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# Composition and the early development of the fecal and oral microbiota in Norwegian Red calves

Sammensetning og tidlig utvikling av den fekale og orale mikrobiotaen hos NRF-kalver

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Class of 2014

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# Prefix

I have long had an interest in One Health and the crossover between animal and human health. That was what originally sparked my interest in a project where I was to gather samples from calves and cows, with the intention to analyze the genetic composition from different samples to see how the microbiota was composed in neonatal calves.

We wanted to investigate if there were any differences between calves that were immediately removed from their mother after birth, and calves that stayed with their mother for days after birth. The microbiota from different body secretions and locations at the body, were to be analyzed at five timepoints up to as long as 16 days after birth. How would the microbiota differ after some time, and would any of the microbiota resemble that of the dams? Could the results possibly be indicative for results in humans were the newborn did not drink breast milk or have any contact with their mother?

As the planned project turned out to be more time consuming than the student paper was supposed to be, it was scaled down to focus on one herd with the same environment and husbandry. In this way we would also avoid some possible confounding factors, but at the same time we would not be able to compare the microbiota in calves that were kept with their mother. We also scaled down the number of timepoints, so the cow only was to be sampled directly after birth at the same time as the calf, and then only the calf two days later. In this way, it would be more manageable for one person to take the samples, and we could avoid a source of error related to the samples being taken with different techniques by different persons. Also, the project would still be able to get an overview of the development of the microbiota within the first two days, with focusing primarily on the oral and fecal samples.

Eventually, the project focused on the development of the fecal and oral microbiota. Additional samples were used to create a databank for further metagenomic studies. Even

though the project had been scaled down, the sampling, extraction and especially understanding and interpreting the analysis, turned out to be more time consuming than initially expected for this student thesis. However, the thesis gave an opportunity to learn about microbiota and metagenomics; a field that has been little explored during my years of studying veterinary medicine.

# Summary

*Title*: Composition and early development of the fecal and oral microbiota in Norwegian Red calves

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To explore the composition and development of the microbiota of the newborn calf, samples of the feces and the oral mucosa were taken from eight calves and their mother at birth. The calves were also sampled two days later. In addition, vaginal mucosal and colostrum samples from the dams were taken at time of birth to create a dataset for future research.

The analysis of the calf and cow fecal and oral mucosal microbiota was done by 16S rRNA amplicon sequencing. Significantly more *Firmicutes* were found in the feces of the dams, while the calf feces at two days of age contained significantly more *Proteobacteria*. The analysis showed that the fecal microbiota of the calves was significantly different from the dams' fecal microbiota at both timepoints of sampling, and the calves' fecal microbiota developed quickly after birth.

There was a change and reduction in richness in the fecal microbiota of the calves during its first two days of life. *Acinetobacter*, *Staphylococcus* and *Bacteroides* were the most abundant genera at birth, while *Escherichia-Shigella*, *Bacteroides* and *Butyricoccus* were the most abundant genera two days later.

The oral microbiota of the calves went through a statistically significant change during the first two days. *Acinetobacter*, *Enterococcus* and *Ruminococcaceae* were the most abundant genera after birth. Two days later there had been a shift in the microbiota, and the oral samples of the calves now resembled the oral microbiota of the dam. *Porphyromonas*, *Neisseria* and *Acinetobacter* were now the most abundant phyla.

# Definitions and Abbreviations

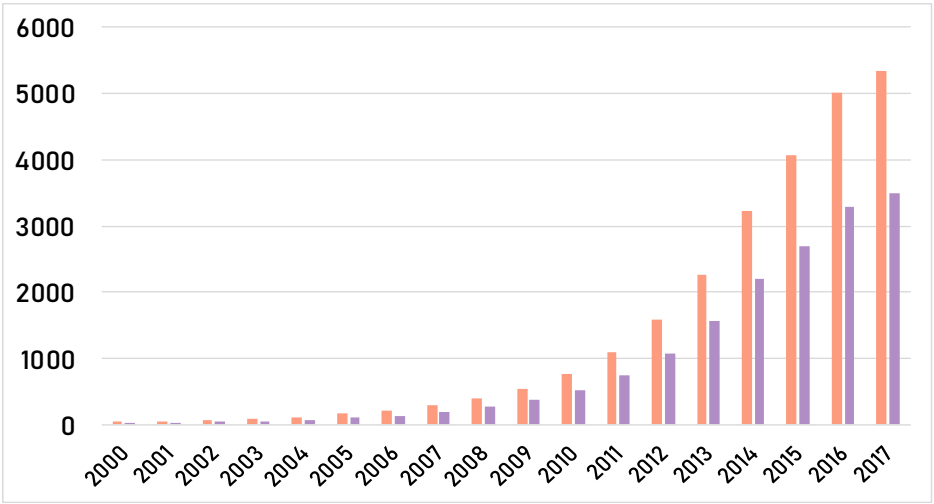
| | |
|---|---|
| Microbiome | The combined genetic material of the microorganisms in a particular environment |
| Microbiota | The microbial community of a specific site or organ |
| OTU | Operational Taxonomic Unit |
| AMS | Automated Milking System |
| NRF | Norwegian Red |
| PCR | Polymerase Chain Reaction |

# Introduction

"Microbiota" is a term that has been used for at least 50 years (1). Already in the 1960s researchers attempted to find specific microbiota and their link with health benefits when using specific pathogen free animals in laboratories (2).

The microbiota in humans has received more attention in recent years, both by the public and in scientific studies, as we learn more about how it affects the health both inside and outside of the gastrointestinal tract (3). The intestinal microbial community that earlier were portrayed as primarily opportunistic by many, has now acquired status as an important contributor to both local and extraintestinal immune status (4), and microbiota dysbiosis is also proven correlated to several immune mediated diseases (5).

Some research and studies have been done on cattle as well, but still there is much we do not know about the development of the microbiota, and especially the intestinal microbiota. The rumen has for decades been in focus, researching the microbial symbiosis and how it affects the production and health of cattle (6-8). As ruminants are linked to environmental issues concerning methane emissions, the rumen and its microbiota have become extremely relevant with the increased focus on feed efficiency and methane production.




*Publications per year for human (red) and animal (purple) microbiomes, as registered in PubMed. From: https://www.risch.ch/wp-content/uploads/2018/10/180925_BKL_PowerPoint_Referat_Rodriguez-Sabrina.pdf*

The microbiota in the specific locations in the animal is of utmost importance for healthy and thriving calves, as the microbiota is linked to both local immunity, the development of intestinal epithelium, the mucosa, lymphoid structures and differentiation of immune cells (9). O'Hara *et al*, 2020 (7), emphasize the importance of investigating the interaction between host performance and gut microbiome in both the rumen and the lower digestive tract. This should give us a better understanding how we can facilitate a sustainable ruminant production.

Sequencing the intestinal microbiota of newborn calves was first performed and published by Alipour *et al.* in 2018 (10), where newborn Ayrshire and Holstein calves in Finland were sampled at different timepoints during the first week after birth. Their findings indicate a diverse low-abundance microbiota in the rectal samples from their newborn calves, that resembled oral samples from the dam. They also found that the fecal samples became less diverse during the first day but were more diverse again by day seven. Klein-Jöbstl *et al*. (2019) (11) sampled fecal microbiota from Holstein-Friesian calves the first two days of life. They found that the fecal microbiota was highly variable at different timepoints, and most similar to the vaginal microbiota of the dam rather than the colostrum or cow feces. They did not sample the oral mucosa of the dam.

The uterine environment and the fetus have for a long time been considered sterile in vertebrates (12), and as a consequence, also the meconium at birth. However, more studies in recent years have claimed to find a uterine microbiome that indicates that the microbial development begins before birth (13-15). There is no scientific consensus to if there is in fact a uterine microbiome that colonizes the fetus before birth, or if the findings of a fetal microbiome can be explained by contamination, misinterpretation or sequencing technology lacking the necessary detection limit for samples with low DNA levels (12).

Although the scientific community is both exploring and questioning the idea of *in utero* colonization, the importance of the developing gut microbiota for gastrointestinal function and development is widely acknowledged (9). As the intestinal microbiota is a result of several factors, both environmental such as antibiotics usage, lifestyle and genetics (16), it is of importance to document the microbiome in cattle in different environment, breed and ages. Further, nearly 40% of the calf diseases recorded in Norway in 2005 were diarrheas, and enteritis is found to be the most common cause of death among calves up to one month of age (17). Therefore, in order to reduce the health challenges regarding calf health, we need more knowledge about the composition and development of a healthy calf's gastrointestinal microbial community in Norwegian Red Breed (NRF).

# Aim of study

The goal of the student thesis was to gather more information about the microbiota of the newborn calf and how the composition of the genetics of the microbial community is compiled at two different timepoints: within a few hours after birth, and 48 hours after birth.

One aim was to characterize the development of oral and fecal microbiota in this time period, with focus on differences and similarities between the two timepoints. Another point of interest was to find out how the calf microbiota resembles samples from the dam. The sample types from the dam included oral and fecal samples taken shortly after birth, in order to make these comparisons. In addition, milk and vaginal vestibulum of the dam were also collected.

All metagenomic data of the fecal and oral samples were analyzed in this study. Metagenomic data from the additional samples (vagina, milk) were added to the final database for use in future research.

