise, Diaz-Lundahl et al [26] found purulent vaginal discharge in only 10 out of 1,648 NR cows when sampling was performed with Metricheck at AI. The reason for the differences in

the abundance of Fusobacteriota might also be partly due to that sampling in the mentioned studies was performed earlier after parturition.

The cytobrush samples and vaginal swabs presented a less even microbiota, which in some of the samples was dominated by only a few taxa, with particularly the class Bacilli highly abundant. *Streptococcus* appeared in almost all cytobrush and vaginal samples, but barely in the biopsy samples. The family Enterobacteriaceae and the genus *Escherichia-Shigella* was detected mainly in the vaginal swabs. Both *Streptococcus* and Enterobacteriaceae have been identified among the most abundant taxa in earlier studies of the vaginal microbiota in the bovine [60]. Wang et al [21] found more *Streptococcus* in healthy cows than SCE positive cows. We did not detect that difference. The taxonomic composition of the vaginal microbiota appears to differ significantly between individuals [8]. This was also indicated in our data. On the genera level, *Bacteroides*, *Aggregatibacter* and *Streptobacillus* are typically highly abundant. *Lactobacillus,* which is the most common vaginal genus in humans, is also commonly detected in the bovine vagina [8]. Interestingly, the biopsies in the present study had some abundance of *Bacteroides*, while it was barely detected in the cytobrush samples, and missing in the vaginal swabs. The same pattern was seen for other taxa of the phylum Bacteroidota. *Aggregatibacter* and *Streptobacillus* were not present in a rich number in our samples in general, although one cytobrush sample had a high abundance of *Aggregatibacter* (85.3%). *Lactobacillus* genus was not represented among the 29 most highly abundant genera. However, there was a high composition of unclassified Bacilli class in the vaginal samples.

One previous study showed that there are differences in the vaginal microbiota in different phases of the estrus cycle in buffalo [61]. Microbial variations throughout the estrus cycle might be a relevant cofounding factor when comparing the microbiota in cows with different fertility outcomes. In our study, the estrus status of each individual was confirmed by milk progesterone measurement, and all

447 animals except for one had a confirmed heat. This animal did not show a deviant pattern in beta
448 diversity or taxonomy.

449 The bacterial composition of the negative extraction controls was dominated by *Massilia* (family
450 Oxalobacteriaceae*)*, *Burkholderia*, *Polaromonas* (which was also highly abundant in other sample types,
451 especially biopsy) and *Flavobacterium* (mainly in negative controls). It is uncertain whether these taxa
452 represent a kit contamination, if they are actually also present in the reproductive tract, or a
453 combination of the two. This question is not only the reality for the current study, but a general concern
454 when studying microbiota in low biomass samples using next generation sequencing. Negative
455 extraction controls can produce a vast number of sequences and represent a high number of taxa due to
456 kit microflora [51, 62]. Well-to-well contamination is common and further complicates the matter, while
457 barcode leakage is indicated to be of lesser importance [63]. An earlier study lists possible
458 contaminating taxa that appeared in different DNA extraction kits over several years, and *Massilia*,
459 *Burkholderia*, *Polaromonas* and *Flavobacterium* are all among the mentioned taxa [64]. Interestingly,
460 Bacteroides and Lachnospiraceae were found in a high abundance in negative extraction controls by
461 Karstens et al. [51]. In the current study, these taxa were present in the biopsies but not in the negative
462 extraction controls. It has been stated that DNA extraction kit contaminants depends on different kits or
463 lots, and also the laboratory in which the samples are processed [64]. We did not observe a clustering in
464 the beta diversity plot, nor any obvious differences in the taxonomy, based on lab extraction round.

465 Of particular concern with low microbial biomass samples, contaminants may play an outsize role, due
466 to less competition from genuine biological material during amplification. We reason that the taxa that
467 distinguish the biopsies from the other sample types, are the ones appearing in the biopsies without
468 detection in the negative extraction controls. Taking this into consideration, Clostridia and Bacteroidia
469 were among the enriched classes in the biopsies compared to the other sample types. Further,

Oscillospiraceae UCG-005, *Bacteroidetes_vadinHA17*, *Marvinbryantia*, *Ruminococcus*, *Bacteroides*, *Alysiella* and three different genera of the family Lachnospiraceae were highly abundant. There is some level of concordance between our results and the results from Knudsen et al [15], who compared the taxonomy in biopsy samples with a superficial uterine sample. They also found a high abundance of Bacteroidia in biopsies at 4- and 7-weeks post-partum, and a higher abundance of *Ruminococcus* in biopsies at week 7. Likewise, they found a high abundance of Streptococcaceae, but only at week 1 post-partum. Even though it was present at a higher abundance in flush samples, it still appeared at a fair abundance in the biopsies, which it did not in the present study. Further, the family Mycoplasmataceae was more abundant in the superficial uterine samples in both studies. The concordance and the fact that these taxa were not present in the negative extraction controls in our study, strengthens the assumption that they are not a result of a random contamination.

