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Characterization of microbiota in the uterus and fallopian tubes of healthy dairy cows and cows with clinical endometritis

Diplomarbeit

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vorgelegt von

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1. Introduction

1.1 Microbiota and animal health

Over the last decades, many scientific efforts have been made to disentangle microbial compositions and their impact on human and animal health. Many of these efforts were based on cultivation and enrichment methods, although most microorganisms, approximately >99% in soil and water, could not be cultivated (1)(2). Although microorganisms developed around 3.8 billion years ago and have been omnipresent in nearly all environmental niches, it was due to advances in high-throughput detection methods arising from 2010 onwards, which have made it possible to get the entirety of microbial genetic content analysable. In the last decade, microbial physiology, motility, cell division mechanisms, pathogenicity, tolerance, persistence, and resilience as well as a possible link to heath parameters could be investigated in detail and with high resolution, all based on high-throughput sequencing analysis (3)(4).

There is ongoing research in all biosciences focusing on the immense diversity of microorganisms that inhabit the Earth. Recent study has found that there are about 10^{12} different microbial species on our planet (5). Besides of bacteria, many other types of microorganisms co-exist, such as fungi (e.g., *Candida*), protozoa (e.g., ciliates), microalgae (e.g., chlorella), and archaea (e.g., *Methanobrevibacter*) (6). The concrete number of different microorganisms remains unknown and is estimated to range from millions to trillions (5, 7). Microbes play key roles in many food chains as creators. However, they also act as destroyers by converting organic material to inorganic matrix. Mammals depend on a range of microbes to keep them healthy and vertical transmission as well as mutualistic exchange of microbes between adults via group living is essential for a homeostatic development of the microbiome (8). The gastrointestinal tract is a very complex niche for microbes and interacts permanently with its micro-inhabitants (9) (10), which are also responsible for a physiological, early anatomical and physiological development (11). Microorganisms have a multitude of essential roles for the gastrointestinal tract (GIT), including food digestion, delivering vitamins and amino acids, supporting critical immune system processes and maintaining intestinal homeostasis and systemic health of the host (12, 13). There are 55 bacterial phyla known until now, from which just three of them dominate the GIT. This gives a hint to the assumption, that mammals have coevolved with these GIT-specialized microorganisms to benefit from symbiosis and intestinal

homeostasis (14). Not only the GIT but also mammals' skin and the reproductive tract are colonized by microorganisms, which shield them from pathogenic microbes and preserve the healthy environment (15, 16). Also the reproductive system of cows is home to a variety of bacteria, such as *Firmicutes* and *Proteobacteria*. The microbiome of the vagina and uterus is influenced by cyclical changes and is significantly shaped by birth cycles and the subsequent postpartum period (17, 18).

1.2 The microbiome in the reproductive tract of cows

The microbiome of the cow's uterus has been described during the last years and commensals, opportunistic bacteria, but also pathogens were associated with this organ (19, 20). Before 2010, all research on the uterine microbiome in cows was culture-dependent and mostly involved cows with clinical endometritis (i.e., with purulent uterine discharge) (21–24). A wide diversity of *Streptococcus* spp*., Staphylococcus* spp., and *Bacillus* spp. were isolated from healthy cows (21, 23, 24, 25). Other bacteria, being classified as commensals or by-passers were affiliated to *Lachnospira, Rikenella, Acinetobacter,* and e.g. *Prevotella*, which are all genera well known for their occurrence in mammal environments (26) (27). In contrast, the occurrence of *Escherichia coli, Fusobacterium necrophorum, Prevotella melaninogenica* and *Bacteroides* species, as well as *Trueperella pyogenes* was consistently linked to clinical endometritis in those trials (21, 23, 24, 25, 28). Also in this field of research, microbiological cultivation greatly improved our knowledge of microbial diversity, but a large set of microorganisms (e.g., slow growers, oligotrophs, fastidious microbes, and dormant-state microbes) remained uncultivable until now (29, 30) and needed molecular-biological high-throughput methods to be elucidated. Since 2010, culture-independent studies have started to investigate the microbiome of healthy cows and cows with metritis and clinical endometritis. These studies have found that *Eschericia coli* was a pioneer pathogen that predisposed cows to infection with *Fusobacterium necrophorum,* which was highly related with metritis, and with *Trueperella pyogenes*, which was strongly associated with clinical endometritis (31) (32). *Eschericia coli* or *Trueperella pyogenes* can damage the uterine wall, interfere with the resumption of ovarian activity after calving, and significantly reduce the likelihood of pregnancy (33, 34). Pathogens that were less abundant belonged to the genus of *Peptostreptococcus, Filifactor, Prevotella, Helcococcus, Peptoniphilus* and *Arcanobacterium* (19) (36) have been found in healthy as well as diseased

animals, although in healthy animals they might be controlled by the occurrence of dominant commensal bacteria (37).

It also has been demonstrated that cows have bacteria in their uterus even before they give birth. *Firmicutes, Bacteroidetes*, and *Proteobacteria* were the three most prevalent phyla found in the uteri of pregnant cows and virgin heifers (38). It is interesting to note that numerous bacterial species, including *Trueperella* spp., *Acinetobacter* spp., *Fusobacteria* spp., *Proteus* spp., *Prevotella* spp., and *Peptostreptococcus* spp. were also found in the uteri of virgin heifers and pregnant cows (38). Due to the open cervix, the uterine lumen of cattle is colonized by a diverse microbiome after calving, and microbial diversity undergoes a dynamic process in the weeks following parturition (39). Cows have an established uterine microbiome shortly after calving, and the microbiome structure is the same in healthy cows and cows who develop metritis until 2 days after giving birth (19). Subsequently, the bacterial structure of cows who developed metritis deviates in favour of greater relative abundance of *Bacteroidetes* and *Fusobacteria* and lower relative abundance of *Proteobacteria* and *Tenericutes* (19). The strongest genus-level associations with metritis are found in *Bacteroides*, *Porphyromonas* and *Fusobacterium* (24, 36, 37, 40). Regarding clinical endometritis, similar results have been presented. The relative abundance of *Bacteroidetes* and *Fusobacterium* is higher in cows with clinical endometritis (41–43). These findings demonstrate that the uterine microbiomes of cows with clinical endometritis and cows with metritis are similar. Dairy cows with clinical endometritis may also have lower fertility (44–47). The precise mechanisms causing this are yet unclear. The ovarian and fallopian tube function, as well as endometrial receptivity, are all crucial for cow fertility (48). These factors are all impacted by a complex interplay of anatomical, hormonal, metabolic, and immunological factors. Microbial imbalance has the potential to interfere with this interaction (49, 50). Nearly half of all dairy cows globally are affected by post-partum microbial infections that cause uterine illness (51). For the farm, uterine diseases hold a great economic significance.

So far, there is no study that comprehensively examines the microbiological colonization of the fallopian tubes in cows with endometritis. There is also no guideline how to take samples in a way to avoid cross-contamination or recontamination, or protocols tested for DNA extraction. This is also due to the difficult accessibility of these organs. With the use of transvaginal

endoscopy, a method has now been developed that allows samples to be routinely obtained from the fallopian tube in the living animal. When this method was used, it was found that this procedure had no negative influence on the further fertility of the animals (52, 53).

1.3 Detection methods for bacteria in clinical microbiology

Over time, microbial detection techniques have steadily evolved. The most significant techniques to date are summarized in the section below.

1.3.1 Classical cultivation methods

Microbiological cultivation is a method of propagating microbial organisms that replicate in a medium under controlled laboratory conditions. Microbial cultivation is an important diagnostic method, even gold standard in many areas of diagnostics and research. However, bacterial growth is influenced by many factors, including atmospheric composition, temperature, incubation time, and nutrient supply. To date, a large proportion of bacteria have not been identified by cultivation methods, as the requirements for conditions vary greatly among species and can be very specific (54). Bacterial cultures allow sequencing of the genome, as well as comparison of transcription under different conditions and the phylogenetic relationship of strains to each other *in vitro* (55). Another use of pure cultures is knock-out experiments, where bacteria can be manipulated and transformed by adding or deleting genes (56). This can be used to analyse the cause of virulence and antibiotic resistance as well as the invasive potential of a microorganism (57). However, bacteria can also enter a viable but non-culturable state (VBNC), this is a form of dormancy. The main characteristic of dormant cells is the shutdown of global metabolism and thus the ability to evade detection in culture and to tolerate stressful environments, including, for example, attacks by the host immune system or drug administration (58). Because it is not possible to detect populations of cells in a resting state in culture media, using such standard culture methods to detect pathogenic organisms always carries risk and is not optimal for describing the diversity of microorganisms (59). At this point, culture-independent techniques allow for faster and more accurate differentiation (60).