To our knowledge, this is the first study of the newborn calf's and dam's microbiota of Norwegian Red cattle in Norway. By gathering this information, we hope to contribute to a wider understanding of how a healthy fecal microbiota develops. More knowledge will give a better basis to determine how we can treat and prevent health issues relating to fecal microbiota dysbiosis in Norway.

# Materials and methods

## The Study Population

The study population was a dairy farm that produces milk for commercial sale. The farm is located close to Oslo in the South-East part of Norway. The milk farm has 69.4 annual average number of productive cows registered for 2019. The cows are of the NRF breed, and most of them are housed in a loose system with an automated milking system (AMS). The cows were inside during the season of the sampling. The farm also has a section were the cows are in tie-stalls. The cows are moved here in the dry period, and they stay here until they again can be milked from the automated milk system in the loose housing. This is at the earliest five days after giving birth.

The health status of the animals was investigated using The Norwegian Dairy Heard Recording System (medlem.tine.no) and by observations during the visits, in addition to interviews with the farmer.

Sixty-one calves were born on the farm during the last twelve months (February 2019-March 2020). During that time, 10 veterinary treatments for six calves were registered in The

Norwegian Dairy Heard Recording System (medlem.tine.no) that were related to the gastrointestinal tract. In total, there were 18 veterinary treatments of calves under the age of six months for different reasons. The number of treatments per six-month-old calf was 0.299 (country average: 0.090).

## Routines on the Farm

### *Feeding of the Dams*

Three to four weeks before calving the cows are fed silage and concentrates, which is the same feed as after calving.

### *Feeding of the Calves*

The calves are given colostrum milked from the mother as soon as possible after birth, which is usually within the first couple of hours, unless the calf is born at night. The colostrum is measured with a density meter to see if the quality is good. If not, or if the mother does not have enough milk, the calf is given frozen, stored colostrum from another cow.

The calves are milk-fed with milk from their own mother for the first five days using a bottle or milk bar two times a day. Within the first couple of days they are offered commercial muesli for calves ("Formel Mysli Start", Felleskjøpet, www.felleskjopet.no) and fine straw hay. The bedding is straw. When sampling the calves 48 hours after birth, they were generally already offered hay and muesli, but they had in general not started to eat it by then, according to the farmer.

All the newborn calves are also given one tube "Pluss Kalvepasta" commercially bought from Felleskjøpet, a supplement with antibodies from eggs, marketed to boost the immune response in neonatal calves. All the tested calves were given this once. The umbilical cord was sprayed with a commercial spray to dry it out soon after birth.

***Housing***

Before calving, the cows are moved from the loose system into the tie stalls for the dry period. The dams give birth in the stalls as there is no calving box on the farm.

The calves are removed from their mother after birth and put in single pens. They are first given the chance to suckle their dam directly after birth, if the dam is susceptible. The dam will at the same time have the possibility to lick the calf.

When sampling after calving, the calf was usually already put in a single pen. On a few occasions the calf was still loose in the barn, as the single pen was not ready yet.

All the calves were born to term and there was no dystocia. None of the calves were observed drinking from their mother, but a few attempted to suckle without managing to drink much. The calves looked and behaved as normal neonatal calves. Two of them had very small fresh blood spots on their feces at two days of age, but they were otherwise healthy.

## The Sampling Plan

The sampling was carried out between 4$^{th}$ of September and 11$^{th}$ of October 2019.

The samples were taken from eight cows and their calves at two timepoints: within the first 6.5 hours after birth (hereafter referred to as “birth”) and 48 hours after birth (+/- 5.5 hours). The cow and calf were selected based on convenience sampling; all cows on the farm giving birth in this time period were chosen for sampling. Two cows were excluded: one was still out on pasture when giving birth, and therefore had a very different environment and management than the other dams and calves. The other cow calved at a time that did not allow for sampling at birth.

The following samples were taken for this project: 1) from the calves at both timepoints: oral (n=16) and feces (n=16) and 2) from the cows (only at birth): oral (n=8) and

feces (n=8). Colostrum (n=9) and vaginal swabs (n=8) were also taken from the dam at birth for use in future research, but not included in the analysis presented here. In addition, negative controls of the farm environment were taken on each visit, in order to control for environmental contamination. The samples were taken in close vicinity to the cow and calf that was sampled.

## The Sampling Procedure

The **sampling of the oral mucosa** was done using sterile swabs. The cows head was held still, and the mouth opened so the sterile swab could be directly applied into the mouth without touching the outside of the cow to prevent contamination with the skin. The swab was then turned against the buccal mucosa ten times. When finished, the swab was placed in a sterile tube.

For the sampling of **colostrum,** the udder was first cleaned with a paper cloth moistened with tempered water, in order to remove soil or shaving, and to stimulate the udder to release milk before the teats were disinfected with 70% ethanol. The sampling tube was tilted in order to minimize the risk of any air borne debris and the first few drops were discarded, before 10 ml colostrum was hand milked into a sterile screw top tube.

It must be noted that one of the colostrum samples was taken directly from a bucket with colostrum that had been frozen, because the mother of the calf did not have enough milk.

For the **sampling of the vagina**, the labia were first cleaned on the outside with temperate water. The labia were then separated digitally using a sterile glove, and the vestibule was sampled by turning a sterile swab 10 times over the mucosa. The swab was then placed in a sterile tube.

**The fecal samples** were taken from the rectum, using sterile gloves, and applied to a sterile swab that was transferred to a sterile container.

**The environmental/negative control samples** were taken by holding a sterile swab up while walking back and forth from the place of equipment to the stall where the cow/calf were kept. In addition, the swab was gently waved for about 10 seconds near each of the place of sampling at the cow and calf (10 seconds near udder, mouth etc.). The swab was then placed in a sterile tube.

All samples were immediately stored in a cooler box and transferred to the laboratory within 8 hours where they were stored at -80°C until batchwise DNA extraction.

## DNA Extraction

DNA extraction was carried out using the ZymoBiomics™ DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA) for fecal swab samples. The extraction was done according to the manufacturer's instruction, except from modification of the bead beating process. The bead beating was performed on a TissueLyser II (Qiagen, Hilden, Germany) at 30 m/s for 3 minutes, before turning the blocks, and bead beating for another 3 minutes.

The colostrum samples were pretreated; 10 ml milk was centrifuged for 15 min at 5000 rpm at 4 °C on an ultracentrifuge (Optima LE-80K, Beckman Coulter, Indianapolis, IN, USA). The supernatant was removed, and the pellet was resuspended in 1 ml of sterilized 2% sodium citrate, and then centrifuged at 13500 rpm for five minutes. The supernatant was then removed, before continuing with the ZymoBIOMICS™ DNA Miniprep protocol. 10 ml milk was centrifuged for 15 min at 5000 rpm at 4 °C on an ultracentrifuge (Optima LE-80K, Beckman Coulter, Indianapolis, IN, USA). The supernatant was removed, and the pellet was resuspended in 1 ml of sterilized 2% sodium citrate, and then centrifuged at 13500 rpm for

five minutes. The supernatant was then removed, before continuing with the ZymoBIOMICS™ DNA Miniprep protocol.

The oral samples and the vaginal swabs were extracted by using the QIAamp DNA Mini Kit protocol with procedure for buccal swabs that contains a lysis step with buffer AL containing guanidine hydrochloride and maleic acid (Qiagen, catalog No. 51306).

In addition to the environmental/negative control samples, three spiked blanks were included to check the performance of the extraction methods: 1) one spiked with ZymoBIOMICS™ Microbial Community Standard (Catalog No. D6300) and extracted with the protocol used for oral samples, 2) one spiked with the ZymoBIOMICS™ Spike-in Control I (Catalog No. D6320) and extracted with the QIAamp DNA Mini Kit extraction protocol used for oral samples, and 3) one also spiked with ZymoBIOMICS™ Spike-in Control I (Catalog No. D6320), but extracted using ZymoBIOMICS™ DNA Miniprep Kit protocol as for the fecal samples.

The DNA concentration was measured using the fluorometric method by Qubit (Life Technologies Corp., Eugene, OR, USA). All DNA samples were initially measured using the Qubit™ dsDNA BR (Broad Range) Assay Kit and if the concentration was too low to yield a result, the Qubit™ dsDNA HS (High Sensitivity) Assay Kit was additionally used.

## DNA Sequencing

Amplicon sequencing was carried out by Novogene Genome Sequencing Company (18) Co., Ltd. (Cambridge, UK) with amplification of two regions of the bacterial 16S rRNA, with library prep, sequencing with >30000 reads per sample.

**Sequencing Preparation**

***Amplicon Generation***

16S rRNA genes of distinct regions (16SV3-V4) were amplified used specific primer (341F (5'- CCTAYGGGRBGCASCAG - 3') and 806R (5'- GGACTACNNGGGTATCTAAT - 3')) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

***PCR Products Quantification and Qualification***

Same volume of 1X loading buffer (contained SYBR green) were mixed with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400bp-450bp were considered apt for library preparation and amplicon sequencing.

***PCR Products Mixing and Purification***

Libraries were generated with NEBNext® Ultra™ DNA Library Prep Kit for Illumina and quantified via Qubit and qPCR and sequenced on the Illumina platform.