Limitations

After this study was initiated, Pascottini et al [65] showed that primiparous cows presented a different composition of uterine bacteria than multiparous cows. This was not considered in the present study and the inclusion of that factor might have affected our outcomes. Another limitation of the study was the low number of SCE positive animals, which made it impossible to draw statistically valid conclusions concerning the microbiota associated with this condition. Hence, that part of the study can only be considered as descriptive.

The most important limitation of this study is related to the use of negative controls. There are large variations in the literature of 16S studies over time and whether this is used or not, and how the results are interpreted, implemented, and presented. The one earlier study investigating the microbiota of uterine biopsies did not mention a negative extraction control [15], and neither did the more recent study by Pascottini et al., although they used sampling blanks as input for Decontam R package to

493 remove ASVs found in control samples from the dataset [20]. Kim et al [11] recommended including
494 negative controls for the full pipeline when investigating low biomass material, and to present the
495 results alongside with the samples. The correct use of sequences appearing in negative controls is still an
496 ongoing discussion [51]. To deal with the issue, some consider all taxa appearing in negative controls as
497 contamination and eliminate them from the dataset. Such an approach might be too strict and might
498 eliminate taxa with a biological relevance . Other methods consist of removing low abundance taxa or
499 taxa that are common contaminants or passing the dataset through programs such as Decontam or
500 SourceTracker. A more recently published method is to use a mock microbial community of known
501 composition as a positive control, which will support the interpretation of possibly contaminating taxa
502 and the findings in a negative extraction control [51, 62]. This approach would have been a good option
503 in the present study. However, the method was published after performing the current study. We did
504 not use a negative sampling control (for instance, a swab in open air in the barn). This is recommended
505 for low biomass samples to be able to detect contamination from the environment [11] and was for
506 example used by Pascottini et al. [20]. Regarding a positive control, we used the swab from the vagina
507 and a positive sequencing control, as suggested by Kim et al. [11] when other types of positive controls
508 are not suitable or cost-effective.
509 In the current study, we filtered the data because we suspected that some sequences were the result of
510 host DNA. Such filtering might skew the outcome and shift the taxonomic composition and the
511 community diversity and presents a common limitation in the study of microbiome data [12]. This might
512 be even more important when analyzing data of low microbial mass. Another important concern is that
513 no region of 16S rRNA can differentiate between all bacteria. Some regions are more likely to better
514 distinguish a certain set of bacteria than other regions, and vice versa [66]. There might be some
515 discrepancies when our results from the V3-V4 region are compared with studies that used other
516 combinations of 16S regions, such as Pascottini et al. [20] (V4) or Knudsen et al. [15] (V1-V2). One study

517 from 2016 suggested that V4-V6 regions are the most reliable to represent the full 16S rRNA [67], but
518 this is also an ongoing discussion within the field of microbiota studies [12].

519 Another concern regarding 16S analyses is that it is not known whether the outcome of such studies
520 comes from viable bacteria or bacterial remnants. As such, relative proportions of viable and non-viable
521 bacteria may differ between both sample types and different anatomical locations. However, Pascottini
522 et al. [20] partly investigated this matter by doing aerobic and anaerobic bacterial culturing of uterine
523 cytobrush samples that were also investigated using 16S rRNA analyses. They found a concordance
524 between the culture results and the most highly abundant bacteria found in 16S analyses [20],
525 suggesting that 16S analysis of these uterine samples probably does reflect mostly live bacteria. On the
526 other hand, the taxonomic composition from the cytobrush samples in the present study had few
527 obvious similarities at the genera level with an earlier study [22] of the cultivable aerobic bacteria at AI
528 in healthy cows, sampled by cytobrush. Nevertheless, both studies showed a high relative abundance of
529 *Streptococcus* in these samples.

530

531 Conclusions and future perspectives

532 The microbiota of endometrial biopsies was qualitatively different and more even than that of cytobrush
533 and vaginal swab samples. It remains to be seen whether microbiota from biopsy samples could be
534 correlated to different disorders and diseases even when superficial cytobrush samples are not.
535 Moreover, the cytobrush samples had a similar taxonomic composition to what could be found in
536 vaginal swabs at estrus, suggesting that vaginal swabs may suffice to sample the surface-layer uterine
537 microbiota, although this conclusion also requires further validation. The current study gave a
538 description of the microbiota in the healthy and SCE positive NR cows at AI. The results from the present

539 study are valuable as we continue to explore the mechanisms for high fertility in NR, and possible

540 further improvements.