1.3.2 PCR-based methods (conventional PCR and qPCR)

The PCR method is based on a duplication principle for nucleotide strands. Fragments up to a length of approx. 3 kbp can be multiplied. To perform PCR, various reagents must be added to

(c)DNA: a primer pair, DNA polymerase, deoxyribonucleoside triphosphates (dNTPs) and a buffer solution. The samples are then run through a specific temperature cycle that is repeated 20 to 50 times. Each cycle consists of three steps. First, denaturation, in which the double-stranded DNA is separated. Second, annealing, where the primers attach to the DNA. And third, elongation, in which the polymerase incorporates the complementary dNTPs into the single strands. The result is innumerable, double-stranded copies of the DNA (61). As PCR is often used in bioscience and diagnostics, this method has been steadily improved over time (62). PCR can be quantified by using quantitative real-time PCR (qPCR). After each amplification cycle, endpoint measurements are performed. Therefore, additional analysis to estimate the quantity by gel electrophoresis is no longer required (63). In contrast to culturing and conventional PCR, the qPCR method is quantitative and often reaches very good sensitivity (64). The low specificity of fluorescent dyes often used in qPCR, however, is a drawback (instead, probes can be used), and with normal qPCR assays, it cannot be distinguished between live and dead cells. Today, though, there are qPCR assays available that employ reagents and dyes to remove free DNA and detect only DNA from viable cells.

1.3.3 Sequencing methods

Dr. Frederick Sanger released his chain termination technique (also known as the didesoxy DNA sequencing technique) in 1977 (65). Continuous development of the didesoxy method has enabled the establishment of new generations of sequencing strategies that are significantly faster and less expensive (66). Microbes that were previously impossible to culture can now be detected and identified with the use of these technologies. Sequencing methods are now used in many areas of science, and they are divided into three generations. Sanger sequencing is the first generation of "first-generation sequencing" (FGS), which served as the foundation for the development of other sequencing technologies known as "next-generation sequencing" (NGS) (67). In recent decades, tremendous progress has been made in terms of speed, read length, and throughput, along with a large reduction in cost per base pair (68). Nowadays, NGS enables microbes to be detected qualitatively and quantitatively in real-time and in vivo, thus providing new insights into their physiology and microbial ecology. A detailed picture of the microbial world is now possible thanks to taxonomic profiling, which were previously impossible (65, 69, 70).

1.3.3.2 Next- Generation- Sequencing (NGS)

At the beginning of the 21st century, next-generation sequencing (NGS) was developed (72). With this technique, which can be divided into different groups (Fig. 1), scientists can sequence thousands to millions of DNA molecules parallel in a single run. NGS includes second-generation sequencing (SGS) and third-generation sequencing (TGS) (Fig 1.). NGS enables genome sequencing at a tremendous speed and at the same time high-cost savings (70). For example, the human genome can be sequenced within one day (73).

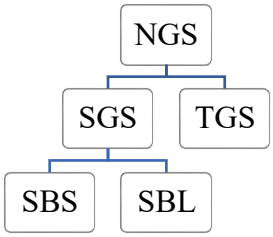


Figure 1: Overview of next-generation sequencing (NGS.) SGS (second-generation sequencing), TGS (third-generation sequencing), SBS (sequencing by synthesis), and SBL (sequencing by hybridization and ligation).

As summarized in Ambardar et al. (2016), the basic principle of second-generation sequencing (SGS) is based on four steps: The first step is fragmentation. In this process, DNA fragments are generated enzymatically, mechanically, or chemically. The next step is adaptation. Here, specific adapters are bound to the previously created fragments. These fragments then bind to a stable surface (e.g., a chip) and the amplification of the DNA can begin (74). Sequencing time can be reduced by grouping identical DNA into so-called clusters. Data analysis is the last phase. Here, the collected data is subjected to bioinformatic analysis. SGS procedures include sequencing by synthesis (SBS) and sequencing by hybridization and ligation (SBL) (74). Sequencing by synthesis (SBS) can be divided into three procedures. The first is pyrosequencing, which is based on the detection of pyrophosphate. The pyrophosphate is formed during DNA polymerization and the release is coupled with a fluorescence signal, which is detected. The second method involves sequencing by reversible termination (Illumina sequencing), which uses fluorescent dNTPs to follow the sequencing in real time (74). Illumina sequencing, which was used for this work, requires amplified sequencing libraries, before sequencing can be performed. Libraries are DNA fragments with integrated adaptors (see

1.3.3.3 Illumina sequencing). The sequencing by ligation (SBL) method is known as SOLiD (Support Oligonucleotide Ligation Detection). Its foundation is two base sequencing. The probes bind at regular intervals successively, always identifying two bases (74). Since there are various approaches, selecting the best SGS frequently depends on the desired coverage (i.e., the frequency of base sequencing within a complete sequence). The higher the coverage, the clearer the assignment to reference sequences (75).

Because the read length of second-generation techniques is constrained, third generation NGS was created. The Pacific Biosciences SMRT (Single Molecule Real Time), Sequencing HelicosTM Single Molecule Sequencing, and Nanopore DNA Sequencing are examples of third generation (TGS) techniques. These procedures enable real-time single molecule sequencing without the requirement for costly and time-consuming pre-generated libraries (75).

1.3.3.3 Illumina Sequencing

For the scientific work conducted in this diploma thesis, Illumina MiSeq sequencing was used. In the first step of the Illumina workflow, the genomic DNA is fragmented (~500 bp) and an adapter ligation, called library production, is performed at both ends. The DNA fragments then bind randomly via the adapters to the surface of an Illumina Flow Cell (special glass slide), which is provided with complementary adapter molecules. As a result of both ends binding to a free adapter on the stationary phase, a DNA bridge is formed. The polymerase starts to synthesize the complementary strand and the so-called bridge PCR runs. Many DNA fragments with identical sequence are produced. These copies are called clusters. In the final step, the polymerase incorporates the dNTPs previously labelled with different fluorescent dyes (https://www.illumina.com/science/technology/next-generation-sequencing/illumina-sequencing-history.html (Access: 13.02.2023)).

High-throughput sequencing of the 16S rRNA gene on the Illumina platform is often used to assess microbial diversity in many environmental samples (76). Illumina sequencing machines offer different throughput rates. The MiniSeq incorporates a wide range of capabilities while maintaining a small footprint and relatively low purchase price. This product is designed to provide access to Illumina for even the smallest laboratories. In 2011, Illumina released MiSeq, a lower-throughput, rapid run instrument aimed at smaller laboratories and for making clinical diagnoses (77). Another instrument is the NextSeq, which combines proven technologies,

including next-generation sequencing and high-resolution Array scanning (https://www.illumina.com/systems/sequencing-platforms.html (Access: 13.02.2023)). Table 1 shows the properties of the different Illumina systems.

	MiniSeq	**MiSeq**	**NextSeq 1000&2000**
Output	1.65- 7.5 Gb	0.3–15 Gb	30-360 Gb
Run time	4-24 h	5–55 h	11-48 h
Reads per run	7- 25 mio	1–25 mio	100 mio- 1.2 bill
Max Read lenght	2 x 150 bp	2 × 300 bp	2 × 150 bp
Samples per run	50	1- 384	30-90

Table 1: Comparison of different Illumina sequencing machines (https://www.illumina.com/systems/sequencing-platforms/comparison-tool.html#/research-use-only/microbiology/small-whole-genome-sequencing (Access 14.02.2023)). Mio = millions, Bill= billions, h= hours, bp= base pairs, Gb= giga base pairs, Tb= tera base pairs.

1.4 Hypothesis and research questions

The objective of this work was to characterize the microbial composition, both qualitatively and quantitatively, in the uterus and fallopian tubes of healthy dairy cows and of cows with clinical endometritis. Modern, cutting-edge bioinformatic technologies are used for the analysis.

Hypotheses: The composition of the uterine microbiome post-partum is the basis for colonization of the fallopian tube. The microbiome of healthy animals differs significantly from the microbiome of endometritis cows in the uterus and in the fallopian tube, specifically in diseased animals there is

i.) a reduced microbial diversity in the uterus
ii.) an increased bacterial load in the fallopian tube and
iii.) a different composition of the microbiome at both locations.

2. Material and methods

The study was carried out at the VetFarm Kremesberg of the University of Veterinary Medicine, Vienna. The Vetfarm has housed around 80 Simmental dairy cows in a free stall barn.

The occurrence of clinical and subclinical endometritis in the herd was determined retrospectively with an average frequency of about 25% each. The study has been approved by the Vetmeduni Vienna ethics committee and the national authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012 – TVG 2012 (GZ 2020-0.228.095).

2.1 Sampling

A total of 22 Simmental dairy cows were included in the study on the day of calving. The animals were gynecologically examined weekly after calving (clinical and gynaecological examination according to Baumgartner, vaginoscopy, endometrial cytobrush smear and transrectal ultrasound). On day 28-35 postpartum (pp) the group was divided into the groups "clinically healthy" and "clinical endometritis". Animals that showed mucopurulent or purulent vaginal discharge on gynaecological examination were defined as having clinical endometritis.

The extraction of cell material from the uterus using a cytobrush (gynobrush, Heinz Herenz, Germany) is equivalent to the technique of artificial insemination and has been used and published in numerous studies by the herd management department of the Vetmeduni Vienna (78, 79). The sterile cytobrush (diameter 0.7 cm), which was in a plastic catheter, was screwed onto a metal rod and was protected by a plastic cover. After dry cleaning of the vulva and wetting the plastic sleeve with NaCl, the cytobrush was inserted vaginally and advanced into the uterus under rectal control. After perforating the plastic sheath and advancing the brush out of the catheter, cell material was obtained by rotating the brush halfway along the uterine wall.

Samples were taken from the fallopian tube using a metal catheter under endoscopic control after application of epidural anaesthesia (4 - 5 ml 2% procaine hydrochloride solution) as described by Papp et al. (2019) (53). The working shaft of the endoscope has a second channel through which the catheter was inserted. The fallopian tubes were then flushed with 0.5 ml PBS solution and then suctioned off again. The rinsing process was repeated once or twice. All samples were stored at -80°C.