## Information Analysis

***Sequencing Data Processing***

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (19), an analysis tool for merging paired-end reads. Quality filtering was carried out to comply with the Qiime (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html) quality controlled process. The merged reads were compared with the reference database (Gold

database, http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (UCHIME Algorithm,http://www.drive5.com/usearch/manual/uchime_algo.html) to detect and subsequently remove chimera sequences (https://drive5.com/usearch/manual/chimeras.html).

**OTU Cluster and Taxonomic Annotation**

Sequence analysis was performed by Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/) using all the filtered merged reads. Sequences with ≥97% similarity were assigned to the same OTU. The representative sequence for each OTU was screened for further annotation using the Mothur software(20) against the SSUrRNA database of SILVA (http://www.arb-silva.de/) (kingdom, phylum, class, order, family, genus, species). To obtain the phylogenetic relationship of all OTUs representative sequences, MUSCLE (Version 3.8.31, http://www.drive5.com/muscle/) was used for multiple sequence alignment and comparison. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences (=45.121 reads). All subsequent analysis of alpha diversity and beta diversity were performed based on this output.

**Grouping of samples**

The fecal and oral samples from the calves were divides into sample groups based on the timepoint and sample type. The feces and oral samples of the dams was grouped according to sample type. The fecal groups were therefore divided into three groups; Calf feces 1, Calf feces 2 and Cow feces. The oral samples were divides into three sample groups as well; Calf oral 1, Calf oral 2 and Cow oral. Some of the analysis and comparisons are also done using groups divides solely by sample types.

### Alpha Diversity

Alpha diversity describes the species richness (or number of taxa) within a sample or sample group and is a measure of different microbial taxa detected in a specific sample or sample group. We used the number of observed operational taxonomic units and Shannon index to describe the alpha diversity and statistical significance was calculated using the Wilcoxon test. The Shannon index also describes the evenness of the samples. The indices were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

### Beta Diversity

The beta diversity describes differences in taxonomic abundance profiles from the different sample groups; Calf feces 1, Calf feces 2, Cow feces, Calf oral 1, Calf oral 2 and Cow oral. Anosim and T-test were performed by R software.

# Results

## DNA concentrations

The concentrations measured from the samples varied considerably, and the samples with concentrations 0.10 – 0.4 ng/ µL (n=4) were excluded (**Table 1A**, numbers in red). That left 55 samples (not counting negative controls/blanks, n=9) with concentrations ranging from 0.6 – 200.0 ng/ µL. Thirty-four of the 55 samples sent for sequencing had DNA concentrations ≤ 12 ng/ µL. The requirements for this type of sequencing is usually a concentration >12ng/µL. Eight of these samples were even below 1 ng/ µL. The fecal samples from the dams and the two-day-old calves had in general higher DNA concentrations than the other sample groups

(on average 52.8 ng/ µL. and 44.2 ng/ µL, respectively). The oral samples for both dam and calves had concentrations ≤ 3.3 ng/ µL.

**1A)**

| Sample | Cow Feces | Calf Feces 1 | Calf feces 2 | Cow oral | Calf oral 1 | Calf oral 2 | Colostrum | Vagina |
|---|---|---|---|---|---|---|---|---|
| 1119 | 58.6 | 1.5 | 106.0 | 0.4 | 1.2 | 0.8 | 187.0 | 26.4 |
| 1375 | 6.5 | 0.3 | 6.4 | 3.3* | 1.9* | 1.0 | 19.4 | 19.2 |
| 1399 | 15.2 | 4.5 | 22.4 | 0.8 | 1.6* | 1.2 | 29.4* | 14.2 |
| 1442 | 93.2 | 0.1 | 132.0 | 1.0 | 0.8 | 1.4 | 24.7 | 58.2 |
| 1460 | 17.3 | 5.0 | 7.1 | 1.3 | 3.2 | 2.4 | 20.8 | 23.8 |
| 1482 | 5.3 | 0.3 | 2.4 | 1.5* | 1.9 | 3.0 | 33.0 | 140.0 |
| 1483 | 200.0 | 5.8 | 60.8 | 0.7* | 1.2 | 3.6 | 49.9 | 30.2 |
| 1539 | 26.6 | 6.1 | 16.6 | 0.6 | 1.2 | 0.6 | 51.9 | 6.5 |
| Extra1539 | | | | | | | 31.3 | |
| Average | 45.5 | 2.9 | 44.2 | 1.2 | 1.6 | 1.8 | 49.7 | 39.8 |

**1B)**

| Negatives | |
|---|---|
| Sample | Conc. (ng/ µL) |
| 1119KN2 | 3.58 |
| 1119N1 | too low |
| 1399KN2 | 0.02 |
| 1442N1* | too low |
| 1483KN1 | 0.03 |
| 1539N1 | too low |

**Table 1A and 1B:** *The **Table 1A** shows the DNA concentrations (ng/µL) measured with Qubit before being sent for sequencing. Some of the samples sent were low in DNA concentration; the ones under 1 ng/ µL are marked in green. The four samples with concentrations in red were excluded because of the very low concentrations (<0.5 ng/µL). Notes: The colostrum and vaginal samples are included for use in the dataset for further research. * The sample failed library preparation. **Table 1B** shows the DNA measurements for the environmental/negative controls. * This sample failed library preparation.*

## Quality control

Out of the 70 submitted samples; seven of the samples (one negative, one milk sample and five oral samples from calf/dam), were considered unsuitable for library preparation. As a result, the final analysis included 63 samples including three controls and five negative

environmental samples. The number of reads were on average 85.611 filtered reads, with the minimum number of reads at 45.121 and the maximum at 133.660 filtered reads.

## Environmental/negative controls and extraction control samples

Six environmental/negative controls were included (**Table 1B**). One of these failed the library preparation. In addition to the environmental controls, there were three controls to check the performance of the extraction. **Figure 1** shows that the environmental/negative control samples contain a broad range of bacteria. The composition of bacteria is also somewhat varying between the different negatives.

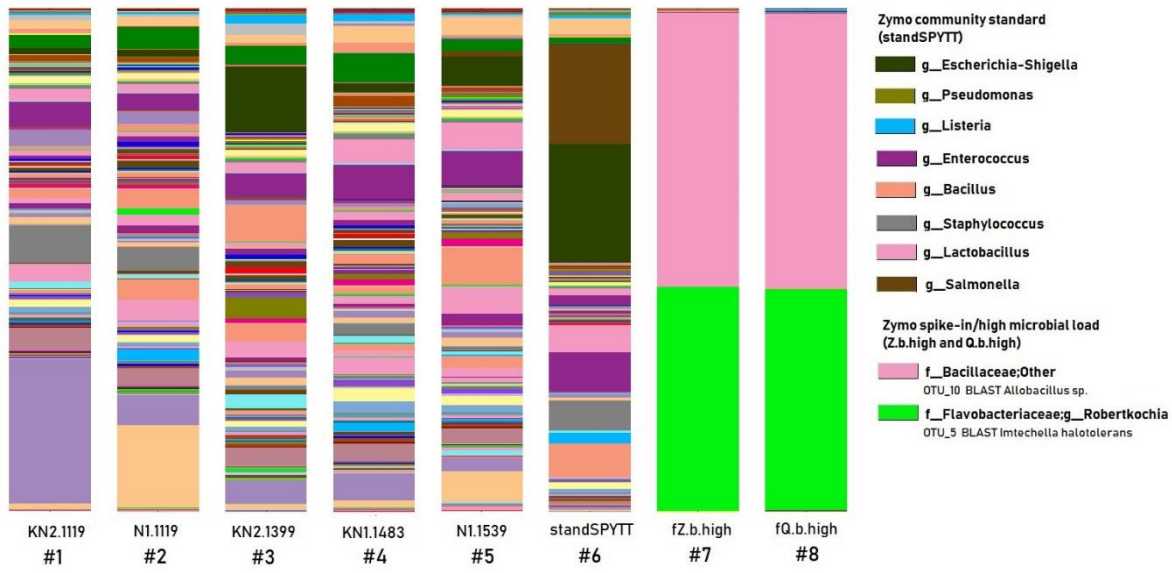



**Figure 1**: *The bar charts illustrate the composition at genus level of the environmental/negative control samples (#1 - #5), #6: blank sample spiked with Zymobiomics™ Microbial Community Standard extracted following the oral protocol, #7: blank sample spiked with Zymobiomics high microbial load spike-in control extracted following the feces protocol,. #8 (blank sample spiked with Zymobiomics high microbial load spike-in control extracted following the oral protocol).*

**Figure 1** shows that the environmental/negative control samples contain a broad range of bacteria. The composition of bacteria is also somewhat varying between the different negatives.

In the microbial community standard named "standSPYTT/#6" extracted using the extraction method for the oral samples, we can see that all the eight different bacteria of the mock community (*Lactobacillus fermentum, Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Salmonella enterica, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa*), are present in the result for the sample. *Escherichia-Shigella* and *Salmonella* (both Gram negative) are the most dominant taxa. In the original community *Lactobacillus fermentum* and *Staphylocccus aureus* (both Gram-positive) were the most abundant.