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Tables and figures

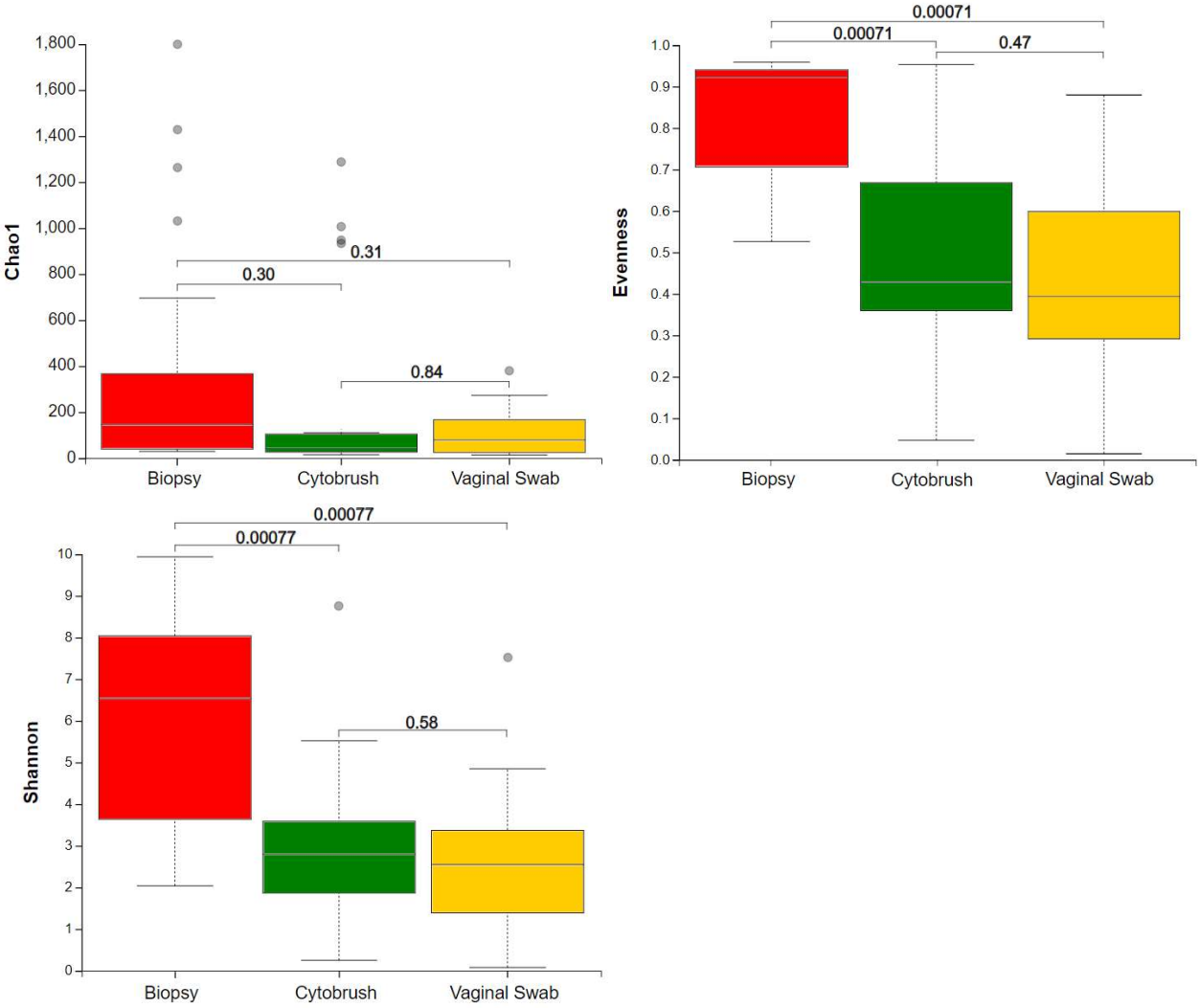
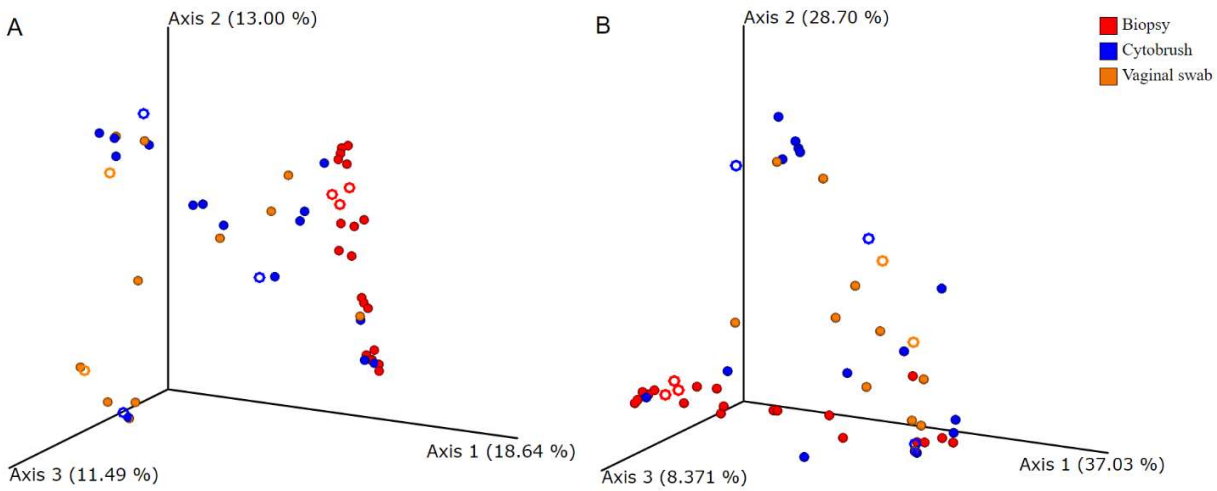