2.2 DNA Extraction

Because samples were expected to be low biomass, a total of 39 negative controls were taken, of which 21 negative controls were taken between cow sampling, 13 PBS negative controls were taken (PBS was used for flushing), and 5 kit extraction controls (blank control) were included. 43 tubal lavage samples and 43 uterine cytobrush samples were extracted.

2.2.1 Fallopian tube flush samples

First, the protocol "Pretreatment for Gram- Positive Bacteria from the dNeasy Blood & Tissue Handbook 07/2020" was applied. Subsequently, the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)" was used. Elution with 2 x 20 µl was used to increase the final DNA concentration in the eluate, but also decreases the overall DNA yield. Samplings were frozen at -20°C after elution. The details of the modified protocols can be found in the Appendix.

2.2.2 Uterus samples

An already published protocol was used for processing the uterus samples (80). For the smear samples from the uterus, the cells retrieved on the cytobrush, were submerged in up to 5 ml of sterile Ringer solution. Samples were vigorously horizontally agitated (5 min) to dislodge cells from the cytobrush by using the MOBIO Vortex adapter tube holder for the vortex. The cells were pelleted by centrifugation at 12,000×g for 10 min at 4°C. After that, the cytobrush was carefully removed, and the centrifugation step was repeated. The pellet was applied to the DNA extraction.

Pretreatment for Gram- Positive Bacteria and the Protocol Purification of Total DNA from Animal Tissues (Spin- Column Protocol) were carried out. The details of the modified protocols can be found in the appendix. Samplings were frozen after elution.

2.3 DNA measurement

The concentration of double-stranded DNA was determined fluorometrically in all samples using a Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Oregon, USA). The QubitDye dsDNA HS reagent is highly selected to double-stranded DNA. The fluorescent dye was used to determine the DNA concentration of the sample. The DNA determination was performed as described in the Qubit® dsDNA HS Assay Kits protocol

(https://assets.fishersci.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf?_ga=2.149549365.241971921.1682267308-169547673.1682267308 (Access: 15.04.2023)).

2.4. Quantitative PCR

Total bacteria counts were quantified for all with a TaqMan qPCR assay (81), that includes a conserved region of the 16S rRNA gene (466 bp). Forward 5′- CCTACGGGDGGCWGCA-3′ and reverse primers 5′- GGACTACHVGGGTMTCTAATC -3′ as well as a probe 6-FAM-5′-CAGCAGCCGCGGTA-3′-MGBNFQ were included in this assay. For quantification of total bacterial counts, we pipetted standard curves by using the same primer-probe combination with all different sample types. DNA concentrations were calculated using a Qubit® 2.0 Fluorometer (Qubit® dsDNA BR Assay Kit; Thermo Fisher Scientific, Vienna, Austria). The standard curves quantities were assessed based on the formula published in Lee, Shannon et al. (2006) (81):

$$DNA\ copies = \frac{(6{,}03 \times 1023) \times \left(\frac{copy}{mol}\right) \times DNA\ amount\ (g)}{DNA\ lenght\ (bp) \times 660 \frac{\frac{g}{mol}}{bp}}$$

Total bacterial counts were extrapolated considering 5 gene copies per bacterium. This estimate was published (82), and is an estimate from rrnDB, a database for RNA operon variation.

The 20µl reaction mixture for qPCR included of 12.33µl diethylpyro-carbonate -treated water, 2.0µl 10×buffer (Invitrogen, Carlsbad, USA), 1.4µl 3.5mM MgCl2 (Invitrogen), 0.36µl of primers (stock concentration 10 µM), 0.45µl of the probe (stock concentration 10 µM), 0.8µl of dNTP Mix (Thermofisher, Vienna, Austria), 0.3µl of Platinum Taq DNA polymerase (5 U/µl; Thermo Fisher Scientific) and 2 µl target DNA. For qPCR reactions we used a Mx3000P qPCR instrument (Strategene, La Jolla Ca USA, MxPro software v.4.10). The program included the following steps: 1. initial denaturation at 95°C for 10 min, 2. 40 cycles of 95°C for 15 s and 3. 60°C for 1 min and negative extraction controls and qPCR controls were included in each qPCR run.

2.4 Sequencing

A total of 24 animal samples (12 tube flushing, 12 uterus cytobrush samples) and 8 negative controls (3 PBS negative controls, 3 negative controls taken between cow sampling and 2 kit

extraction controls) were sequenced with Illumina High-throughput Sequencing. From the 24 animal samples, half of them were taken from healthy and the other half from endometritis associated cows. The presence of sufficient biomass was first verified by 16S rRNA gene PCR using primers (27F (5'- AGAGTTGATCMTGGCTCAG-3') and 1492R (5′-GGYTACCTTGTTACGACTT-3'). For high-throughput sequencing, libraries for amplification of the 16S rRNA gene (V3/4 region) were prepared based on the recommendations for preparation of the Illumina 16S Metagenomic Sequencing Library. Primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') (83) were used together with Illumina adapter sequences (5' CGTCGGCAGCGTCAGATGTGTATAAGAGACAG and 5' GTCTCGTGGGCTCGGAGATGTATAAGACAG, respectively) for amplification. Libraries were prepared by ligating sequencing adapters and indexes onto purified PCR products using the Nextera XT Sample Preparation Kit (Illumina). Equimolar amounts of each of the purified amplicons were pooled and sequenced on an Illumina MiSeq sequencer using a 300-bp "paired-end" read protocol. Generation and sequencing of 16S rRNA gene amplicon libraries was performed at Vienna Biocenter Core Facilities.

2.5 Bioinformatic analysis and statistics

All sequencing analysis were done by using the version qiime2, 2019.7 (QIIME=Quantitative Insights Into Microbial Ecology 2 (84) and visualization of the results was done with qiime2view (https://view.qiime2.org/). All group differences were calculated with the Mann–Whitney U test and a P-value <0.05 was considered significant.

3. Results

3.1 qPCR analysis

The standard curves for the FAM 16S rRNA gene assay showed a very good R^2 value (0.998) with a good efficiency score (93.4%) (Figure 2). The standard curves were pipetted throughout an 8 log scale.

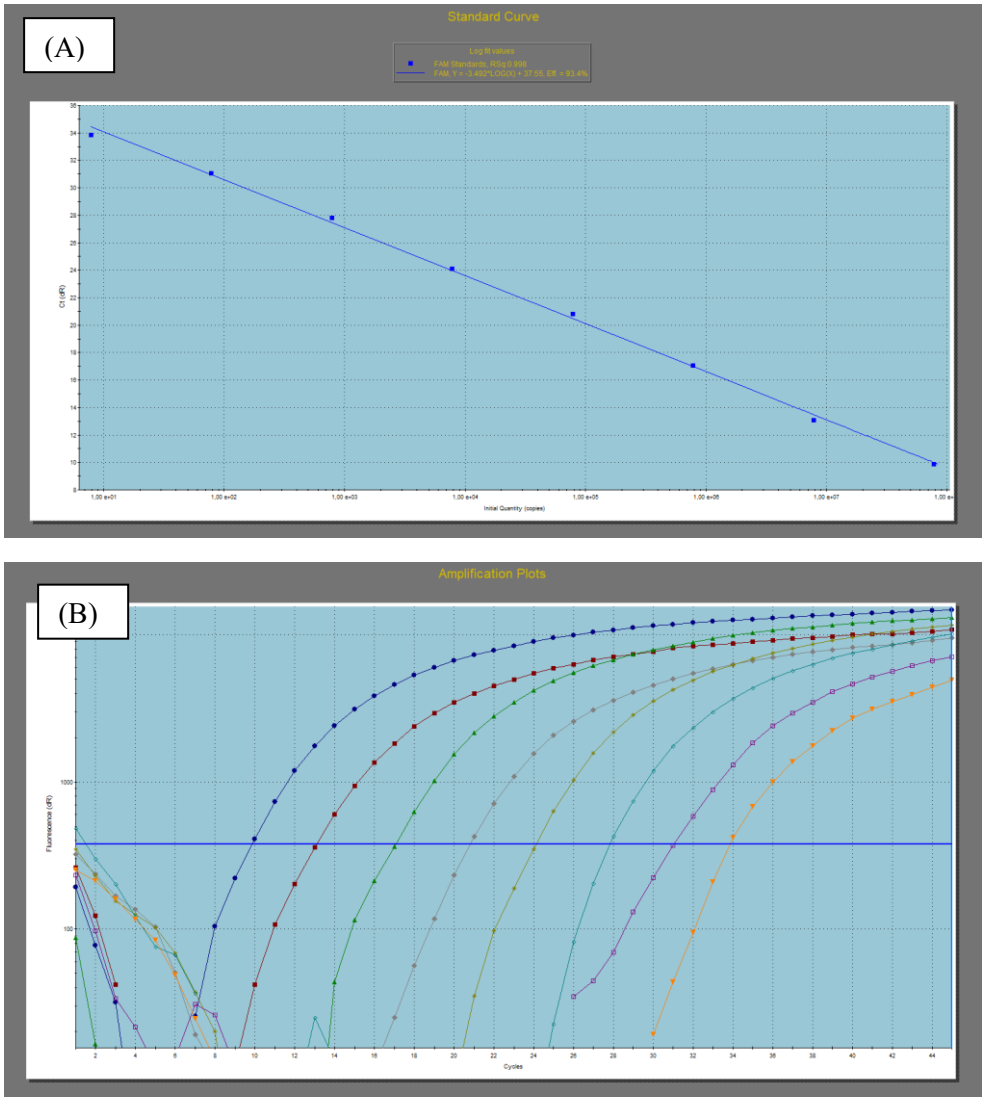


Figure 2: (A): Log fit values of the FAM Standard curve, which was pipetted for 8 log scales (B): Amplification plots of the standard curve and Ct-treshhold.