The Zymobiomics high microbial load spike in controls extracted with the oral (QIAamp DNA Mini Kit) and fecal protocol (ZymoBiomics DNA Miniprep Kit) originally contain equal cell numbers of *Imtechella halotolerans* (Gram-negative) and *Allobacillus halotolerans* (Gram-positive). The bar chart of the two samples extracted with the two protocols shows that the two bacteria seem to be at similar abundance. There is a small amount of other bacteria in the samples, but at very small abundance.

## Relative abundance

The oral and fecal samples from both dams and calves showed a relative abundance dominated by *Proteobacteria* and *Firmicutes* when organized by phylum (**Figure 2**), but at somewhat different distributions in the different sample groups.

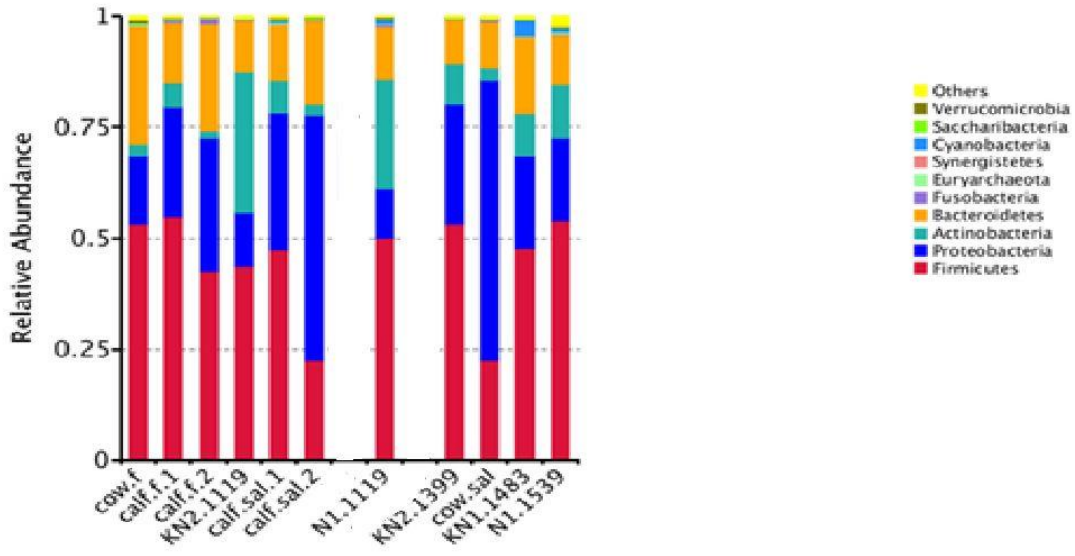



**Figure 2:** *Relative abundance of the 10 most abundant phyla in the sample groups, including negative controls. The individual samples are grouped according to origin of sample and timepoint of collection. The groups are as follows: cow.f = feces dam; calf.f.1 = feces calf at birth, calf.f.2 = feces calf two-days old, cow.sal = oral dam, calf.sal.1 = oral calf at birth, calf.sal.2 = oral calf two-days old, groups containing "N" = environmental controls.*

## Fecal samples

The fecal samples from calves at birth (n=5) had a total of 3.185 observed OTUs with an average of 1.524 OTUs. In the calves' first feces the most abundant genus was *Acinetobacter* (11.1%), followed by *Staphylococcus* (7.2%) and *Bacteroides* (5.1%). The group entailing "Others" made up a total of 11.1% of the reads.

There were in total 2.844 OTUs found in the fecal samples from the two-day-old calves (n=8), with an average of 1.263 OTUs per sample. While the genus *Escherichia-Shigella* was the eighth most abundant genus (3.7%) in the calves' feces at birth, it was the most abundant genus (22.0%) from the feces two days later. *Bacteroides* was the second most abundant genus (19.7%) followed by *Butyricicoccus* (8.2%).

For the dams, 4.148 OTUs were observed in total (n=8), with 1.806 OTUs on average. Analysis of the relative abundance in the dam's fecal sample groups showed that *Ruminococcaceae UCG-005* (12.7%), *Ruminococcaceae UCG-010* (5.6%) and *Bacteroides*

(5.5%) was the most abundant genera in this sample group. The collection group containing "Others" accounted for 16.3%.

### Oral samples

The total observed OTUs for the oral samples of the calves at birth were 3.731 (n=6), with 1.774 on average. The most abundant genera in the first oral samples of the calves were *Acinetobacter* (13.8%), *Enterococcus* (6.1%) and *Ruminococcaceae USG-005* (5.7%), while 12.8% were comprised of genera categorized as "Others".

Two days later the total OTU-count were 3.096 for the calves (n=8), with 1.318 OTUS on average. Analysis of the oral samples from two days old calves showed that the collection group of "Others" measured a total of 37.9%. The top three genera were *Porphyromonas* (9.0%), *Neisseria* (5.8%) and *Acinetobacter* (4.6%).

The total observed OTUs for the dams were 2.802 (n=4), with an average on 1.445. In the oral samples from the dams, *Moraxella* (8,7%), *Neisseria* (6.0%) and *Acinetobacter* (5.6%). The group "Others" were measured to 36.1%.

## Alpha diversity analysis

### Distribution of operational taxonomic units

#### *Fecal samples*

The analysis showed that the feces of the calves at birth and 48 hours of age shared 2.233 operational taxonomic units (OTUs) (**Figure 3A**); which means that 70.1% of the observed fecal OTUs at birth could also be found in two-days-old calves. Here, the shared OTUs were 78.5% of the fecal sample at the second timepoint, as the feces at the second timepoint had a

slightly lower diversity than at the first sampling. While the fecal samples at birth had 3.185 OTUs in total, the number of OTUs in feces from two days old calves was reduced to 2.844.

By comparison, the number of OTUs in the dams' fecal samples were 4.148, and 2.802 in the oral samples of the dams. 1.736 of the OTUs were shared with all the four sample groups in **Figure 3A**. The operational taxonomic units that were specific for the first feces in the diagram, amounted to 12.5 % of the fecal sample group from the newborn calves.

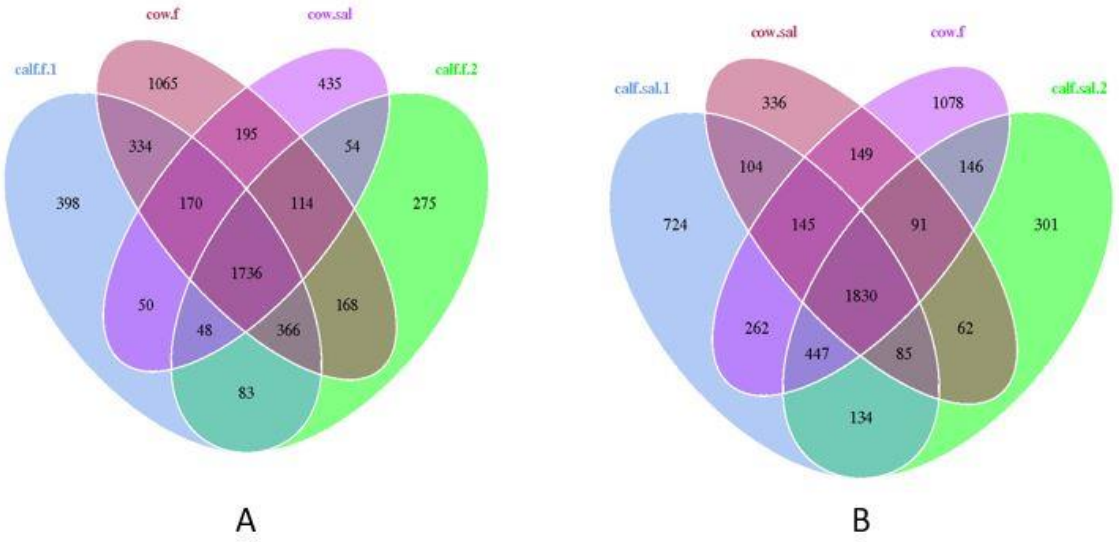



**Figure 3A and 3B:** *OTU distribution shown in Venn diagram. Calf.f.1: Feces from calf birth. Calf.f.2: Feces from calf at two days of age. Cow.sal: Oral sample from the dams. Cow.f: Feces samples from the dam. Calf.sal.1: Oral sample of calf at birth. Calf.sal.2: Oral samples of calves at two days of age.*

The calf feces at the first timepoint shared 2.606 of the OTUs (81.8 % of the calves' OTUs) with the feces of the dam. Of the OTUs in the first feces of the calves, 62.9 % of the OTUs were also found in the oral samples of the dams.

At the second timepoint, 83.8 % of the OTUs found in the calf feces were OTUs also found in the dam's feces, while 68.6 % of the OTUs were also found in the oral samples of the dam.

Although the OTUs in some extent overlap at all the four sample types, nearly 26% of the dam's fecal microbiota are OTUs that cannot be found in the oral or the fecal samples of the calves.

***Oral samples***

The oral samples from the calves taken at different timepoints shared 2.496 OTUs. In total, the samples first taken from the calves had a total OTU-count of 3.731, while the count had decreased to 3.096 two days later. The oral samples of the grown cow were even less diverse, with 2.802 OTUs.

In the oral samples at birth, 58.0 % of the observed OTUs were shared with the oral samples from the dams, while 19.4 % of the OTUs was specific for this group (**Figure 3B)**.