Figure 1: *Alpha diversity for bacterial ASV's from uterine biopsy and cytobrush, and vaginal swab, sampled from NR cows at AI.*



738

739 ***Figure 2:*** *Beta diversity (principal coordinate analysis) of the microbiota in uterine biopsies and cytobrush, and vaginal swabs,*
740 *sampled from NR cows at AI. A: Bray Curtis. B: Weighted Unifrac. Samples from SCE positive animals are marked with a ring.*

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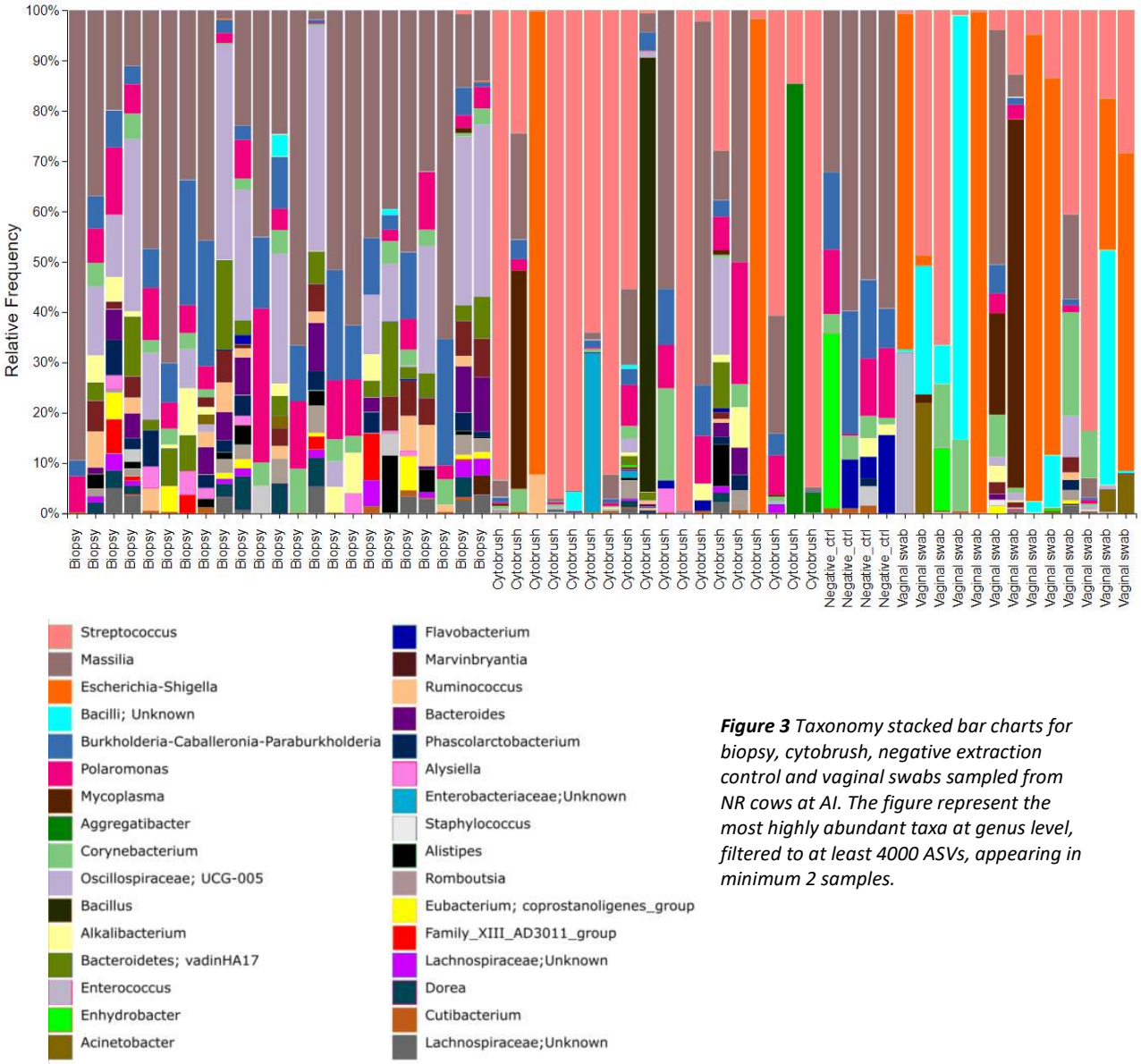