According to the results of the all-bacteria targeted qPCR, the microbial load ranged from 2x 10^1 to 9x 10^5 (Table 2). The microbial load in the fallopian tubes did not substantially differ between the endometritis-affected animals and the healthy animals (p=0.43 for flushing and p=0.42 for cytobrush samples). A trend towards higher bacterial load was observed in the

diseased animals at the second sampling time point (day 56) compared to the first sampling time point (day 28) (p=0.06), but there was no significant difference in the microbial load between the healthy group and the diseased group from days 28 to 56 (p=0.46).

Cow name	Sampling day	Tube flushing BCEs	Uterus cytobrush BCEs	Health status
Guave	28	$5.14x10^3$	$9.31x10^5$	healthy
Guave	56	$3.91x10^3$	$1.44x10^4$	healthy
Lysan	28	8.79x10	2.15x10	healthy
Lysan	28	$5.32x10^2$	No Ct	healthy
Lysan	56	$1.32x10^3$	$2.51x10^4$	healthy
Norli	28	$1.74x10^2$	5.26x10	healthy
Norli	56	$4.27x10^4$	No Ct	disease
Norli	56	$1.05x10^3$	$2.08x10^3$	disease
Zambra	28	$1.05x10^3$	$1.70x10^2$	disease
Zambra	56	$7.46x10^4$	$3.82x10^5$	disease
Nurmi	28	$3.56x10^2$	$1.19x10^2$	healthy
Nurmi	56	$2.17x10^5$	$9.37x10^4$	disease
Lisabon	28	$8.11x10^4$	$7.34x10^2$	healthy
Lisabon	56	$4.26x10^3$	$5.06x10^2$	disease
Los Angeles	28	$2.29x10^3$	$9.67x10^3$	healthy
Los Angeles	56	$4.83x10^4$	$8.04x10^3$	disease
Levi	28	$4.18x10^3$	$7.18x10^3$	disease
Levi	56	$6.25x10^2$	$1.07x10^3$	healthy
Namibia	28	$7.77x10^2$	$7.42x10^3$	disease
Namibia	56	$8.33x10^2$	$8.22x10^2$	healthy
Zipora	28	$6.69x10^2$	$6.31x10^3$	disease
Zipora	56	$1.04x10^3$	$3.73x10^2$	healthy
Tzaziki	28	$4.46x10^2$	$1.99x10^3$	disease
Tzaziki	56	$2.72x10^5$	3.7210^4	healthy
Zucchini	28	$1.63x10^3$	$1.64x10^2$	disease
Zucchini	56	$5.54x10^2$	$2.80x10^4$	disease
Golfi	28	$1.28x10^3$	$1.80x10^4$	healthy
Golfi	56	$9.97x10^3$	$2.40x10^3$	disease
Löwin	28	$1.39x10^4$	$7.49x10^3$	disease
Löwin	56	$9.07x10^2$	$7.14x10^3$	disease
Sina	28	$7.80x10^2$	$7.43x10^3$	healthy
Sina	56	$5.66x10^3$	$9.70x10^3$	disease
Beauty	28	$3.85x10^3$	$3.25x10^5$	disease
Beauty	56	$6.58x10^2$	$3.06x10^4$	disease
Hanna	28	$2.26x10^2$	$2.08x10^3$	healthy

Hanna	56	$1.78x10^4$	$6.50x10^4$	healthy
Nika	28	$3.25x10^3$	$3.28x10^4$	disease
Nika	56	$2.96x10^3$	$8.46x10^3$	disease
Nora	28	$1.97x10^3$	$1.64x10^3$	disease
Nora	56	$9.93x10^2$	$2.16x10^3$	disease
Zirm	28	$3.80x10^2$	$7.76x10^2$	healthy
Zirm	56	No Ct	$1.85x10^2$	healthy
Genia	28	No Ct	$6.05x10^3$	healthy
Genia	56	$1.01x10^3$	$1.86x10^3$	healthy
Granny	28	1.77x10	$3.63x10^2$	healthy
Granny	56	1.69x10	$1.25x10^3$	healthy

Table 2: Sample designation and Bacterial Cell Equivalents (BCE) for tube flushing and uterus cytobrush samples are listed. "No Ct" stands for biomass below the detection limit.

The microbial load in the negative controls varied from 2x 10^2 to 1x 10^5, and they did not differ significantly from the tube flushing (p=0.25) or the uterus cytobrush samples (p=0.51). We found highest levels of BCEs in negative PBS controls (mean= $1.4x10^5$), followed by negative control samples that were taken between the sampling of cows (mean=$5.4x10^4$).

3.2 Bioinformatic analysis

A box plot of the quality score distribution is shown for each position in the input sequences in Figure 3. Forward and reverse read plots were used to identify strong decreases in quality and were the basis for choosing truncation and trimming parameters (Figure 3). 31.559 sequences were generated on average per sample, with an average of 66% (20.919 sequences) remaining for subsequent analyses after the initial filtering, denoising, and chimera check steps (Table 3).

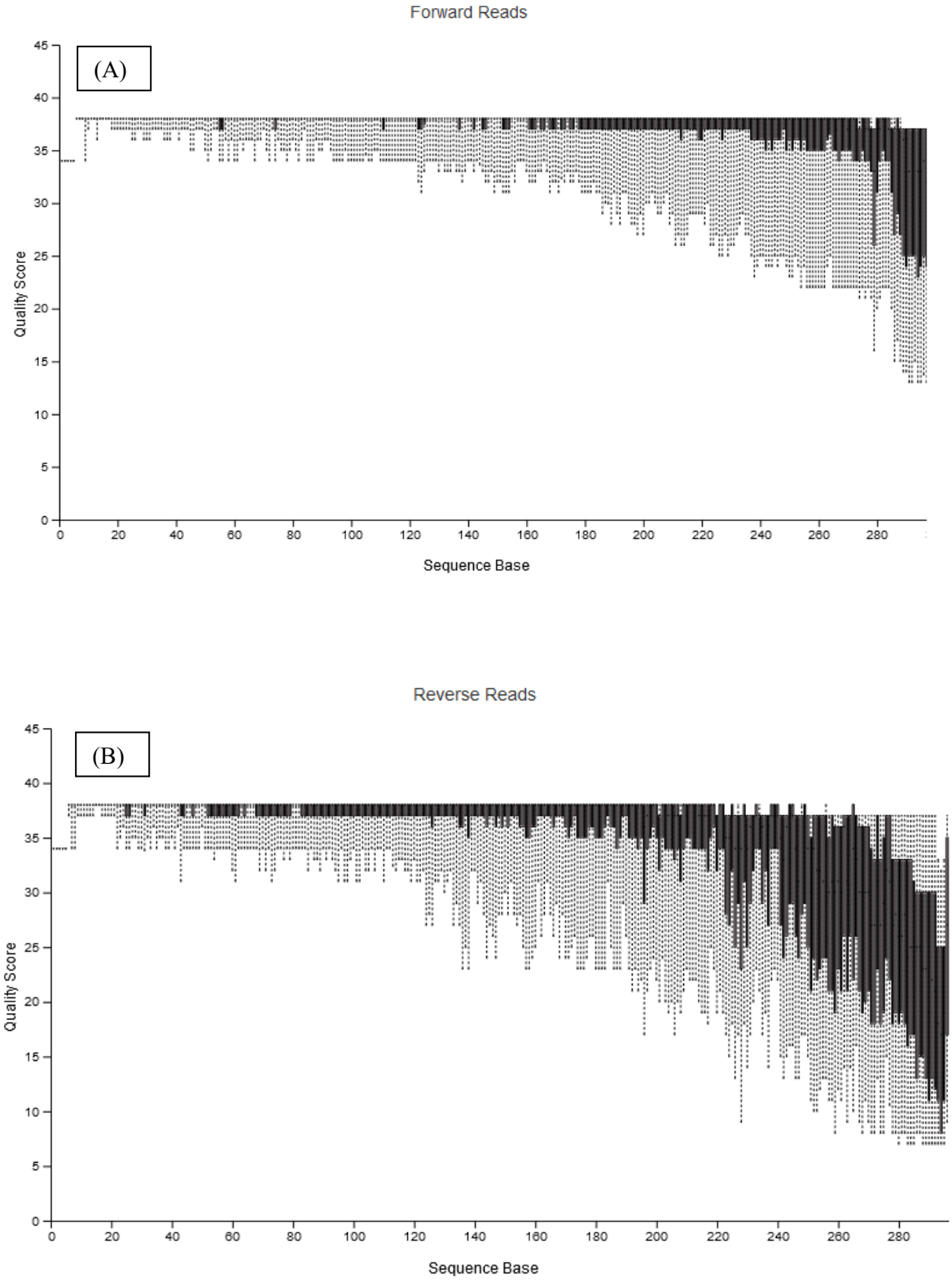


Figure 3: A) and (B) Forward and reverse read plots were generated using a random sampling of 10000 sequences.