Two days after birth, 66.9 % of the OTUs from the first timepoint was still present in the sample, making up 80.6 % of the OTUs in the oral samples.
Around half (51.3 %) of the operational taxonomic units presented in the first oral samples also can be found both two days later and in the oral samples of the dam.

## Alpha diversity indices

While the number of observed OTUs tells us something about the richness of the microbiota of the samples, it does not present the distribution or abundance of the OTUs in the samples. The Shannon diversity index gives us more information as it considers both the richness and evenness of the samples, and the difference in this diversity can be calculated using a statistical test. Here we used Wilcoxon test for testing if the differences seen between groups

in both number of observed OTUs and Shannon diversity index, and *p*-values below 0.05 was considered significant (**Table 2**).

| Groups | p-values calculated by Wilcoxon test for | |
|---|---|---|
| | **Shannon Diversity indices** | **Number of observed OTUs** |
| Calf feces 1 – calf feces 2 | **0.0117** | 0.0979 |
| Calf feces 1 – cow feces | **0.0143** | 0.0628 |
| Calf feces 2 – cow feces | **<0.0001** | **0.0001** |
| Calf feces 1 – cow oral | 0.8024 | 0.8522 |
| Calf feces 2 – calf oral 1 | **0.0007** | **0.0006** |
| Calf feces 2 – cow oral | **0.0356** | 0.1785 |
| Cow feces – calf oral 2 | **0.0001** | **0.0014** |
| Calf oral 1 – calf oral 2 | **0.0385** | **0.0041** |
| Calf oral 1 – cow oral | 0. 3335 | 0.0819 |
| Calf oral 2 – cow oral | 0.4037 | 0.4421 |
| Feces (all groups) – oral (all groups) | 0.9103 | 0.9439 |
| | | |

**Table 2:** *The table above presents differences in alpha diversity between the compared groups. Fecal groups are marked in red, and oral groups in blue. The number of observed OTUs and Shannon diversity indices for the groups were analyzed with the Wilcoxon test, statistically significant p-values (p <0.05) are shown in bold. The number "1" and "2" refers to sampling timepoint; "1" at birth, "2" at two days of age.*

## Richness and evenness within groups

### *Comparisons between sample sites*

The analysis of the two different sample groups divided by sample types (feces and oral, independent of age and timepoint) showed that there were no statistically significant ($p \geq 0.05$) differences between the two sample sites (**Table 2**) when it comes to only the richness (number of observed OTUs; p=0.9439) and the richness and evenness of operational taxonomic units (Shannon diversity index; p=0.9103). The same results apply when comparing the calves' feces at birth and the dams' oral microbiota; the richness and evenness of operational taxonomic units is not significantly different (Shannon diversity p=0.8024) (**Table 2**).

### *Fecal samples*

The observed number of OTUs in the different fecal sample groups is shown in the box plot in **Figure 4**. The feces of the dams had the largest number of OTUs, while the OTU-count showed a less diverse fecal microbiota in the newborn calves that decreases even further in the feces of the two-day-old calves. The richness of the samples (number of observed OTUs) and the richness and evenness (Shannon diversity index) differed significantly (**Table 2**) between the feces of the dams and the two-day-old calves ($p=0.0001$ and $p<0.0001$, respectively).

In addition, there was a significant difference in richness and evenness (Shannon diversity index, $p=0.0143$) between the first calf feces and that of the dam. There was also a statistically significant difference regarding species richness and evenness (Shannon diversity index, $p=0.0117$) between the calves' feces at birth and two days later.

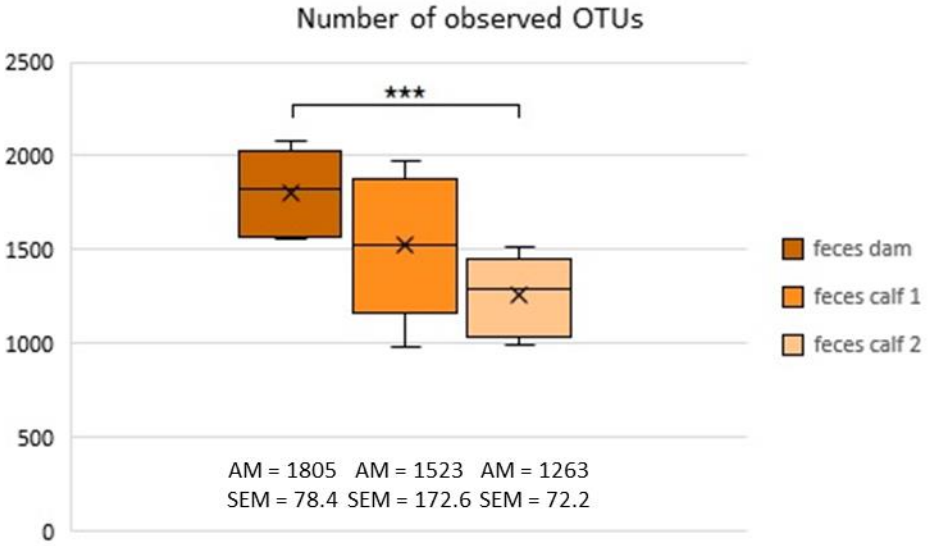



**Figure 4:** *Box plot shows the comparison of the α-diversity by number of observed OTUs in the fecal samples from calves at the first (calf1) and second timepoint (calf2) and the fecal samples from the dams. Also including average of the mean (AM) and standard error of the mean (SEM). Statistical significance for calf feces 2 and cow feces is indicated (***p< 0.001).*

### *Oral samples*

While the fecal microbiota of the dams and the calves differ in richness and evenness (**Table 2**), there was no observable statistically significant difference between the oral microbiota of the dams and the calves at either of the two timepoints (**Table 2**). However, the oral microbiota of the two-day-old calves was significantly different from the oral microbiota of the newborn calves when it came to richness (number of observed OTUs, p=0.0041) and richness and evenness (Shannon diversity index, p=0.0385) (**Table 2**, **Figure 5**).

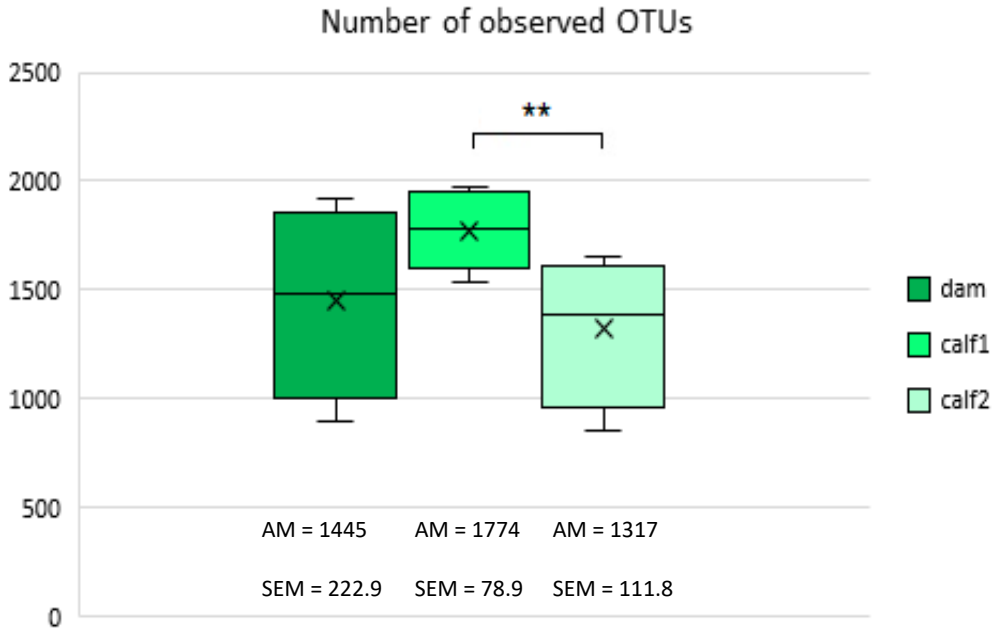



**Figure 5:** *The box plot shows the comparison of the α-diversity by number of observed OTUs in the oral samples from calves at birth (calf1) and two days later (calf2) and the oral samples from the dams. Also including average of the mean (AM) and standard error of the mean (SEM). Statistical significance is indicated (**p < 0.01). There is a significant difference in the number of OTUs and the Shannon diversity index (**Table 2**) of the oral microbiota in the calf the first two days after birth (p=0.0041 and p=0.0385, respectively).*

## Beta Diversity indices

The beta diversity expresses the difference in the microbial composition between two groups, measured by the distribution of taxa specific for the compared groups and the abundance of the taxa in the groups.