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Figure 3 *Taxonomy stacked bar charts for biopsy, cytobrush, negative extraction control and vaginal swabs sampled from NR cows at AI. The figure represent the most highly abundant taxa at genus level, filtered to at least 4000 ASVs, appearing in minimum 2 samples.*

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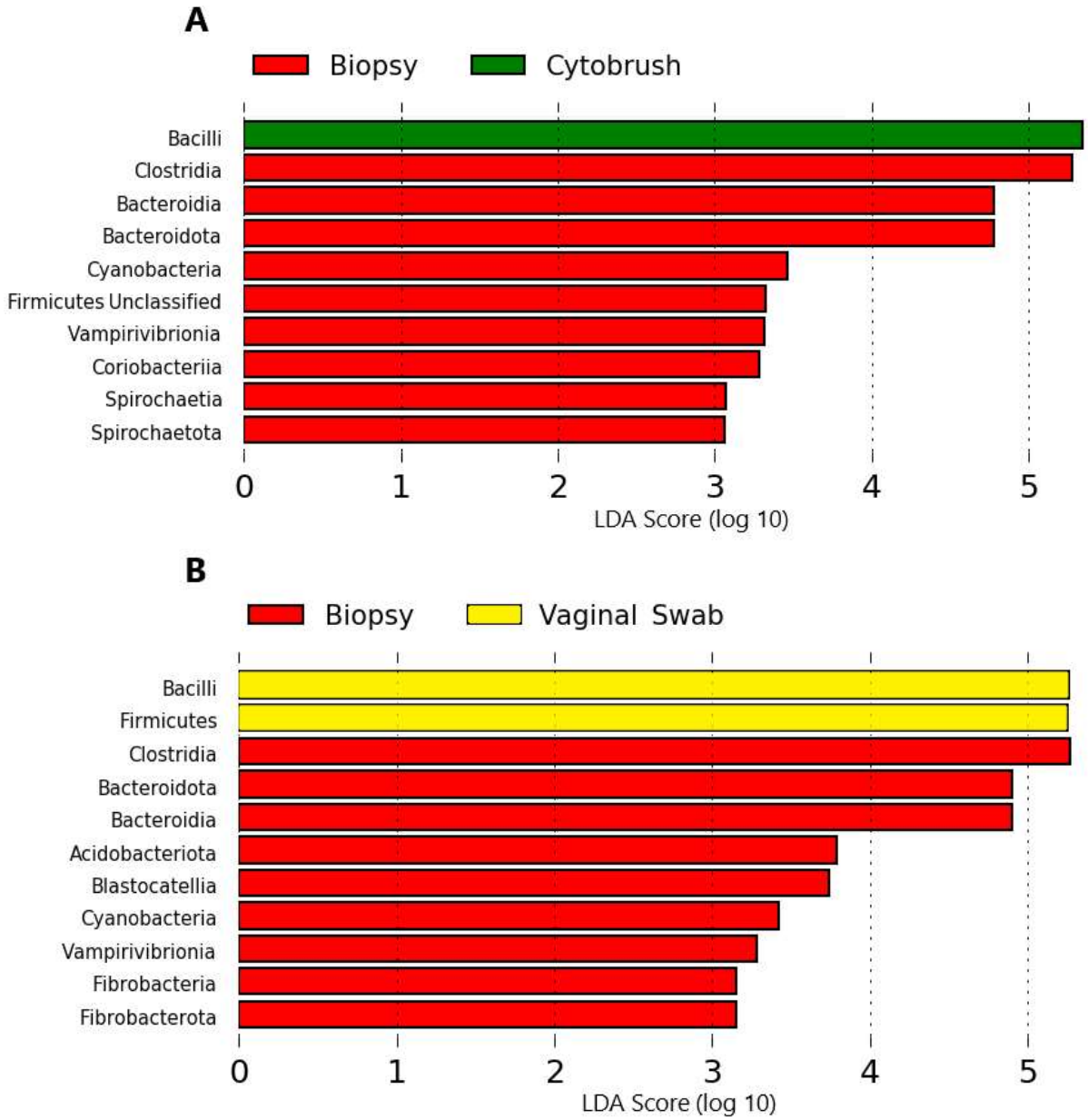

Figure 4: *Logarithmic LDA score for biopsy vs cytobrush (A) and biopsy vs vaginal swab (B) sampled from NR cows at AI.*

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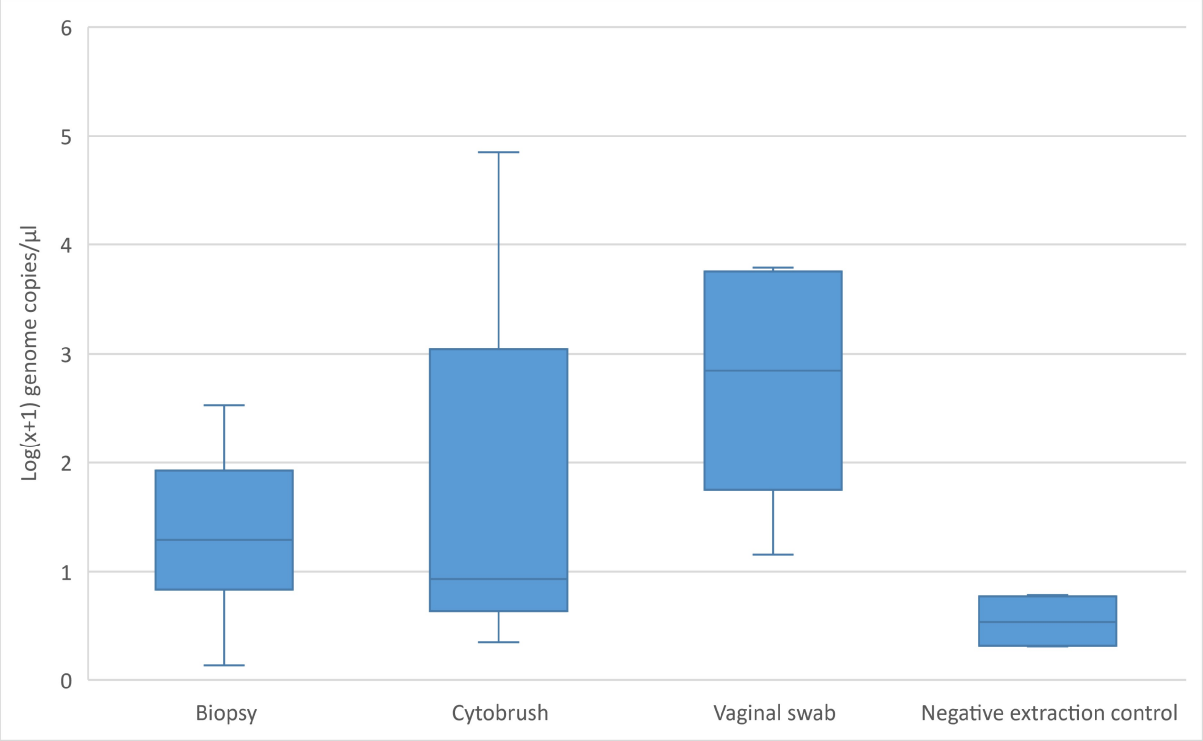
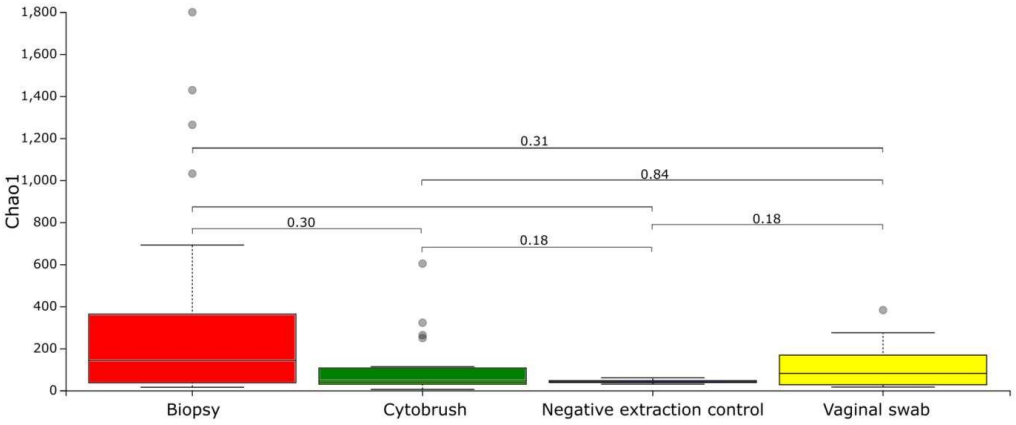


Supplementary Figure 1: *Log(x+1) of 16S rRNA genome copies per µl in a subgroup of 30 samples (20 biopsies, 5 cytobrush samples and 5 vaginal swabs) sampled from NR cows at AI. One cytobrush sample with a copy number of 78048.1 was detected as an outlier and was removed from the figure.*

762



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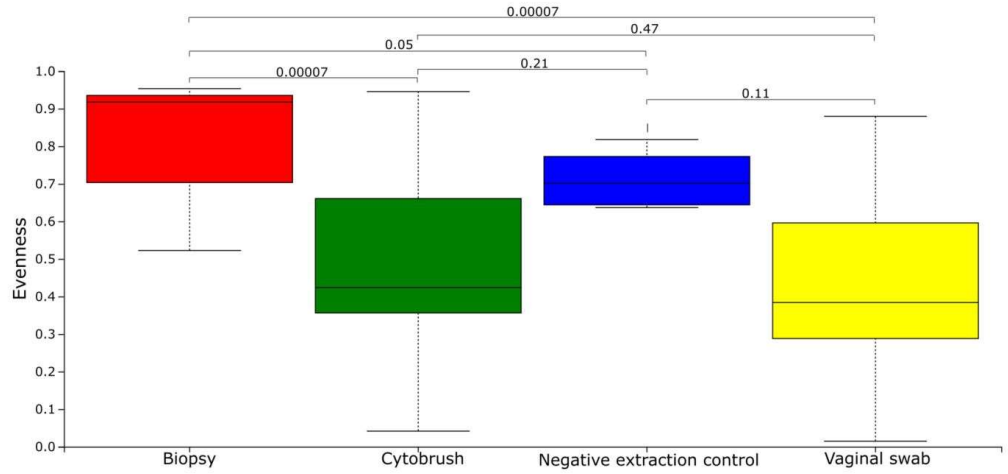

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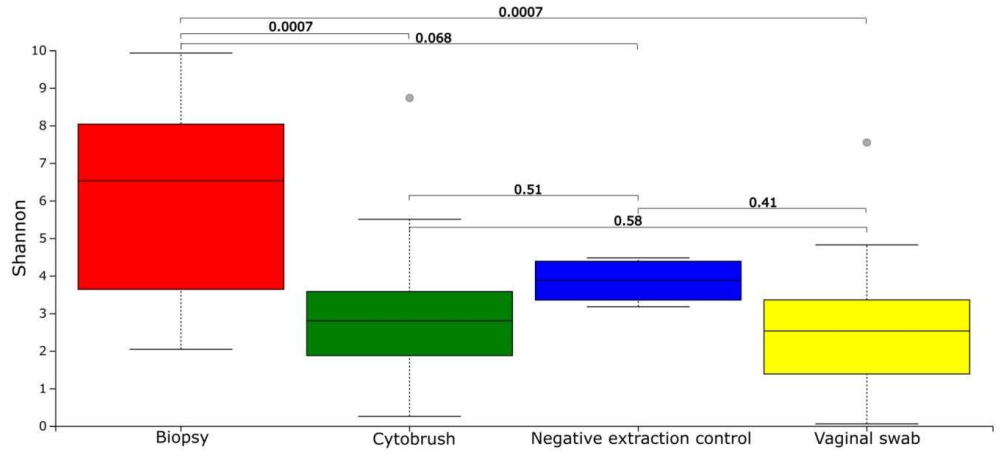

765 ***Supplementary figure 2*** *Alpha diversity for bacterial ASV's for uterine biopsy and cytobrush, negative extraction control and*
766 *vaginal swab, sampled from NR cows at AI.*

767

Supplementary table 1 *Descriptive statistics for the sample types that represented each cow, the diagnosis of subclinical endometritis (SCE) and the number of polymorphonuclear cells (PMN) when counting 300 cells from a cytology slide. Three different sample types were collected from NR cows at AI: B = endometrial biopsy, C = Cytobrush from the endometrium, V = Vaginal swab*

Sample type	Number of animals	Number of SCE positive	PMN count per SCE positive animal
B	23	3	39, 12, 12
C	18	3	39, 12, 12
V	13	2	39, 12
B and C	18	3	39, 12, 12
B and V	12	2	39, 12
B, C and V	9	2	39, 12

Supplementary Table 2: *Pairwise comparison of the alpha diversity between sample types or SE-diagnosis, with Kruskal-Wallis. H=diversity value (or, effect size). q-value= Benjamini Hochberg corrected p-value.*

Metric	Group 1	Group 2	H	p-value	q-value
Shannon	Biopsy	Cytobrush	12.577640	0.000390	0.000767
		Vaginal swab	12.072946	0.000512	0.000767
		Negative extraction control	5.359420	0.020611	0.068702
	Cytobrush	Vaginal swab	0.314103	0.575174	0.575174
		Negative extraction control	0.360000	0.548506	0.506729
	Vaginal swab	Negative extraction control	0.930769	0.334663	0.418329
Chao1	Biopsy	Cytobrush	2.739369	0.097903	0.293710
		Vaginal swab	1.608620	0.204686	0.307029
		Negative extraction control	6.626928	0.010045	0.100448
	Cytobrush	Vaginal swab	0.040105	0.841276	0.841276
		Negative extraction control	2.290258	0.130188	0.179939
	Vaginal swab	Negative extraction control	3.181356	0.074483	0.179939
Evenness	Biopsy	Cytobrush	17.228433	0.000033	0.000071
		Vaginal swab	16.544066	0.000048	0.000071
		Negative extraction control	5.869565	0.015405	0.051350
	Cytobrush	Vaginal swab	0.641026	0.519231	0.471170
		Negative extraction control	1.777778	0.182422	0.202692
	Vaginal swab	Negative extraction control	4.069231	0.043671	0.109178
Shannon	SCE positive	SCE negative	0.048024	0.826539	0.826539
Chao1	SCE positive	SCE negative	0.432345	0.51084	0.510840
Evenness	SCE positive	SCE negative	0.261462	0.609117	0.609117

Supplementary table 3 *Pairwise comparision of the beta diversity calculated by PERMANOVA between sample types or SCE-diagnosis. q-value= Benjamini Hochberg corrected p-value.*

SCE diagnosis, pairwise						
Metric	**Group 1**	**Group2**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
Weighted Unifrac	SCE positive	SCE negative	54	0.563282	0.678	0.678
Bray-Curtis	SCE positive	SCE negative	54	0.974616	0.465	0.465
Sample type, pairwise						
Metric	**Group 1**	**Group2**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
Weighted unifrac	Biopsy	Cytobrush	41	8.275838	0.001	0.0015
		Vaginal swab	36	9.377037	0.001	0.0015
		Negative extraction control	27	9.792818	0.001	0.0033
	Cytobrush	Vaginal swab	31	1.038841	0.353	0.3530
		Negative extraction control	24	4.912167	0.004	0.0080
	Vaginal swab	Negative extraction control	19	5.185061	0.002	0.0050
Bray-Curtis	Biopsy	Cytobrush	41	3.623646	0.001	0.0015
		Vaginal swab	36	6.056833	0.001	0.0015
		Negative extraction control	27	2.183685	0.027	0.0540
	Cytobrush	Vaginal swab	31	1.832312	0.045	0.0450
		Negative extraction control	24	2.961306	0.001	0.0033
	Vaginal swab	Negative extraction control	19	6.167003	0.001	0.0033
Sample type all (biopsy, cytobrush, vaginal swab)						
Weighted unifrac			53		0.001	
Bray-Curtis			53		0.001	

Author contribution

Sofia Diaz-Lundahl: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft

Simen Foyn Nørstebø: Conceptualization, Data curation, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing - review and editing.

Thea Blystad Klem: Conceptualization, Investigation, Methodology, Resources, Writing - review and editing.

Gregor Duncan Gilfillan: Data curation, Formal analysis, Methodology, Resources, Writing - review and editing.

Marianne Dalland: Data curation, Formal analysis, Resources, Writing - review and editing.

Per Gillund: Conceptualization, Funding acquisition, Project administration, Resources, Writing - review and editing.

Anette Krogenæs: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing - review and editing.