Sample-id	input	filtered	denoised	merged	Non-chimeric	% reads for downstream analysis
Gr_CB_28d	27.609	22.893	22.641	21.610	18.954	68.7%
Gr_CB_56d	28.511	23.620	23.364	22.292	19.292	67.7%
Gr_FL_28d	25.828	20.582	23.364	19.207	16.266	63.0%
Gr_FL_56d	39.907	25.505	24.625	22.903	20.855	52.3%
Ha_FL_28d_C05	40.194	29.667	29.139	28.251	25.432	63.3%
Ha_FL_28d_C07	41.106	22.133	21.537	20.332	16.434	40.0%
Ha_FL_56d_C06	32.926	25.745	25.209	24.147	21.773	66.1%
Ha_FL_56d_C08	33.529	27.067	26.717	25.772	22.779	67.9%
Lo_CB_28d	26.210	20.427	20.141	18.973	15.763	60.1%
Lo_CB_56d	29.911	24.356	23.975	22.949	21.330	71.3%
Lo_FL_28d	25.041	19.982	19.615	19.027	16.847	67.3%
Lo_FL_56d	30.908	24.513	24.208	23.154	20.224	65.4%
Nu_CB_28d	25.592	18.594	18.341	16.978	14.339	56.0%
Nu_CB_56d	32.702	26562	25.479	22.810	21.796	66.7%
Nu_FL_28d	32.073	23.586	23.240	22.165	20.070	62.6%
Nu_FL_56d	33.493	28.217	27.995	27.363	26.598	79.4%
Zi_CB_28d	33.848	20.964	20.294	19.111	15.166	44.8%
Zi_CB_56d	26.796	21.545	21.249	20.269	16.917	63.1%
Zi_FL_28d	25.370	20.732	20.558	19.425	14.596	57.5%
Zi_FL_56d	25.194	20.749	20.445	19.867	17.717	70.3%
Zu_CB_28d	27.245	20.534	20.263	19.021	16.886	62.0%
Zu_CB_56d	45.127	35.299	34.857	34.252	33.379	74.0%
Zu_FL_28d	24.476	18.879	18.592	17.439	15.905	65.0%
Zu_FL_56d	32.440	26.292	26.066	24.732	22.640	69.8%
LK_1	30.776	25.944	25.928	25.911	25.877	84.1%
LK_2	31.859	27.119	27.079	26.958	26.197	82.2%
Neg_1	38.216	31.137	31.072	30.916	30.432	79.6%
Neg_2	19.729	8677	7821	5352	5274	26.7%
Neg_3	37.654	31.193	31.051	30.743	30.046	79.8%
Neg_4	37.503	30.980	30.896	30.704	30.657	81.7%
Neg_5	39.009	31.643	30.240	26.754	24.821	63.6%
Neg_6	29.110	24.616	24.470	24.258	24.145	82.9%

Table 3: Sample-id included an abbreviation of the cow name, CB=uterus cytobrush sample, FL= tube flushing sample, and the day of the sampling, LK= negative control DNA-extraction- kit, Neg_1- Neg_3= negative controls between sampling, Neg_4- Neg_6= negative controls PBS. Overview of sequence statistics is shown. Input sequences= number of sequences in the raw data, filtered & denoised= filtered number of sequences after the quality check, merged= number of sequences where the forward read could be merged with the reverse read, non-chimeric= number of sequences after the chimera check.

3.3 Alpha diversity analysis

In the alpha diversity analyses, the highest numbers of species found (observed OTUs) were in the uterus cytobrush samples (Figure 4). The flattening curve indicates that the overall diversity was detected with the selected sequencing depth. The number of OTUs detected in tube flushing samples was below the negative control samples.

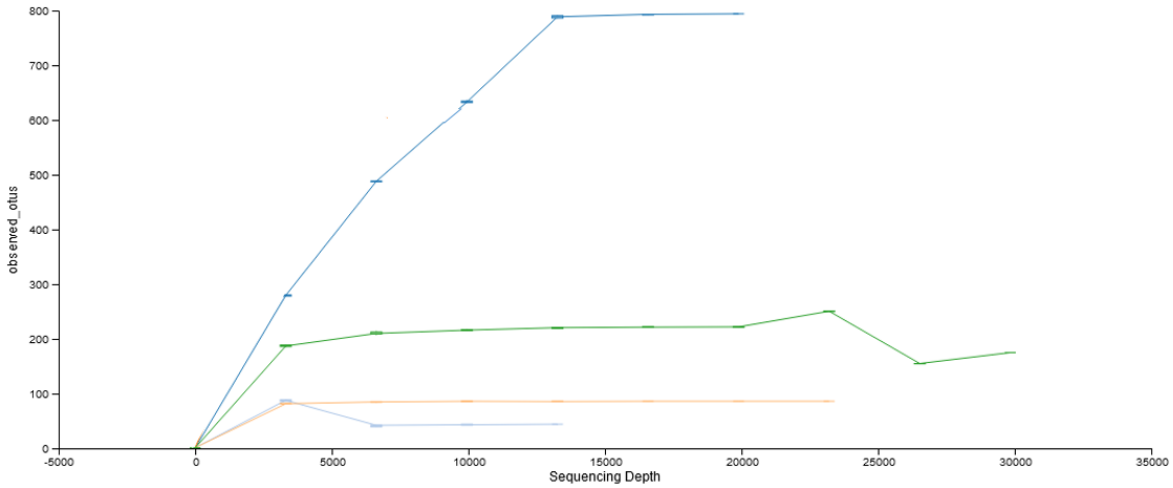


Figure 4: Total number of detected species (OTUs) in the uterus cytobrush samples (dark blue), process controls (green), negative controls (orange), and tube flushing samples (light blue).

3.4 Taxonomic composition

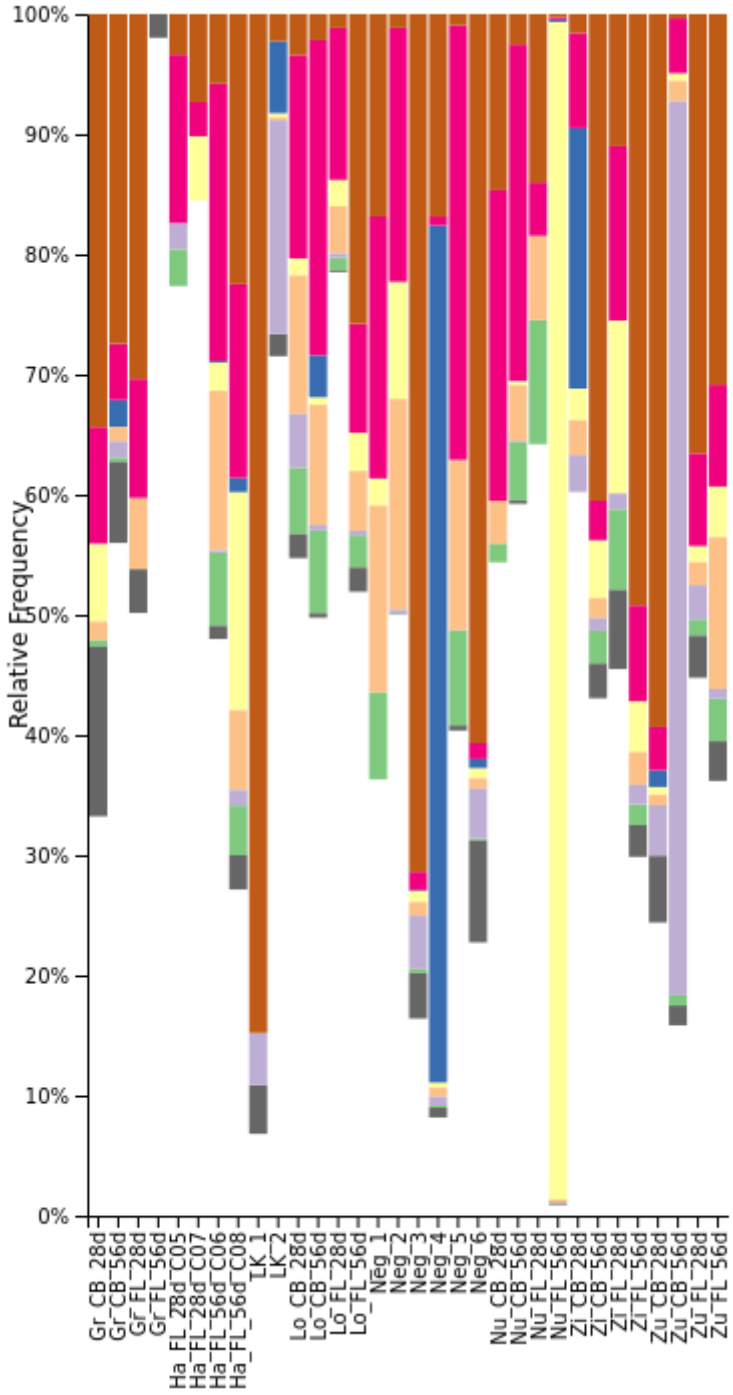
In total, 5.262 ASVs were detected, which were classified based on a taxonomy database. A total of 25 phyla were detected throughout all samples, with the 10 most abundant listed in Table 4.