### ANOSIM: Analysis of similarity

| Comparison | R-value | p-value | Interpretation |
|---|---|---|---|
| Cow feces – calf feces 1 | 0.5474 | 0.003 | variation among groups is significantly larger than variation within groups |
| Cow feces – calf feces 2 | 0.9113 | 0.001 | variation among groups is significantly larger than variation within groups |
| Calf feces 1 – calf feces 2 | 0.6408 | 0.004 | variation among groups is significantly larger than variation within groups |
| Calf feces 1 – cow oral | 0.5688 | 0.01 | variation among groups is significantly larger than variation within groups |
| Calf feces 2 – cow oral | 0.9651 | 0.004 | variation among groups is significantly larger than variation within groups |
| Cow oral – calf oral 1 | 0.6151 | 0.003 | variation among groups is significantly larger than variation within groups |
| Cow oral – calf oral 2 | **0.1893** | **0.142** | variation among groups is **not** significantly larger than variation within groups; the R-value is not significant. |
| Calf oral 1 – calf oral 2 | 0.6124 | 0.001 | variation among groups is significantly larger than variation within groups |
| Cow feces – calf oral 1 | 0.5979 | 0.001 | variation among groups is significantly larger than variation within groups |
| Cow feces – calf oral 2 | 0.865 | 0.002 | variation among groups is significantly larger than variation within groups |

**Table 3:** *The table shows the results of the ANOSIM test. The variation among groups is significantly larger than variation within groups (p<0.05), except for the group "cow oral– calf oral 2", that has a non-significant R-value and therefore inconclusive ANOSIM test. The sample groups named "1"is at birth, while "2" is sampled at two days of age. An R value close to 1 suggests dissimilarity between groups while an R value close to 0 suggests an even distribution within and between groups.*

The ANOSIM analysis ranks the similarity or dissimilarity between and within groups. **Table 3** shows that oral microbiota belonging to the two-day-old calves and the mother's oral

microbiota has an R-value close to zero (0.1893), which indicates low separation between the two groups, and therefore similarity and the R-value is not significant (<0.2).

The remaining comparisons between age specific groups have a higher R-value and a p-value below 0.05. The variation among the groups are significantly larger than the variation within these groups.

**Between Group Variation Analysis T-test**

The t-test of the beta diversity was performed on the groups by analysis of between-group variation. This test (**Figure 6A**) shows that there is significantly more *Proteobacteria* in the feces from the two-day-old calves, than in the feces of the dams, and that there is significantly more *Firmicutes* in the dams' feces than in the feces of the two-day old calves. At genus level (**Figure 6B**), the t-test shows significantly more *Escherichia/Shigella*, *Bacteroides* and *Butyricicoccus* in calf feces two days post-partum compared to in the dam. There is also significantly more *Ruminococcaceae*, *Rikenellaceae* and *Alistipes* in the dams' feces.

6A)

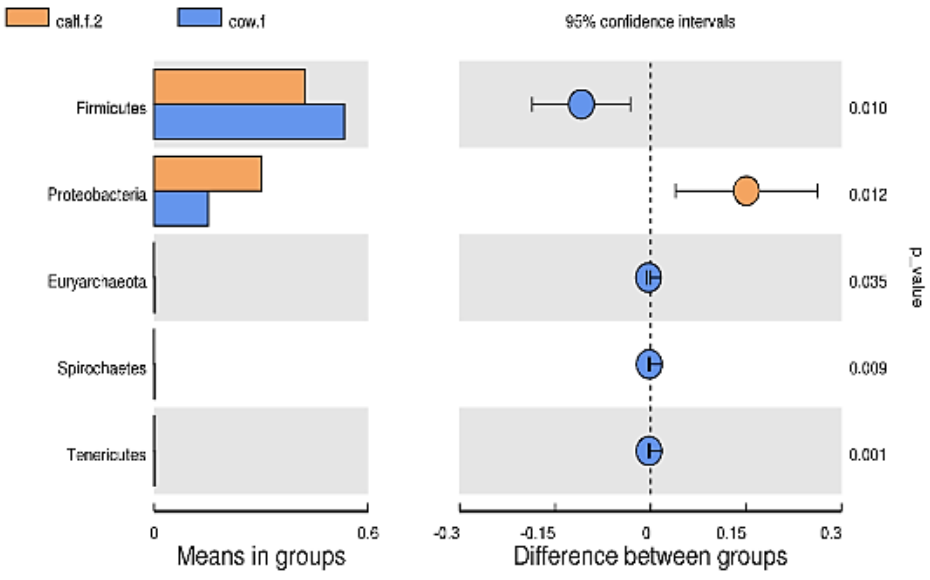
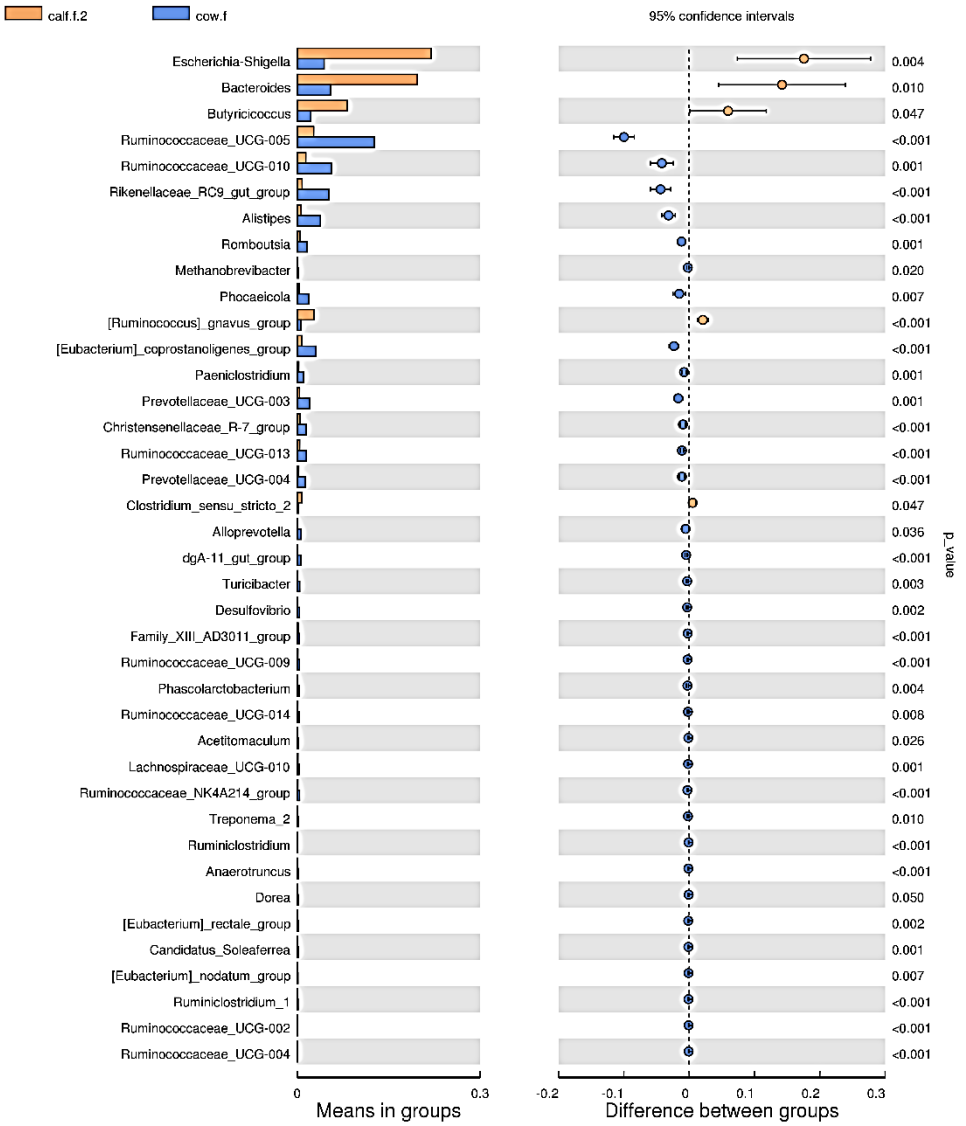



**Figure 6A:** *Phylum level T- test between calf feces at timepoint two and cow feces (above). Significantly more Proteobacteria in calves, and more Firmicutes in cows. The left panel is the abundance of species showing significant difference between group (p-value < 0.05). Each bar represents the mean value of the abundance in each group of the species showing significant difference between group. The right panel is the confidence interval of between group variation. The left-most part of each circle stands for the lower limit of 95% confidence interval, while the right-most part is the upper limit. The center of the circle stands for the difference of the mean value. The color of the circle represents the group. The right-most value is the p-value of the significance test of between group variation.*

6B)




***6B**: Genus level t-test between calf feces at timepoint two and cow feces (above). The figure is presented the same way as in **Figure 6A**, but organized by genera.*

The t-test analysis of the beta diversity also compared the calves' fecal microbiota at two-days-old and the oral microbiota of the dam (**see Figure 7A and 7B**). The test at phylum level showed that there are significantly more *Firmicutes* and *Bacteroides* in the feces of the calves, than in the oral microbiota of the dams. The oral microbiota of the dam contained more *Proteobacteria* than the fecal microbiota of the calves.