	Healthy, uterus	Disease, uterus	Healthy, fallopian tube	Disease, fallopian tube	neg controls*
Proteobacteria	36.93%	42.23%	38.12%	34.17%	50.36%
Firmicutes	41.30%	32.71%	37.21%	40.98%	36.22%
Bacteroidetes	7.77%	10.57%	12.58%	12.10%	8.35%
Actinobacteria	9.37%	7.36%	4.27%	9.86%	3.16%
Fusobacteria	0.00%	4.79%	3.48%	0.00%	0.01%
Tenericutes	3.14%	1.17%	1.98%	0.68%	0.54%
Patescibacteria	0.47%	0.39%	1.43%	0.15%	0.58%
Verrucomicrobia	0.00%	0.04%	0.54%	0.04%	0.24%
Spirochaetes	0.02%	0.57%	0.08%	0.04%	0.27%
Acidobacteria	0.63%	0.00%	0.00%	0.00%	0.03%
Sum of the 10 most abundant	99.63%	99.84%	99.70%	98.02%	99.76%

*Table 4: The 10 most abundant phyla throughout all samples.*please note, that the relative abundances cannot be interpreted quantitatively*

The family level is shown in the bar charts in Figure 5. High diversity is evident in all samples. The cytobrush sample of cow Granny (healthy) shows one dominant genus, *Rhodanobacteraceae* (30%), followed by *Staphylococcus* (6%) and *Ruminococcaceae* (5%) at day 28. In addition to the dominant genus, 25 other genera are present in this sample. *Rhodanobacteraceae* are dominant on day 56 (20%), followed by *Enterococcus* (14%), *Lactobacillus* (12%) and *Rhodanobacter* (6%). Flushing samples show very low diversity, but also had an overall lower number of sequences being generated in the run. *Rhodanobacteraceae* were also found dominant in these samples, followed by *Clostridia* (Cluster XI) and *Bifidobacteriaceae*. The other healthy cows Hanna and Zirm showed a very similar picture, with higher diversity in the Cytobrush samples. Also here, *Rhodanobacteraceae* were detected abundantly (between 2% and 50%), *Clostridia* (cluster XI), *Staphylococcus* and *Ruminococcaceae* were also prevalent. *Enterobacteriaceae* were detected in the healthy samples, but with low abundance values (max. 1%). The cow Los Angeles, which was healthy on day 28 but had endometritis on day 56, had an elevated *Pasteurellaceae* level on day 56 (increase from 0.5% to 12.3%). *Leptotrichiaceae*, belonging to the *Fusobacteria*, were

detectable exclusively on day 56 (2.5%). The cow Nurmi, which was healthy but had endometritis on day 56, also had *Leptotrichiaceae* (3.8%) on day 56 in the cytobrush samples. In addition, *Fusobacteriaceae* were highly abundant (14%), which were not yet detectable on day 28. *Actinomycetaceae*, which were not detectable on day 28, were also highly abundant (8.8%) on day 56. The cow Zucchini, which had endometritis on both days, had *Enterobacteriaceae* (4.4%), *Burkholderiaceae* (5.5%), and *Mycoplasmaceae* (5.1%) in the microbiota profile on day 28, while *Enterobacteriaceae* increased massively (70.9%) on day 56 (Figure 5).


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
Gr_CB_28d
Gr_CB_56d
Gr_FL_28d
Gr_FL_56d
Ha_FL_28d_C05
Ha_FL_28d_C07
Ha_FL_56d_C06
Ha_FL_56d_C08
LK_1
LK_2
Lo_CB_28d
Lo_CB_56d
Lo_FL_28d
Lo_FL_56d
Neg_1
Neg_2
Neg_3
Neg_4
Neg_5
Neg_6
Nu_CB_28d
Nu_CB_56d
Nu_FL_28d
Nu_FL_56d
Zi_CB_28d
Zi_CB_56d
Zi_FL_28d
Zi_FL_56d
Zu_CB_28d
Zu_CB_56d
Zu_FL_28d
Zu_FL_56d

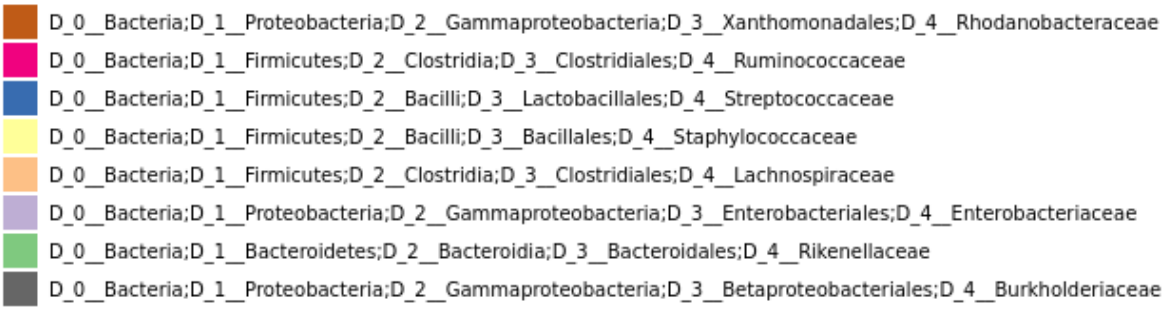


Figure 5: The taxonomic profile on family level, in the legend the 8 most abundant bacterial families are listed. Per cow was one cytobrush and one flushing sample taken on day 28 and 56.

4. Discussion

The aim of the study was to test methods for the detection of microbiota, both qualitatively and quantitatively, in the uterus and fallopian tubes of healthy cows and cows with clinical endometritis. Because the oviductal flushing samples often contain just a limited amount of sample material and a small number of bacteria, 16S rRNA gene sequencing was not only used for samples, but also for negative and process controls (85). There are already many studies published for environmental samples that a large diversity but low biomass of bacteria, where a high-throughput sequencing strategy targeting the 16S rRNA gene on an Illumina platform was utilized to detect the full content microbial diversity (86). Wherefore we tested this method together with real-time PCR, to quantify BCEs reliably (sequencing is semiquantitative and should not be used for the quantification of bacterial loads). A total of 29 negative controls (NEG PBS and NEG cows from the barn) for method establishment, 43 tubal lavage samples and 43 uterine cytobrush samples were extracted. One blank control was included in each extraction. A total of 24 animal samples were sequenced with Illumina High-throughput Sequencing (12 flushing, 12 cytobrush samples), 8 of which were endometritis associated. In addition, 6 negative controls (process controls) and two blank controls were sequenced for the control of the method. The implementation of negative controls is crucial to detect gaps in the analytical chain and to avoid false positive interpretation of microbial profiles.

The fact that there weren't many bacteria in the oviduct resulted in low bacterial DNA load. Since flushing dilutes oviductal fluid, it is also possible that not enough material could be collected during the sampling process. Another possibility is that the bacteria in the excluded samples adhered to the epithelia rather than accumulating well in the oviductal lumen, and as a

result, were not present in sufficient numbers. Future research could avoid this issue by obtaining the samples in a different way, such as avoiding severe dilution during sampling or massaging the oviduct while flushing to help the bacteria detach from the wall.

A main challenge in this project was the microbial load in the negative controls and the process controls, which was even higher than in some of the samples. Due to this greater microbial load in the negative controls, it was difficult to identify and eliminate the contaminants in the samples. Contamination in negative and process controls can come from multiple sources. We expect the PBS reagent used to be not sterile, or to include some DNA residues, as PBS controls show highest microbial cell loads. Also, the lysozyme used in the DNA extraction and the DNA extraction kit itself could be a minor source of contamination. The sampling technique should be optimized in ongoing projects and improved so that in the future as little contamination as possible occurs.

In the alpha diversity analyses, the highest numbers of species found were in the uterus cytobrush samples. The flattening of the rarefaction curve indicated that it was sequenced deep enough to cover the whole diversity of microbes in the samples.

4.1 Comparison of our results with literature about the uterine microbiota

The five most abundant bacteria found in the healthy uteri were *Firmicutes* (41.30 %), *Proteobacteria* (36.93 %), *Actinobacteria* (9.37 %) *Bacteroidetes* (7.77 %) and *Tenericutes* (3.14%). Whereas in the diseased uteri are *Proteobacteria* (42.23 %), *Firmicutes* (32.71 %), *Bacteroidetes* (10.57%), *Actinobacteria* (7.36 %) and *Fusobacteria* (4.79%) in the top 5. According to Jeon et al. (2015) and Santos et al. (2012) *Bacteroidetes, Fusobacteria, Firmicutes*, and *Proteobacteria* were the most prevalent bacteria in the healthy uterus (19) (32). Comparing these results with our study, *Bacteroidetes, Firmicutes,* and *Proteobacteria* are also among the most abundant bacteria in the healthy uterus. According to Sicsic et al. (2018), *Actinobacteriota* are found in the uteri of healthy cows (20). *Fusobacteria* didn´t play a big role in the healthy uteri, however *Fusobacteria* were among the top 5 in the diseased uteri. *Proteobacteria* and *Tenericutes* were numerically more abundant in healthy cows, than in cows with metritis (19) (24) (87). According to Machado et al. (2012), who studied cows with clinical endometritis, we found a high percentage of *Tenericutes* in the diseased uteri too (41). These

findings demonstrate that, cows with endometritis have a greater relative abundance of Tenericutes, than cows with metritis.

The most common bacteria in our study of the diseased uteri were *Proteobacteria, Firmicutes and Bacteroidetes.* Most frequently, uterine disorders like endometritis or metritis are associated with *Bacteroides* (88).

Machado et al. (2012) published that healthy cows had higher relative abundances of *Firmicutes*, whereas cows with clinical endometritis had higher relative abundances of *Bacteroidetes, Tenericutes, Fusobacteria*, and *Actinobacteria* (41). Others later confirmed that clinical endometritis-affected cows had higher relative abundances of *Bacteroidetes* and *Fusobacterium* (42)(43). We found in our study a higher relative abundance of *Bacteroidetes* and *Fusobacteria* in the diseased uteri too.

Nevertheless, *Firmicutes* can be potential uterine pathogens. But it can be discovered in both healthy and uterine diseased cows (88). Additionally regarded as possibly harmful are *Proteobacteria*. They have been linked to metritis in certain studies (37) and to healthy uteri in others (19, 32). For example the cow Granny, who was healthy on day 28 and 56, had a high percentage of *Rhodanobacteriaceae* (30%) on day 28 and 20% on day 56. *Rhodanobacteriaceae* was present between 2-50% also in healthy cows Hanna and Zirm. In all three cows, other potential pathogens such as *Staphylococcus* and *Ruminococcaceae* also played a role. Despite these potentially pathological bacteria, they were healthy. It´s also interesting to note that the microbiome in the uteri was similar to the microbiome in the flushing samples. The uteri contained potentially dangerous microorganisms, however not all cows had clinical illness. The tremendous variability of the microbiome may be one possible explanation for the lack of a connection between isolated bacterial results and health status. Clinical disease only appears to arise when a pathogenic bacterium clearly dominates and heterogeneity is reduced (24).

4.2 Comparison of our results with literature about the oviduct microbiota

The four most abundant bacteria found in the healthy fallopian tubes were *Proteobacteria* (38.12 %), *Firmicutes* (37.21 %), *Bacteroidetes* (12.58 %) and *Actinobacteria* (4.27 %). In the fallopian tubes with disease dominated *Firmicutes* (40.98 %), *Proteobacteria* (34.17 %), *Bacteroidetes* (12.10 %), *Actinobacteria* (9.86%).

Using culture-dependent techniques, Sadeghi et al. (2022) examined bacteria in the oviduct of healthy cows and cows with salpingitis that were taken in a slaughterhouse (89). According to this study 37.5 percent of the healthy oviducts had a detectable load of bacteria. There, *Eschericia coli, Clostridium perfringens, Bacillus subtilis, Pseudomonas aeruginosa,* and *Rhodococcus equi* were discovered by the researchers. Along with these results, we found in our study a high number of *Proteobacteria, Firmicutes* and *Actinobacteria* in cows with healthy fallopian tubes. We were not able to detect the remaining bacteria or only in very low quantities in our study. However, because the two investigations used different detection strategies, it is challenging to compare them. Culture-dependent approaches are less effective at detecting bacteria. Just three healthy oviducts could be confirmed to have bacteria in the investigation, significantly limiting the study's representativeness and the ability to compare it to our findings (89). Bacteria were present in all the oviducts with salpingitis. *Eschericia coli, Trueperella pyogenes, and Fusobacterium necrophorum* were the most prevalent bacteria growing on the plates, which was also seen in other investigations (90, 91). In our study *Fusobacteria* wasn´t detected in the diseased fallopian tubes. Nevertheless, *Fusobacteria* was detected 3.48 % in healthy fallopian tubes and 4.79 % in the diseased uteri samples. *Actinobacteria*, like *Trueperella pyogenes*, were with 9.86 % in the diseased fallopian tubes and 7.36 % in the diseased uteri among the top ten phyla. In chronic endometritis, where they co-dominate with *Bacteroides* as the primary pathogens (92), *Fusobacteria* play a significant role. It's interesting to note that cows with endometritis appear to have *Fusobacteria* in their oviducts as well. In a study, bacteria were discovered in the lumen or epithelium of 76.20% of the oviducts from slaughtered cows with pyometra. *Fusobacterium necrophorum* and *Trueperella pyogenes* were discovered using fluorescence in situ hybridization in 76.20% of the samples (90). In a different investigation, *Trueperella pyogenes* could be found in most samples with severe salpingitis utilising culture-dependent methods for bacterial identification. However, only one out of 14 oviducts was found to contain *Fusobacterium necrophorum* by other authors (91). Our study

included both healthy cows and cows suffering from endometritis. Thus, *Fusobacteria* would be expected to play a greater role in diseased fallopian tubes. Interestingly, the percentage of *Fusobacteria* in the negative control was higher than in the diseased oviduct samples. This suggests an urgent need for improvement in sample collection and processing.

4.3 Possible microbial compositions of the vagina, uterus, and fallopian tubes leading to endometritis

The uterine microbiome of cows with uterine infection was shown to include the highest concentrations of *Bacteroides and Fusobacterium* at various postpartum periods (87). The most prevalent phyla in the uterus of metritic cows shifted during the first 6 days postpartum (DPP) quickly from *Proteobacteria* to *Bacteroidetes* and *Fusobacteria* (19). A higher abundance of OTUs of *Bacteroides, Porphyromonas,* and *Fusobacterium* was seen in diseased cows, which was correlated with lower uterine bacterial richness and diversity indices (19, 93). Increased relative abundances of the aforementioned genera and a concomitant decline in bacterial diversity in the uterus were linked to the inability to treat metritis, either naturally or with antibiotic therapy (93). The four most prevalent OTUs in uterine fluids were discovered to belong to *the Fusobacteriaceae, Bacteroidaceae, Pasteurellaceae,* and *Porphyromonadaceae* families, according to a study that examined uterine fluids taken from cows with pyometra who were slain at least 22 DPP (94). The microbiota of uterine lavages from cows with severe endometritis at 35 days in milk (DIM) also showed a higher proportion of *Bacteroides* and *Fusobacterium* than either the healthy group or cows with mild endometritis (41). In another study, the three most prevalent families in endometrial biopsies from healthy cows at 4 weeks postpartum (WPP) as well as from both healthy and endometritic cows at 7 WPP were *Ruminococcaceae, Bacteroidaceae*, and an unclassified family that belonged to class *Bacteroidia* (40). The cow Nurmi, who was healthy on day 28 PP in our study, but had endometritis on day 56 showed a higher percentage of *Fusobacteriacea* (14%), which wasn´t detected on day 28 in her uterus samples. Her flushing samples from day 28 showed *Proteobacteria, Bacteroidetes* and *Firmicutes* as the top 3 on family level. On day 56, diversity decreases significantly and *Firmicutes* strongly dominate. The extent to which this result can be trusted is questionable, since we unfortunately have positive negative samples due to contamination. Nevertheless, one can see a strong dysbiosis. The cow Los Angeles, who was healthy on day 28, had on day 56 a high level of *Pasteurellaceae* (12.3%) and *Leptotrichiacea*

with 2.5% on day 56 in her uterus samples. Both flushing sample from day 28 and day 56 had the 3 Top families *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. Comparing the profiles of both cows at the family level, we see that *Proteobacteria, Firmicutes* and *Bacteroidetes* are similarly represented in both uterus and oviduct, so we have a shared microbiome. Further studies with improved sampling and processing are needed to better understand and compare the exact relationship between the uterus and the fallopian tube and to identify key bacteria leading to endometritis.

Miranda-CasoLuengo et al. (2019) demonstrated the existence of a diverse microbiome in the vaginas of healthy cows at 7 DPP, using pyrosequencing, and a dysbiotic microbiome in cows with postpartum endometritis at 21 DPP (42). The vaginal microbiome of healthy cows has a high *Firmicutes* content, a high *Firmicutes* to *Bacteroidetes* ratio, and a high diversity index, among other distinguishing characteristics. An elevated abundance of OTUs of the genera *Bacteroides, Helcococcus,* and *Fusobacterium*, among others, was associated with a significant decrease in the vaginal bacterial diversity of cows that later had postpartum endometritis.

Other research, however, has produced conflicting findings. For instance, the most notable distinction between cows with metritis and healthy cows was an increased rate of *Eschericia coli* isolation in infected cows (95). This contrasted with dramatic alterations in the microbiome of cows that go on to develop postpartum endometritis, which were identified in this work. The scientists hypothesised that the bovine vagina lacked a stable microbiota and concluded that vaginal bacteria were probably contaminants from many sources, such as skin, faeces, and/or the environment. In our study the cow Zucchini, who had endometritis on both sampling days, had a high rise of *Enterobacteriaceae*. On day 28 *Enterobacteriacea*e were present with 4.4% and on day 56 it was massive with 70.9% in the uterus. It is possible that the number of *Enterobacteriaceae* in the vagina was also high, so that the bacteria migrated to the uterus and gained the upper hand there. It would have been interesting to have samples from the vagina to determine how the microbiome is similar or different to the uterus.

In the postpartum phase, Miranda- CasoLuengo et al. (2019) revealed a common community in the uterus and vagina (42). When the uterine and vaginal microbiomes were most similar, at 7 DPP, changes connected to subsequent postpartum endometritis development were seen.