7A)

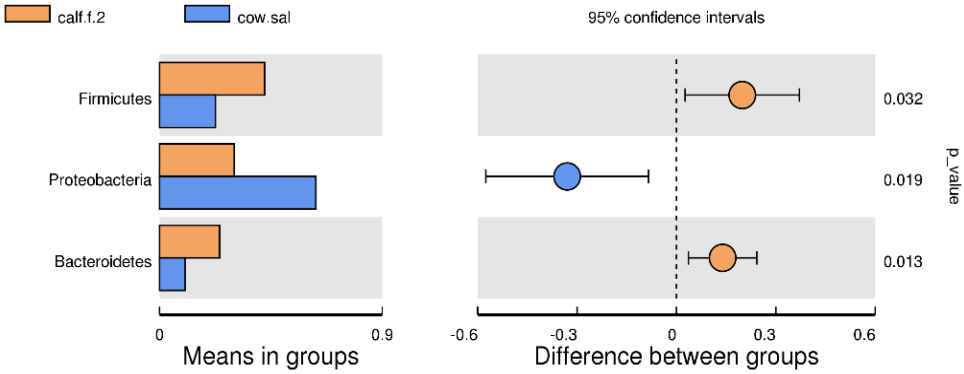
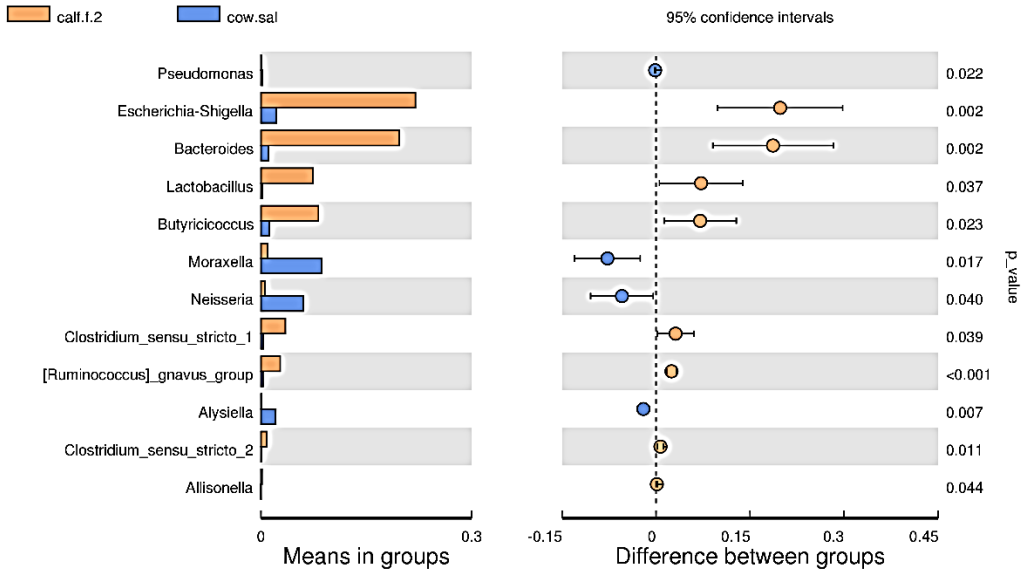



**Figure 7**
**7A**: *Phylum level t- test between calf feces at timepoint two and cow oral (above). The figure is presented in the same way as in figure 6, but the feces of the calves are here compared to the oral microbiota of the dam. There are significantly ($p < 0.05$) more Firmicutes and Bacteroides in calf feces, and more Proteobacteria in the adult oral microbiota.*

7B)




***7B**: Genus level t- test between calf feces at timepoint two and cow oral (above). The figure is presented in the same way as in **Figure 6B**, but the feces of the calves are here compared to the oral microbiota of the dam. There are significantly ($p < 0.05$) more Firmicutes and Bacteroides in calf feces, and more Proteobacteria in the adult oral microbiota.*

At genus level the feces of the two-day-old calves contained significantly more *Escherichia-Shigella*, *Bacteroides*, *Lactobacillus* and *Butyricoccus* compared to the oral microbiota of the

dam. While the oral microbiota of the dams contained significantly more *Moraxella, Neisseria* and *Alysiella* than the feces of the calves.

# Discussion

In this study, the aim was to learn more about the development of the oral and fecal microbiota of the calf in its two first days of life; how does this microbiota develop during these days, and how does it compare to the oral and fecal microbiota of the mother?

## Comparisons of Collection Groups

When comparing the collection groups (all fecal and all oral), there were no significant difference in richness and evenness. However, these two sample groups are very crude, as the fecal and oral samples are not divided between newborn calf, two-day-old calf or the dam. In this sense, the use of these groups does not provide the information that this study aims to learn more about, which is the differences and equality between these subgroups.

## The Fecal Microbiota in the Calf

The fecal samples of the calves at the two different timepoints had statistically significant differences in both the richness and evenness, and analysis of similarity that is based on abundance.

The first feces of the calves were more diverse than in the two-days-old calves, with 1.524 OTUs on average, and with *Acinetobacter* as the most abundant genus. At the second sampling the feces had become somewhat less diverse, with 1.263 OTUs on average per sample. Now *Escherichia-Shigella* was the most abundant genus.

It is not surprising that there is a certain difference, as the first feces of the calf is meconium, and as such affected by the uterine environment. When sampling feces two days later the calf has been exposed to an entirely different environment and feeding.

## Calf Fecal Compared to Fecal Dam

When comparing the fecal samples from the calves and the dams, the analysis showed a significant difference in the richness and evenness between all the fecal sample groups (**Table 2),** indicating that the feces undergoes a significant change in the first few days. There is also changes in the fecal microbial composition at a later stage, before the feces develops into the fecal microbiota of the grown animal (**Figure 6A and 6B**).

The T-test showed significantly more *Proteobacteria* in the newborn feces at two days of age, than in the feces of the dam. The dams had significantly more *Firmicutes* than the calves.

The ANOSIM of the beta diversity rated the variation between the two groups as significant, compared to the differences within the groups.

## Calf Fecal Compared to Dam Oral Microbiota

We found that in the first feces of the sampled calves, the most abundant phyla were *Firmicutes, Proteobacteria and Bacteroidetes* (**Figure 2***)*. The same phyla were found to be dominating in the samples of newborn calves in studies in 2018 and 2019 (10, 11), and they also found that *Acinetobacter* ranked as the third most abundant phylum. In this study *Acinetobacter* was the fourth most abundant phylum.

Two days after birth, the feces of the calves had shifted, with *Escherichia-Shigella* as the most abundant genus, but still with Firmicutes as the dominant phylum. These findings are

also in line with the results from Alipour *et al* (2018), where they found fecal samples from the calves increasing in *Escherichia/Shigella* alongside with *Clostridia* during the first day.

The oral microbiota of the dams was found to have *Proteobacteria* as the most abundant phylum, followed by *Firmicutes* and *Bacteroidetes* (**Figure 2**).No statistically significant difference in richness and richness and evenness between the first feces of the newborn calves and the dams' oral microbiota was observed (**Table 2**).

However, the analysis of similarity (**Table 3**) of the feces of the calves at each timepoint compared to the oral microbiota of the dam, concluded that the variation between the feces of the calves and the oral microbiota of the dam was significantly larger than the variation within the groups. So even though earlier findings (10) emphasize similarities between the microbial community in the newborn feces and the oral microbiota of the cow, we could not find statistically significant similarities in this analysis of similarity that takes the relative abundance into account.

Indeed, the t-test of the beta diversity showed significantly more *Firmicutes* and *Bacteroidetes* in the feces of the calves at two days of age compared to the oral microbiota of the dams. The adult oral microbiota in our results contained more *Proteobacteria* than the newborn fecal microbiota.

## The Oral Microbiota of the Calf

The oral microbiota of the newborn calves is significantly different from the oral microbiota of the two-day-old calves when it comes to richness and evenness (**Table 2**), and the variation between the two groups are significantly larger than within (**Table 3**).

The oral microbiota of the two-day-old calves is less diverse than in the newborn calves, measured in number of observed OTUS (**Figure 5**). After birth, 19.4 % of the OTUs are specific for the newborn oral microbiota, in the sense that these OTUs are not shared with

the oral samples from the dam or the oral microbiota two days later. While *Acinetobacter* was the most abundant genera after birth (13.8 %), followed by *Enterococcus* and *Ruminococcaceae USG-005* the oral microbiota had shifted to *Porphyromonas* and *Neisseria* as the most abundant genera two days later, with *Acinetobacter* as number three (4.6 %).

## Oral Microbiota in Calf and Mother

When assessing the diversity by analysis of similarity, it was only the comparison of the oral sample of the two-day old calves (**Table 3**) and the mother that trended towards a similarity in diversity. ANOSIM found a significant difference in diversity between the other compared groups. Moreover, alpha diversity assessed by number of OTUs and Shannon index showed no significant differences between these two oral sample groups. This corroborates a similarity of the two groups.

By considering the relative abundance of the calves and the dams, we can also see how the single sample is composed. When comparing the ten most abundant genera in the two sample groups, we can find that the mothers and the two-day old calves have the same eight genera as the ten most abundant.

Of course, these findings must be interpreted with some caution, as only four out of the eight oral samples from the dam were of high enough quality to be analyzed. This sample group is unfortunately the smallest of the groups, and therefore there is more uncertainty linked to these results, as individual results will have a large impact on the mean of the samples. This can also potentially explain that the R-value is not significant between these two groups in the ANOSIM analysis.

## Interpretation of the Analysis

The number of OTUs tells us how many bacterial taxa are present in the sample and we can compare the OTUs between sample types by using Venn diagrams (**Figure 3A and 3B**). The relative abundance presents the microorganisms and how common or rare they are in the sample. In that way we can compare the composition and abundance to the other sample groups. To take the evenness of OTUs into consideration, we compared beta diversities using ANOSIM and a t-test.

## Quality and Number of the Samples

The DNA measurements were done using Qubit dsDNA Broad Range Assay Kit, and we had several low biomass samples, especially from oral swabs.

Four of the samples with the lowest DNA concentrations (0.1 – 0.4 ng/ µL) were excluded, based on the low biomass in the samples. Still, eight of the samples sent for sequencing were below 1 ng/ µL. The measured DNA concentrations from the samples were of low quantities (**Table 1**) before sending these to the sequencing laboratory for sequencing and analysis. The commercial provider usually requires at least 20 µL of a sample with a DNA concentration above 12 ng/µL; 34 of the samples had DNA concentrations below this value.

Seven of the samples failed library preparation. Many of these were samples of low biomass (oral n= 5, negatives n=1), but also a milk sample with a fairly decent quantity of DNA.

| Sample types | Number of samples |
|---|---|
| Feces calf 1 | 5 |
| Feces calf 2 | 8 |
| Feces dam | 8 |
| Oral calf 1 | 6 |
| Oral calf 2 | 8 |
| Oral dam | 4 |
| Environment negative | 5 |
| Standards | 3 |
| Colostrum | 8 |
| Vagina | 8 |

**Table 4:** *Number of samples that were of good enough quality and were analyzed.*

In consequence, the number of samples in each group were not equal (**Table 4**), and fewer than expected. The smallest sample group were the oral samples from the dam, that only counted four samples. Since the data is derived from a small study population (n=8), and the number of samples has somewhat declined during the process, the results must be interpreted with caution. This makes it especially challenging to draw biological conclusions, as individual differences and contamination can potentially have a large impact on results with this sample size.

## Contaminants and Negative Controls

In addition to the samples taken directly from the animals, we sequenced and analyzed five environmental samples, two spike-in negatives and one mock community standard.

### Environmental Samples

The environmental/negative control samples can show the composition from the environment, what we need to subtract from the other samples and what can have contaminated the samples during DNA extraction and sequencing. The environmental samples were diverse, but they

had some differences in how abundant the different bacteria are (see **Figure 1 and 2)**. Unfortunately, since the bioinformatic analysis was done externally, no additional filtering was applied. A possible problem here could also be contamination during DNA extraction, which is also supported by finding other bacteria than those supplied in the community standard in the STANDspytt sample (see below).

**Community Standard**

The mock community in the microbial community standard can be used to assess the quality of extraction, possible bias and errors in the process. Here, we found all eight microbes from the mock community in the sample. The extraction and analysis had been successful for identifying these microbes.

However, the results for the samples showed that the relative abundance of the different microbes were not composed in the same way, as *Escherichia-Shigella* and *Salmonella* were the most abundant taxa in the results. This could indicate a bias in either the extraction method or the amplification process towards Gram-negative bacteria. There were also genera present that cannot stem from the Community Standard indicating a possible laboratory contamination during the extraction process and raising concerns about the suitability of the environmental/negative controls.

**Spike-in Control**

The ZymoBiomics spike-in control I (high microbial load) is comprised of equal cell numbers of two known bacterial strains; *Imtechella Halotolerans* (Gram-negative) and *Allobacillus halotolerans* (Gram-positive). This can potentially reveal bias during the extraction when comparing the content of the two bacterial strains after analysis with the known bacterial load in the spike-in sample.

In these results, we could see a distribution of both the two taxa very close to 50 % of the entire sample. The results are almost identical for the two extraction protocols. This is an indicator that the extraction was successful for both the Gram-negative and Gram-positive bacteria with both protocols. In addition, very few contaminants were present. It must be noted that these two extractions were carried out by another person.

**Contaminants**

Controlling for contaminants in samples with low biomass, such as many of the samples in this study, is of importance, but is also problematic. A recent article (21) discusses the challenges with contamination in low biomass samples sequenced with 16S rRNA metagenomics. The authors recommend to use negative controls and a mock microbial community in order to identify the contaminants, which has been done in this student study.

However, in the article it was moreover recommended to include a dilution series of the mock microbial community consisting of at least high, medium, and low concentrations, to be able to determine the filter level for bioinformatic analysis.

The authors found negative control filter unsatisfying, while other computational strategies worked well under certain conditions to control contaminants; nevertheless, when the contaminants consisted of one third or more of the sample, as was the case for our samples, all filter methods seemed to be unsuccessful (21).

More controls taken by different persons would be needed to determine whether for our samples an inter-person bias is present. There is currently also a lack in the literature regarding the importance and correct downstream processing of environmental samples. This study highlights the importance of proper controls and the need for more research into correct environmental controls from the farm environment.

## Extraction Methods and Possible Sources of Errors

The extraction method is an important consideration when comparing sample groups, as this will probably have influenced the generated metagenomes.

In our study, the oral samples were extracted with a different method than the fecal samples. The Qubit measurements implied that the ZymoBiomics DNA Miniprep Kit (feces samples) was more efficient in extracting DNA from the samples, as the standards from ZymoBiomics showed a higher DNA concentration than those from QiaAmp DNA Mini Kit (oral samples).

The blank extractions with both kits did not indicate a significant difference in performance; however, ideally, all samples to be compared should be extracted with the same protocol and same kits. Potential bias introduced by the different protocols can give systematical errors and lead to wrong results when comparing sample groups.

It cannot be ruled out that significant differences between sample groups can be attributed to the different methods used. For example, the protocol for ZymoBiomics DNA Miniprep Kit included a bead beating process. In the QIAamp DNA Mini Kit protocol for the oral samples, there was no bead beating. The potential different sensitivity of the two kits can also affect the results in the other direction; there is a possibility that there is a larger variation between groups than what our results show.

The samples of the same type were always extracted with the same protocol, independent of the timepoint. The comparisons within these groups of the same sample type can therefore be considered more trustworthy. As an example, comparing oral samples at birth with oral samples two days later, is in this sense more reliable than comparing between fecal and oral sample groups.

The human factor is also a possible source of error. The author of this student thesis did not have any experience with DNA extractions and was doing this for the first time when extracting the DNA for library preparation. When lacking experience in this field, the possibility of errors related to practical steps as exact pipetting, measuring and handling of the samples, hygiene and a systematical overview and workflow, increases.

# Conclusion

In this student study the aim was to learn more about the development and composition of the microbiota in Norwegian red calves.

The findings indicate that the fecal microbiota of calves has a less diverse composition than the microbiota of the adult cow, and that the microbial composition develops quickly during the two first days of life that is dominated by *Escherichia-Shigella* two days after birth. There were significantly more *Proteobacteria* in feces of the two-day-old calves than in the mothers' feces, and significantly more *Firmicutes* in the feces of the dam.

The abundance of taxa in the feces of the calves is similar to what is described in earlier studies, with *Firmicutes* being the most abundant phyla. The fecal microbiota of the calves at birth and the oral microbiota of the dams have previously been described as similar; yet, we only found these two groups similar in terms of alpha, but not beta diversity.

The oral microbiota of the calves also develops quickly. The calves' oral samples after birth and two days later statistically differed in diversity. Only two days into the life of the calves, the oral microbiota was similar to the oral microbiota of the dams (**Table 3**), with *Porphyromonas* as the most abundant phyla.

The few oral samples from the dams (n=4) is a factor that increases the uncertainty in the study. The results from the community standard also indicates a possible laboratory

contamination during the extractions that needs to be taken into account when assessing the results, as this can have rendered the environmental/negative controls ineffective.

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# Sammendrag

*Tittel*: Sammensetning og tidlig utvikling av den fekale og orale mikrobiotaen hos NRF-kalver.

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Åtte kyr og deres kalver ble prøvetatt for å kartlegge sammensetning og utviklingen av den fekale og orale mikrobiotaen. Kalvene og kuene ble prøvetatt for spytt og fecesprøver like etter fødsel, og kalvene to dager senere. Det ble også tatt prøver av vaginalslimhinnen og råmelken hos kuene til en database for videre forskning.

Sekvensering av 16S rRNA amplicons og analyse av resultatene viste at den fekale mikrobiotaen utviklet seg raskt etter fødsel, og var betydelig annerledes hos den nyfødte kalven enn hos kua. Det var signifikant mer *Firmicutes* i avføringen til kua enn hos kalven ved to dagers alder. Kalven hadde derimot mer *Proteobacteria*.

Det var også et betydelig skifte i tarmfloraen fra fødsel til todagers alder hos kalven. Mens genera som *Acinetobacter*, *Stafylococcus* og *Bacteroides* dominerte det fekale mikrobiomet ved fødsel, var det to dager senere *Escherichia-Shigella*, fulgt av *Bacteroides* og *Butyricoccus*. Vi kunne også se en nedgang i artsmangfoldet.

Den orale mikrobiotaen til kalven undergikk også en betydelig utvikling. Phyla som *Acinetobacter* dominerte ved første prøvetaking, etterfulgt av *Enterococcus* og *Ruminococcaceae*. To dager senere var det en signifikant forskjell i artsmangfoldet, hvor den orale mikrobiotaen til kalven lignet mest på kuenes orale mikrobiota. Nå var *Porphyromonas*, *Neisseria* og *Acinetobacter* de mest mangfoldige phyla.

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