These included the disappearance of bacterial OTUs found in healthy cows and the emergence of a subcommunity linked to postpartum endometritis development. Miranda-CasoLuengo et al. (2019) suggests that the compartmentalisation of the reproductive system is lost at calving, leading to the dispersal and mixing of microbial communities from the uterus and vagina (42). Differentiation between the vaginal and uterine microbiomes was easily visible at 7 DPP in the healthy group, in contrast to cows that had postpartum endometritis, as diagnosed at 21 DPP. The development of postpartum endometritis could be associated with a delayed differentiation of vaginal and uterine microbiomes early postpartum (42).

Many questions remain as to how and when colonization of the reproductive tract occurs. In this context, interesting Studies have also been carried out on heifers. For instance, *Firmicutes* (40.00%), *Bacteroidetes* (36.00%), and *Proteobacteria* (10.00%) were found in healthy heifer uteri (38). *Tenericutes, Proteobacteria, Fusobacteria*, and *Firmicutes* were found to be the most prevalent phylotypes in the uterus of heifers (96).

Firmicutes, Tenericutes, Bacteriodotes, and *Proteobacteria* were the most prevalent types of bacteria found in the vagina of healthy heifers. Like that, these were typically discovered in heifers that had uterine conditions including metritis, preclinical endometritis, or clinical endometritis. Heifers with metritis had *Fusobacterium, Bacteroides, Porphyromonas,* and *Prevotella* pathogens detected. In addition, a wider variety of distinct bacterial strains have been found in healthy heifers (19).

Because heifers have not yet given birth or been mated, the oviduct is likely relatively sterile and undeveloped, which accounts for the large difference in the total number of phylotypes between cows and heifers (97). Further studies would be interesting to clarify the associations between bacterial colonization and subsequent disease with endometritis. The extent to which the vagina, uterus and especially the fallopian tubes play a role is not yet clear.

4.4 Possible limitations of the study and future improvements

The cytobrush samples from the uterus were discovered during the data analysis to be more appropriate for microbiota studies than the flushing samples from the fallopian tube. The reason for this could be a mixture of the expandable technology and the oviduct as a low abundance site. The variety of the microbial population and the amount of DNA that could be extracted were both higher in the cytobrush samples. Given that there are numerous abundant bacteria present in the process controls as well, the included process controls demonstrated that contaminations that may have occurred in the analytical chain are significant for the analysis itself. In consultation with the staff of the herd care, follow-up initiatives will optimise the work processes. Here, the controls will also be expanded to allow for the analysis of each phase in a separate process control. Due to the extremely low bacterial counts in the fallopian tube, this is necessary. There is literature available that already describe the importance of sterile sampling, sterile pipetting in the lab and contamination of equipment, reagents and fluids (98–100).

5. Conclusion

In conclusion, our findings indicated that the uterus and oviduct of healthy cows contain a wide variety of microorganisms. In contrast to cows with endometritis, healthy cows tended to have a different microbiome, but further investigations are needed.

The contamination during sample extraction and preparation posed the most challenge to the investigation because it was difficult to distinguish between commensals and contaminants. Additionally, there is a significant risk of contamination when working in the barn. By utilising just single-use items in future experiments, this can be prevented. Additionally, as many negative samples as feasible should be used so that any (and inevitable) contamination may be differentiated in the statistical analysis.

Nevertheless, we invented oviductal sampling from live cows with this study. As a result, our study provides a strong foundation for future research that will strengthen its techniques. This work also demonstrates the enormous potential for future investigation of the oviduct microbiome. By using next-generation sequencing, it might be demonstrated that there are bacteria in the oviduct's lumen. The uterus and oviduct are anatomically connected, which suggests that uterine infections may spread to the oviduct and affect fertility. Therefore, a

deeper comprehension of the oviduct's microbiome may help us better comprehend uterine disorders and subfertility.

6. Summary

The objective of the study was to precisely describe the microbiome, both qualitatively and quantitatively, in the uterus and fallopian tubes of healthy cows and of cows with clinical endometritis. Despite the important role of the oviduct for fertility, to our knowledge, no study has examined bacteria in the oviduct of living cattle by repeated sampling. In our study were 22 Simmental dairy cows included and sampled on day 28 and 56 postpartum. A cytobrush sample from the uterus and an oviduct flush sample via transvaginal endoscopy were taken respectively. After the samples were processed in the lab, qPCR was carried out. 16S rRNA sequencing was used to determine the bacteria's taxonomy. The uterus and oviduct of healthy cows contain a wide variety of microorganisms. In contrast to cows with endometritis, healthy cows tended to have a different microbiome.

The four most abundant bacteria found in the healthy uteri were *Firmicutes*, *Proteobacteria, Actinobacteria*, and *Bacteroidetes*. Whereas in the diseased uteri were *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* in the top 4. The four most abundant bacteria found in the healthy fallopian tubes were *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. In the fallopian tubes with disease dominated *Firmicutes*, *Proteobacteria, Bacteroidetes* and *Actinobacteria*. A main challenge in this project was the microbial load in the negative controls and the process controls, which was even higher than in some of the samples. Due to this greater microbial load in the negative controls, it was difficult to identify and eliminate the contaminants in the samples. Nevertheless, our study provides a strong foundation for future research that will strengthen its techniques. This work also demonstrates the enormous potential for future investigation of the uterus and oviduct microbiome.

7. Zusammenfassung

Ziel dieser Studie war es, das Mikrobiom im Uterus und in den Eileitern von gesunden Kühen und von Kühen mit klinischer Endometritis qualitativ und quantitativ zu beschreiben. Trotz der wichtigen Rolle des Eileiters für die Fruchtbarkeit hat unseres Wissens nach keine Studie die Bakterien im Eileiter von lebenden Kühen untersucht. In die Studie wurden 22 Fleckviehkühe

aufgenommen und am 28. und 56. Tag postpartum beprobt. Von jeder Kuh wurde eine Cytobrush-Probe aus der Gebärmutter und eine Eileiter-Spülprobe mittels transvaginaler Endoskopie entnommen. Nach der Verarbeitung der Proben im Labor wurde eine qPCR gemacht. Zur Bestimmung der Bakterientaxonomie wurde eine 16S rRNA Gen-Sequenzierung durchgeführt. Die Gebärmutter und die Eileiter gesunder Kühe enthalten eine große Vielfalt an Mikroorganismen. Im Gegensatz zu Kühen mit Endometritis haben gesunde Kühe ein anderes Mikrobiom. Die vier häufigsten Bakterien, die in den gesunden Gebärmüttern gefunden wurden, waren *Firmicutes*, *Proteobacteria, Actinobacteria* und *Bacteroidetes.* Im Gegensatz dazu befanden sich in den kranken Gebärmüttern *Proteobacteria*, *Firmicutes*, *Bacteroidetes* und *Actinobacteria* unter den Top 4. Die vier wichtigsten Bakterien in den gesunden Eileitern waren *Proteobacteria*, *Firmicutes*, *Bacteroidetes* und *Actinobacteria*. In den kranken Eileitern dominierten *Firmicutes*, *Proteobacteria, Bacteroidetes* und *Actinobacteria.* Eine große Herausforderung bei diesem Projekt war die mikrobielle Belastung in den Negativkontrollen und den Prozesskontrollen, die teilweise höher war, als in den Proben. Aufgrund dieser höheren mikrobiellen Belastung in den Negativkontrollen war es schwierig, die Kontaminanten in den Proben zu identifizieren und zu eliminieren. Nichtsdestotrotz bietet unsere Studie eine solide Grundlage für künftige Forschungsarbeiten zur Verbesserung der methodischen Verfahren. Diese Arbeit zeigt auch das enorme Potenzial für zukünftige Studien des Mikrobioms von Gebärmutter und Eileiter.

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9. List of figures and tables

10. Supplement

Use of the DNeasy Blood & Tissue Handbook 07/2020

Protocol: Pretreatment for Gram- Positive Bacteria

Procedure

1. Harvest cells (maximum 2 x 109 cells) in a microcentrifuge tube by centrifuging for 10 min at 5000 x g (7500 rpm). Discard supernatant.

2. Resuspend bacterial pellet in 180 µl enzymatic lysis buffer.

3. Incubate for at least 30 min at 37°C.

After incubation, heat the heating block or water bath to 56°C if it is to be used for the incubation in step 5.

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4. Add 25 µl Proteinase K and 200 µl Buffer AL (without ethanol). Mix by vortexing.

Note: Do not add Proteinase K directly to Buffer AL. Ensure that ethanol has not been added to Buffer AL (see “Buffer AL”, page 19). Buffer AL can be purchased separately (see ordering information starting on page 59).

5. Incubate at 56°C for 30 min.

Optional: If required, incubate at 95°C for 15 min to inactivate pathogens. Note that incubation at 95°C can lead to some DNA degradation.

6. Add 200 µl ethanol (96–100%) to the sample and mix thoroughly by vortexing.

It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy Mini spin column. This precipitate does not interfere with the

DNeasy procedure.

7. Continue with step 4 of the protocol “Purification of Total DNA from Animal Tissues SpinColumn Protocol)”, page 30.

Protocol: Purification of Total DNA from Animal Tissues (Spin- Column Protocol)

3. Vortex for 15 s. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing.
It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.
A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.
Change: Zentrifugation 30 seconds 500 G

4. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.*

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.*

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000rpm).

7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 20 µl DEPC- H2o directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute.