RECTAL MICROBIOTA DYNAMICS IN PREWEANED DAIRY CALVES DEPENDING ON
COLOSTRUM INTAKE, PRESENCE OF DIARRHEA, AND
ANTIBIOTIC TREATMENT

By

LETIZIA TOMASSINI

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College of Veterinary Medicine

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To the Faculty of Washington State University:

The members of the committee appointed to examine the dissertation of LETIZIA TOMASSINI find it satisfactory and recommend that it be accepted.

____________________________________
William M. Sischo, Ph.D., Chair

____________________________________
Thomas E. Besser, Ph.D.

____________________________________
Douglas R. Call, Ph.D.

____________________________________
Stephen S. Lee, Ph.D.
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RECTAL MICROBIOTA DYNAMICS IN PREWEANED DAIRY CALVES DEPENDING ON COLOSTRUM INTAKE, PRESENCE OF DIARRHEA, AND ANTIBIOTIC TREATMENT

Abstract

by Letizia Tomassini, Ph.D.
Washington State University
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Chair: William M. Sischo

The mammalian intestine microbial community carries out many functions contributing to host’s defense from infectious diseases, nutrition, growth, immunodevelopment and immunomodulation. Several factors such as diet, age, antibiotics, and disease can affect microbiota composition and biodiversity.

Neonatal diarrhea continues to be a major challenge in dairy calf raising. Diarrhea represents one of the most common causes of calf mortality, and the major cause of morbidity and use of antimicrobial treatment. Contributing to the persistent presence of calf neonatal diarrhea in dairy production is a farm management’s flaw in not being able to ensure adequate passive immunity to calves through colostrum feeding.

Very little is known on calves’ gut microbiota composition, and nothing is known on the impact colostrum has on microbiota; the relationship between microbiota and presence of diarrhea; and the impact of antimicrobial treatment on microbiota. The goal of this study was to explore the effect of colostrum and antimicrobial treatment on calves’ fecal rectal microbiota and evaluate microbiota as risk factor for diarrhea and antimicrobial treatment.
In a cross-sectional study we assessed the dominant taxa in microbiota from dairy calves during their first month of life and found an age-dependent succession of the dominant microbial community. From a data analysis perspective, principal component analysis was a useful tool to present community structure collected as relative abundance (proportion of taxa in a sample) to subsequent multivariate analyses.

In a longitudinal study we investigated age and colostrum effect on the microbial community from dairy calves during the first two weeks of life. This field-based investigation demonstrated significant colostrum-age interaction effect on intestinal microbiota in preweaned dairy calves.

We also examined microbiota from 1 and 3 day old calves as risk factor for neonatal diarrhea and antimicrobial treatment. We found significant likelihood of diarrhea and treatment associated with calves’ microbiota structures.

Finally, effect of antibiotic treatment on microbiota structure and diversity, and microbiota resilience to antibiotic treatment were examined. Antibiotics impacted microbiota structure and biodiversity and the effect was detected immediately after antibiotic administration. Microbiota profile from treated animals returned similar to controls’ microbial community by fourteen days after treatment.
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Dedication

To my mother who passed on to me the value of education as important for personal growth.
GENERAL INTRODUCTION

Mammalian intestinal microbial cells are by far more numerous than the other commensal microbial communities associated with the animal body. Moreover, the gut microbial cell population is ~10 times greater than the host’s somatic and germ cells. The number of the genes contained in the gut microbiome is estimated to be 100 times greater than the number of the genes contained in the human genome. Being a substantial component of the gut, intestinal microbiota could be considered a metabolic “organ” beneficially contributing to host’s health and defense. A fair amount is known about the dynamics of the human gut microbiota including its early development in the neonate and the effects of diet, antibiotics administration, and disease status on its composition. Little is known about the biota composition in calves and its dynamics, particularly how colostrum intake, presence of diarrhea, and antibiotic treatment affect microbiota structure and diversity.

Raising healthy dairy calves continues to be a challenge to our dairy industry. A 2007 USDA national survey reported mortality in preweaned dairy calves to be 7.8%, with neonatal diarrhea (scours) accounting for more than 50% of these deaths per year. Farm management should ensure calves receive adequate transfer of passive immunity (ATPI) through colostrum feeding. However, the USDA national survey reported that at least 20% of heifer and bull calves on U.S. farms did not receive ATPI. The combination of preweaned calves’ exposure to pathogens from their environment and failure to transfer passive immunity (FTPI) contribute to an increased likelihood of calf diarrhea and mortality. In addition, calf neonatal diarrhea is the leading cause of antimicrobial treatment use on farms.
The literature with a focus on humans has documented a beneficial role for the intestinal microbiota in host health and defense. However, little is known about the biota dynamics in earliest stages of calves’ life, and nothing is known on the impact that colostrum intake, antibiotics, and diarrhea have on the composition and development of calves’ microbiota. Through use of 16S rRNA gene sequencing technologies, the objective of this study is to fill this knowledge gap by investigating the newborn calves’ rectal microbial community and microbiota changes related to colostrum intake, presence of diarrhea, and antibiotic treatment.

The first chapter describes a study of microbiota from preweaned dairy calves enrolled in a cross-sectional study. A pool of samples from 1 week, 2 week, and 4 week old calves was collected from a total of 164 calves. DNA was extracted and 16S rRNA gene sequenced by Sanger sequencing technology. Microbiota structures from these calves across ages and Total Serum Protein (TSP) levels were explored. Possible approaches to microbiota data analysis through use of multivariate data analysis were also considered. This chapter was submitted to Journal of Dairy Science and formatted accordingly.

The second chapter is an investigation of colostrum and age effect on fecal rectal microbiota from preweaned dairy calves. Next generation 454 sequencing technology was used to obtain compositional data on microbial taxonomy. Calves were enrolled in a cohort study and were sampled at five time points during the first two weeks of life. Microbiota biodiversity across ages and TSP levels was explored. High dimensionality of microbiota compositional data was reduced by use of multivariate data analysis techniques, and effect of covariates on microbiota structures were tested by regression analysis. This chapter was submitted to the ISME journal and formatted accordingly.
The third chapter evaluates microbiota structure as risk factor for calves’ neonatal diarrhea and antibiotic treatment. Rectal fecal samples were collected from 1 and 3 day old dairy calves and microbial community data obtained. Calves were followed up for two weeks from time of sampling to monitor diarrhea and treatment. Multivariate data analysis was used to reduce microbiota data dimensionality; regression and cox proportional hazard models were used to test for microbiota as risk factor for diarrhea and treatment. This chapter will be submitted to the ISME journal and formatted accordingly.

The fourth chapter investigates antibiotic treatment effect on preweaned dairy calves’ microbiota structure and biodiversity. Rectal fecal samples from preweaned dairy calves were collected at one day before treatment, the day of treatment, the day after treatment, and 14 days after the last day of treatment. Treatment effect on microbiota biodiversity was explored through use of richness and evenness diversity measures. Moreover, microbiota data dimensionality was reduced through use of multivariate data analysis, and treatment effect on microbiota structure was tested by regression analysis.
CHAPTER ONE

Molecular and statistical analyses of the fecal bacterial microbiota of preweaned dairy calves

L. Tomassini*, T. E. Besser†+, D. R. Call†+, J. K. Harris†, K. A. Ross§, S.S. Lee#, W. M. Sischo*†1

*Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman WA 99164
†Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman WA 99164
+Paul G. Allen School for Global Animal Health, College of Veterinary Medicine, Washington State University, Pullman, WA 99164
‡Department of Pediatrics, Division of Pulmonology, University of Colorado Denver, School of Medicine, Aurora, CO 80045
§Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309
#Department of Statistical Science, College of Science, University of Idaho, Moscow, ID 83844
1William M. Sischo, College of Veterinary Medicine, PO Box 646610, Pullman, WA 99164-6610

Telephone: 509-335-7495 • Fax: 509-335-0880 wmsischo@vetmed.wsu.edu

Email addresses:

LT: tomassini@vetmed.wsu.edu
TEB: tbesser@vetmed.wsu.edu
DRC: drcall@vetmed.wsu.edu
KAR: rossk@colorado.edu
JKH: jonathan.harris@ucdenver.edu
SSL: stevel@uidaho.edu
WMS: wmsischo@vetmed.wsu.edu
INTERPRETIVE SUMMARY

Molecular and statistical analyses of the fecal bacterial microbiota of preweaned dairy calves. Tomassini. This field-based investigation on dairy calves during their first month of life detected the dominant rectal bacterial taxa and found an age-dependent succession in the bacterial community composition. There were no apparent associations between the dominant bacterial taxa and passive immunity (estimated by TSP) or farm. Rarefaction analysis showed our study design and sample size described the dominant microbial taxa and was not designed to describe all the diversity in the microbial community. Our results and others suggest that a greater sampling effort to define taxa richness would help to understand the complete population structure and how it changes with age and management inputs. From a data analysis perspective, principal component analysis was a useful tool to present community structure collected as relative abundance (proportion of taxa in a sample) to subsequent multivariate analyses. The method reduces taxa dimensionality, removes correlation between taxa, and removes the problem introduced by the numeric constraint of relative abundance (range 0-1).
ABSTRACT

**Background:** Relatively little is known about the bacterial microbiota of the neonatal dairy calf gastrointestinal tract. The objective of this study was to assess the structure of the dominant fecal bacterial microbiota in preweaned dairy calves using culture-independent methods combined with data transformation and multivariate analytic approaches. **Methods:** We collected rectal fecal samples from dairy calves housed on four commercial dairy farms in the Pacific Northwest, United States. Samples were collected from 1-, 2-, and 4-week old calves with total serum protein (TSP) values ≤5.0 or ≥5.2 g/dl. From the fecal samples, DNA libraries based on 16S small subunit (SSU)-rRNA gene sequences were constructed using DNA pooled by farm, age, and TSP category. The effects of farm, age, and TSP on microbiota structure were assessed using Principal Component Analysis (PCA), Multivariate Analysis of Variance (MANOVA), and cluster analysis. **Results:** A total of 164 calves were enrolled in the study and 1824 SSU-rRNA gene sequences from 20 DNA libraries were obtained. Age affected the dominant community composition, but no TSP or farm effects were observed. These relationships were consistent across the different quantitative analyses. Microbes commonly used as probiotics to support calf health were a minor component of neonatal calf bacterial community. **Conclusions:** This field-based investigation demonstrated significant age-dependent shifts in the dominant intestinal microbiota in preweaned dairy calves.

**Key words:** dairy calf, bacterial microbiota, colostrum, statistical methodology
INTRODUCTION

In mammals, the gut microbial population outnumbers the host’s somatic and germ cells by 10-fold (Savage, 1977). The total genetic content of the gut microbiome is estimated to be 100 times greater than the number of the genes contained in the human genome (Backhed et al., 2005). Being a substantial component of the gut, the microbiota is an irreplaceable “organ” that contributes to host’s health and defense. Until recently, the composition of the intestinal microbiota was largely undefined and little known about its compositional dynamics.

The application of 16S small subunit (SSU)-rRNA gene technology to bacterial taxonomy has transformed our view of microbial communities. The SSU-rRNA gene is ubiquitous in bacteria and contains the genetic information for encoding the ribosomal RNA found in the small ribosomal subunit (Pace et al., 2012). The gene contains approximately 1,500 nucleotides characterized by the presence of taxonomic-dependent variable regions flanked by conserved regions (Huysmans and De Wachter, 1986). The conserved regions are used as primer binding sites for PCR amplification of variable regions (Lane et al., 1985). The development of Sanger and subsequent sequencing technologies created the opportunity to use these variable regions for phylogenetic analyses (Lane et al., 1985; Cai et al., 2003; Petrosino et al., 2009). This technology has revealed the existence of numerous bacterial taxa that have resisted cultivation, and are numerically significant members of many microbial communities (Hugenholtz and Pace, 1996). In particular, characterization of the intestinal bacterial microbiome in people and cattle, and evaluating the link between microbiome composition and health is an emergent research area (Eckburg et al., 2005; Leser and Molbak, 2009; Braegger et al., 2011; Oikonomou et al., 2013).

Calf health results from the interaction between host, pathogen, and environmental factors. One of the most important host factors affecting calf health is the delivery of colostrum
to the neonate. Colostrum is derived from the first post parturient milk of the dam and contains high concentrations of antibodies. The effective delivery of colostrum (quality and quantity) to the calf within 24 hours post parturition creates a passive immunity that supports the calf’s developing active immune system (Besser et al., 1988; Godden, 2008). Failure to transfer passive immunity from dam to calf is the most important predictor of calf mortality and diarrhea in the first four weeks of life (Besser and Gay, 1994; Berge et al., 2005).

Colostrum is not only high in IgG but compositionally distinct from milk both in nutrients and immunoactive molecules (Blum and Hammondate, 2000). These components likely create an ecological environment that is suitable for the physiological establishment of the intestinal microbiome. While the importance of colostrum feeding in the establishment of effective calf passive immunity is well known, the impact of colostrum on development of the bacterial microbiome is unexplored.

DNA sequence technology applied to SSU-rRNA gene assessment has rapidly developed and created challenges for analysing and organizing the resulting data. These challenges include high level dimensionality, the use of relative rather than actual values to measure abundance, and high level correlation between bacterial taxa. A number of approaches for handling these types of data are suggested in the literature (Yeung and Ruzzo, 2001; Lattin et al., 2003) but their application in the analyses of community microbiota structure is still being evaluated.

Using culture-independent methods based on SSU-rRNA gene variability, our study objectives included: characterize the dominant fecal bacterial microbial community in neonatal dairy calves from commercial dairy farms, explore the effect of farm, age and TSP on the structure and dynamics of the dominant rectal microbiota, and examine methods of data transformation and multivariate statistics to describe microbiota dynamics.
MATERIALS AND METHODS

Farms, Animals, and Sample Selection

Four Pacific Northwest (USA) dairy farms were selected for this cross-sectional study. This convenience sample consisted of dairy farms that reared calves on farm and did not use antibiotics in their feeding system. On participating farms, calves were enrolled into one of two total serum protein (TSP) categories (≤5.0 and ≥5.2 g/dl) and one of three age categories (2-5 days old, 12-17 days old, and 26-30 days old). Eligible calves were identified with the aid of farm personnel. On two farms, TSP category was based on farm records used to monitor their colostrum program. On the other two farms, study personnel removed 10 ml of blood via jugular venipuncture into a serum tube (BD Vacutainer, Franklin Lakes, NJ) (IACUC-04021-055). The blood was allowed to clot and serum removed. Serum protein was measured using a temperature-compensating refractometer previously calibrated with distilled water. From each enrolled calf, a 5 g fecal sample was collected by digital rectal stimulation. The samples were individually transferred into sterile bags and transported at 4°C to the laboratory for further processing.

DNA Extraction and Pooling

DNA was extracted from fecal samples using a stool DNA kit (QIAamp DNA Stool Mini Kit, QIAGEN, Valencia, CA). Briefly, from each calf a 200 mg fecal sample was transferred to a 2 ml micro-centrifuge tube. 1.4 ml of Buffer ASL was added to the tube and the contents mixed. The suspension was heated for 5 minutes at 70°C, mixed, and centrifuged to pellet the stool particles. A 1.2 ml aliquot of the supernatant was transferred into a new 2 ml micro-centrifuge tube. One InhibitEX tablet was added to each sample and the tube contents mixed to suspend the tablet, incubated for 1 minute at room temperature, and centrifuged. The supernatant was transferred into a 1.5 ml micro-centrifuge tube, centrifuged for 3 minutes after which 200 µl of
the supernatant was transferred to a 1.5 ml micro-centrifuge tube with 15 µl (>600 mAU/ml) of proteinase K and 200 µl of Buffer AL. The solution was mixed for 15 seconds and incubated at 70°C for 10 minutes. Following incubation, 200 µl of ethanol (96-100%) was added and thoroughly mixed. All the lysate was transferred into a QIAamp spin column that had been previously placed into a 2 ml collection tube. After centrifugation for 1 minute, the spin column was placed in a new 2 ml collection tube and 500 µl of Buffer AW1 was added to the spin column and centrifuged for 1 minute. The spin column was placed into a new collection tube and 500 µl of Buffer AW2 was added to the spin column and centrifuged for 3 minutes. The spin column was transferred to a new 2 ml collection tube and centrifuged again for 1 minute. Finally, the spin column was transferred to a 1.5 ml micro-centrifuge tube and 200 µl of Buffer AE was added to the column, the tube was incubated for 1 minute at room temperature, and centrifuged for 1 minute to elute the DNA. The extracted DNA was stored at 4°C if processed within 7-days or stored at -20°C for later processing. Pooled samples of extracted DNA were assembled based on age, farm, and passive transfer status by combining 20 µl from each vial containing individual unstandardized concentrations of calf extracted DNA.

**SSU-rRNA gene Amplification, Libraries Construction and Sequencing**

Using previously published methods (Frank et al., 2007), the DNA from each pool was assayed for bacterial community diversity by amplifying a segment of the SSU-rRNA gene. Two universal primers, 27F (5’ AGA GTT TGA TCC TGG CTC AG3’) and 805R (5’ GAC TAC CAG GGT ATC TAA T3’) corresponding to the V1-5 fragment were used for the SSU-rRNA gene PCR. The PCR amplification mixture included 1.5 µl of template DNA and 37.5 ng of each primer with a final volume of 25 µl (Illustra™ puReTaq Ready-To-Go PCR Beads, GE Healthcare, Buckinghamshire, UK). The PCR protocol was: initial denaturation at 94°C for 4
min, 20 cycles of 94°C for 30 sec, 65°C-45°C (1°C decrement in annealing temperature per cycle) for 30 sec, 72°C for 30 sec, amplification with 14 additional cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 60 sec, and final extension at 72°C for 20 min. Agarose gel electrophoresis (1.5% agarose gel in Tris-borate EDTA stained with ethidium bromide) was used to qualitatively assess the presence of the PCR products (800 base pair (bp) products). Negative and positive controls were included to monitor for external contamination and confirm amplification, respectively.

The SSU-rRNA gene PCR products from each DNA library were cloned using a TA-vector and transformed into TOP10 chemically competent E. coli cells (TOPO TA Cloning® Kit for Sequencing, Invitrogen™, Carlsbad, CA) per manufacturer’s instructions. The transformed E. coli cells were plated for isolation on LB agar with 100 µg/ml ampicillin and incubated overnight at 37°C. Ninety-six transformants from each culture plate were selected by convenience and transferred to individual wells in a 96-well culture plate containing 150 µl of LB broth with 100 µg/ml ampicillin per well. Plates were incubated overnight at 37°C. After incubation, LB broth (2 µl) was removed from each well and transferred to a 96-well PCR plate to be used as DNA template in a PCR assay. The 96-well PCR plates were stored at -20°C and shipped overnight with dry ice for PCR clean-up and Sanger sequencing (Functional Biosciences, Inc., Madison, WI).

**Sequence Data Analysis**

Nucleotide sequence data (fasta and quality files) were derived from chromatograms generated by Sanger sequencing. Base-calling was performed using phred software embedded in the open-source XplorSeq package and contigs were assembled with the phrap functionality in XplorSeq (Frank, 2008). Quality filtering of the sequences with XplorSeq removed nucleotides
with mean quality scores of $Q < 20$ over a 10-nucleotide window at the 5’ and 3’ ends. Sequences with greater than one ambiguous base were discarded. Non-bacterial sequences were removed from the dataset by requiring a close match with a bacterial secondary structure rRNA model within Infernal (Nawrocki et al., 2009). Sequences identified as potential chimeras by ChimeraSlayer (Haas et al., 2011) were also removed from the dataset. Taxonomic identities were assigned to sequences using the Ribosomal Database Project (RDP) Classifier (Wang et al., 2007). The taxonomic information provided by the RDP Classifier was used to construct sequence groups with identical taxonomic rank, which were used to calculate ecology statistics for each sample. A total of 1,635 sequences (average length, 745 nucleotides) were deposited in GenBank with accession numbers KJ421217 - KJ422851 and are retrievable from NCBI (http://www.ncbi.nlm.nih.gov/).

**Bacterial Microbiome Richness Analyses**

Species richness (Magurran, 2004a) was estimated using sample-based rarefaction (Gotelli and Colwell, 2001) and unconditional confidence intervals based on Chao1 richness estimator. A rarefaction curve was computed using a bootstrap size of 100 and 20 knots (Colwell, 2000).

**Statistical Analysis**

The analysis unit was taxa composition within each DNA library. To adjust for the between-library variability in the numbers of recovered sequences, taxa composition was standardized to relative abundance, i.e. proportion of sequences assigned to each bacterium within a library. From these data we assessed and reported the association between library bacterial composition and farm, age and TSP.
Prior to assessing population structure dynamics, data dimensions were reduced and normalized using Principal Component Analysis (PCA) (Lattin et al., 2003). All multivariate analyses were based on the PCA dimensional space. Multivariate Analysis of Variance (MANOVA) was used to determine if the mean vectors of the principal components were conditional on farm, TSP and age categories at the significant level ($P \leq 0.05$). Specifically, principal components instead of direct bacteria proportions were used as dependent variables to minimize the number of dependent variables in the MANOVA model and to meet the MANOVA’s normality requirement on dependent variables distribution.

Cluster analysis was performed to describe the structural dynamics of microbiota associated with the risk factors that were significant by MANOVA model (Rencher, 2002). For clustering, initial hierarchical clustering analysis was used by calculating Euclidean distance and Ward’s algorithm (R, version 3.0.0) and from this we identified patterns of possible clusters describing the data. This initial clustering, combined with significant risk factors from MANOVA analysis, and PseudoF parameter were used to decide on number of clusters for running K-means clustering analysis with 50 randomizations (R, version 3.0.0). As a result, each DNA library was assigned to unique taxonomic clusters (Xu and Wunsch, 2005). The library-based clusters were visualized by frequency tables where cluster membership was independently cross-classified by the significant risk factors from MANOVA and tested for homogeneity using a Fisher’s exact test (Daniel, 2005). Microbiota structure determining our clustering solutions was characterized, and the link between microbial profiles and calves study groups described.

RESULTS

We obtained fecal samples from 164 calves housed on four commercial dairy farms. From these cross-sectional samples, 20 pools of extracted DNA were created. A result of this
field-based sampling was between-pool variability in the number of animals contributing DNA to a pool, i.e. the number of eligible animals on a farm at the time of a visit varied. We compensated for this variability by follow-up farm sampling and created ‘replicate’ farm-based age and TSP status libraries. In addition, not all enrolled farms yielded calves for all TSP and age categories (Table 1).

From these 20 pools we obtained 1,824 SSU rRNA gene sequences that were trimmed and screened. From these sequences, 1,635 met quality control standards and were used in the final analyses (range of 70-90 sequences per library). Across all libraries, the RDP-based taxonomic analyses identified 59 taxa; 48 taxa (81%) corresponded to genus level and represented 89% of the evaluated sequences.

**Taxa Diversity**

Across the 20 DNA libraries, 4 taxa (7% of total number of taxa) accounted for greater than half of the observed SSU-rRNA gene sequences with the remaining 55 taxa having a rapidly diminishing contribution to the remaining taxa richness. For example, 38% of taxa were observed as singletons (Figure 1). Using sample-based rarefaction analyses and the Chao1 richness estimator, we expect an average 89 taxa (95% CI, 69-147) within a pooled sample (Figure 2). These results indicate that we detected and defined the dominant taxa from our samples, but our sampling “depth” was not sufficient to fully capture the taxonomic richness of the calf rectal microbial community.

**Multivariate Analyses of Associations between Taxa Diversity and Library Attributes**

The PCA transformation of DNA library-based relative abundance reduced the dimensions of the data from 59 taxa to 20 principal components and the first 15 principal components explained 95% of the dimensional variability. These 15 PCA components were used
in subsequent analyses. From the MANOVA, there was a significant age effect ($P=0.005$) but no significant influence of TSP ($P=0.89$) or farm ($P=0.12$) on microbiota structure. From K-Means clustering, 3 microbiome clusters were identified and cross-classified with age (Tables 1 and 2). An age effect on microbiome structure was observed; all 2-5 day old SSU-rRNA gene libraries were found in single cluster, 12-17 day old based libraries were split between 2 clusters, and the 26-30 day old based libraries were also split between two clusters, one of them being an outlier cluster containing one observation from the ‘single animal’ DNA library (Fishers Exact Test, $P=0.002$). The dominant members of the three clusters differed. The cluster describing the taxa recovered from the youngest calves and shared by 12-17 day old calves was dominated by \textit{Bacteroides} (37%), \textit{Faecalibacterium} (17%), and \textit{Fusobacterium} (9%). The cluster shared by 12-17 day old calves and 26-30 day old calves was dominated by \textit{Bacteroides} (32%), \textit{Prevotella} (13%), and \textit{Prevotellaceae} (11%). A single 26-30 day old library was defined by the remaining cluster and was dominated by two taxa, \textit{Prevotella} (54%) and \textit{Bacteroides} (12%).

*A Prevalence of Taxa with Health and Public Health Implications*

Bacteria that are components of probiotics supporting calf health (Signorini et al., 2012), were minor components of the dominant microbiome. \textit{Lactobacillus}, \textit{Bifidobacterium}, and \textit{Enterococcus}, were detected rarely or not at all in 2-5 day old calf libraries (1.1%, 0.1%, and 0.1%, respectively) or 12-17 day old calf libraries (4.2%, 0%, and 0.1%, respectively). They were not detected from 26-30 day old calf libraries. A similar prevalence and age-dependent pattern was observed for \textit{Escherichia} and \textit{Campylobacter}, detected from 2-5 day old (2.2% and 0.1%, respectively) and 12-17 day old calf libraries (0.1% and 0.4%, respectively) but not from 26-30 day old calf libraries.
DISCUSSION

This research focused on characterizing the population structure of the dominant fecal microbiota of preweaned calves housed on commercial dairy farms. We used a culture-independent method based on SSU-rRNA gene sequences and employing Sanger sequencing technology. We obtained 1,635 SSU-rRNA gene sequences from 164 calves sampled across four farms. We focused on the dominant members of the microbiome by pooling fecal microbial DNA by farm, TSP category, and age to create 20 independent DNA libraries. We found significant age-dependent effects on intestinal microbiome structure but no effect of TSP or farm on that structure.

The age-dependent trends we observed are consistent with a previous culture-independent study that followed Holstein calves through 12 weeks of age (Uyeno et al., 2010). They observed genus-level age-dependent shifts in the dominant taxa with Bacteroides/Prevotella (combined as single group) and Faecalibacterium being the most prevalent in week 1. In their study, while Bacteroides/Prevotella remained the dominant group in all age groups, Faecalibacterium decreased as animals aged. A field-based study also using culture-independent methods sampled calf cohorts across seven consecutive weeks beginning at one-week of age. Sixty-one animals were sampled across 7 weeks and the authors observed shifts in taxa prevalence across ages (Oikonomou et al., 2013). Based on discriminant analyses they reported that there were genus level differences across age groups though they did not report taxa composition beyond the phyla level for these findings. While the results of our study demonstrate an age-dependent change in the microbiome for calves in a commercial dairy setting, our field-based, cross-sectional farm sampling resulted in undersampling the 26-30 day old calves (calves were not available on the
sampling dates on 3 of 4 farms) and impacts our estimate of community structure of that age group.

Successful transfer of passive immunity based on TSP did not influence the dominant fecal microbiome detected from neonatal calves. We evaluated TSP as an independent effect with age and farm, and could not assess the interaction of TSP and age because of sample size constraints. Consequently, the limitations of the current study did not allow us to fully evaluate the potential effect of colostrum intake on the dominant fecal microbiome as a function of age.

Similarly, farm source did not impact the structure of the dominant fecal microbiome. This suggests that age factor effect on dynamics and succession of the dominant bacteria in the microbiome outweighs local management and environmental factors’ effect.

Three bacteria *Lactobacillus*, *Bifidobacterium* and *Enterococcus* are cited as having important roles in gut and calf health (Signorini et al., 2011) and are often included in probiotic formulations. In our study, *Lactobacillus*, *Bifidobacterium* and *Enterococcus* were detectable but minor members of the calf fecal microbiome in the early neonate period and undetectable by week 4. Another culture-independent study of calves also found this trend (Uyeno et al., 2010). These findings differ from culture-dependent studies that suggest these taxa are dominants in young calves (Hartman et al., 1966; Watase and Takenouchi, 1978; Vlkova et al., 2006). These differences are fundamental to the two methods and have implications for understanding the relationship between the microbiome and health. Culture-dependent methods discover only those taxa that can be cultivated but provide true measures of abundance within the cultured microbiome. Culture-independent methods based on SSU-rRNA genes, are effective at defining gene-based taxa richness though there are known SSU-rRNA primer biases that will under-represent taxa (Sim et al., 2012). Relative abundance estimates are also biased because the copy
number of the SSU-rRNA gene varies across taxa (Klappenbach et al., 2001). Our study was
designed to focus on the dominant taxa and combined with the SSU-rRNA technology it is likely
that the failure to detect *Lactobacillus*, *Bifidobacterium* and *Enterococcus* as significant
members of the community is inherent to the study implementation or that their role in health is
not as a dominant taxa but through more subtle metabolic interactions in the gut (Taschuk and
Griebel, 2012).

By creating risk-factor based DNA libraries from pooled fecal samples, the study was
designed to bias our microbiome assessment towards detecting dominant bacterial members of
the microbiome. Rarefaction, an analysis which estimates biological diversity in ecological
niches, was used to assess our taxa coverage of the microbiome (Magurran, 2004b). This
analysis is based on richness diversity index which, through bootstrap sampling, estimates the
unobserved taxonomic richness. While we clearly sampled sufficiently to describe the dominant
taxa, we did not sample sufficiently to describe the complete diversity of the calf microbiome or
the impact risk factors (age, TSP (colostrum intake), or farm) might have on that diversity. Our
study and a recent study (Oikonomou et al., 2013) provide guidance for study designs and
sample size to more completely describe microbiome diversity.

One of the challenges for effectively using sequence-based microbiome data is to
accurately describe community membership and its dynamics. There are three important issues
for analyses of these data: accounting for the dependent nature of community structure, i.e. the
presence and absence of taxa are unlikely to be independent, understanding and meeting the
assumptions for the quantitative methods, and recognizing the impact of taxa dominance on the
methods used to organize microbiome structure. Many studies evaluate the microbiome as a
series of independent taxa. Our analyses focused on community structure dynamics by using
multivariate statistical methods that explicitly account for multiple dependent variables (MANOVA) and a clustering approach that optimized both similarities and differences in the variation of the overall community membership. Because most quantitative approaches used to analyze microbiome dependent structure require uncorrelated and normalized data, we used PCA. PCA allowed us to reduce the complexity or dimensions of the microbiome taxa, remove dependencies among the taxa, and remove the inherent distribution constraint of relative abundance. The final quantitative issue of dominance was not addressed in this study. We designed the study to define dominance and found that relatively few taxa accounted for the majority of detected sequence. Other studies not biased towards detecting dominant taxa also indicate that a few taxa dominate the microbiome in the calf gut. There are standardization and transformation techniques that need to be considered to understand the relative roles that dominant and less dominant taxa have on population-based health and productivity outcomes.

CONCLUSIONS

This field-based investigation on dairy calves during their first month of life detected the dominant rectal bacterial taxa and found an age-dependent succession in the bacterial community composition. There were no apparent associations between the dominant bacterial taxa and passive immunity (estimated by TSP) or farm.

Rarefaction analysis showed our study design and sample size described the dominant microbial taxa and was not designed to describe all the diversity in the microbial community. Our results and others suggest that a greater sampling effort to define taxa richness would help to understand the complete population structure and how it changes with age and management inputs. From a data analysis perspective, principal component analysis was a useful tool to present community structure collected as relative abundance (proportion of taxa in a sample) to
subsequent multivariate analyses. The method reduces taxa dimensionality, removes correlation
between taxa, and removes the problem introduced by the numeric constraint of relative
abundance (range 0-1).
ACKNOWLEDGMENTS

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Pullman). This project was funded by USDA Animal Health Formula Fund.
REFERENCES


Table 1 Animal and farm attributes of 20 DNA pools used to generate SSU-rRNA gene libraries. Each row corresponds to a DNA pool and its associated microbiome-based, K-means cluster.

<table>
<thead>
<tr>
<th>FARM</th>
<th>AGE (days)</th>
<th>TSP (g/dl)</th>
<th>Number of Animals Contributing to the pool</th>
<th>Cluster membership</th>
</tr>
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<tr>
<td></td>
<td>12—17</td>
<td>≤5.0³</td>
<td>8</td>
<td>1</td>
</tr>
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</table>

Total 164

¹TSP= Total Serum Protein.
²TSP detected on the day of visit to the farm.
³TSP detected at 1-4 day old (farm’s records).
Table 2 Twenty pooled DNA libraries from dairy calves were examined for differences in rectal microbiota composition across three age categories. Microbiota composition proportional data were transformed into principal components and used in k-means clustering analysis. Clusters’ frequencies were cross tabulated against age levels. Fishers Exact Test, $P=0.002$

<table>
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<tr>
<td>3</td>
<td>9</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\): Sample contained DNA from a single animal.
Figure 1 Relative abundance (proportions) of the ten most dominant taxa in preweaned calves’ fecal microbiota (n= 20 DNA pools from 4 farms).*

*Remaining 49 taxa in calves rectal microbiota were 1% for the following: Sutterella, Odoribacter, Oscillibacter, Clostridium, Gallibacterium, Cetobacterium, Parabacteroides, Escherichia; <1% for the following: Clostridiales, Ruminococcaceae, Parasutterella, Bacteroidales, Butyrificimonas, Paraprevotella, Roseburia, Alphaproteobacteria, Campylobacter, Porphyromonadaceae, Barnesiella, Clostridiaceae1, Fusobacteriaceae, Actinobacillus, Bacteria, Collinsella, Enterococcus, Anaerotrichus, Subdoligranulum, Succinivibrio, Bifidobacterium, Bacteroidetes, Anaerophaga, Tannerella, Hallella, Leuconostoc, Syntrophococcus, Peptostreptococcus, Acetanaerobacterium, Anaerobilis, Ruminococcus, Anaerobacter, Anaerovorax, Howardella, Peptococcus, Allisonella, Anaerovibrio, Dialister, Psychrobacter, Victivallis, Treponema (cumulative proportion=0.20).
Figure 2 Rarefaction curve estimating taxa richness of the rectal microbial community in preweaned dairy calves. Richness estimates and 95% confidence limits are based on Chao1 predictor sample-based methods (n=20) and 1,635 SSU-rRNA gene sequences.
CHAPTER TWO

Age and colostrum effects on the intestinal microbiota of preweaned dairy calves.

Letizia Tomassini*, Jonathan K. Harris‡, Charles E. Robertson§, Stephen S. Lee#, William M. Sischo*1

*Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA 99164

‡Department of Pediatrics, Division of Pulmonology, University of Colorado, School of Medicine, Aurora, CO 80045

§Division of Infectious Diseases, University of Colorado, School of Medicine, Aurora, CO 80045

#Department of Statistics, College of Science, University of Idaho, Moscow, ID 83844

1William M. Sischo, College of Veterinary Medicine, PO Box 646610, Pullman, WA 99164-6610

Telephone: 509-335-7495 • Fax: 509-335-0880 wmsischo@vetmed.wsu.edu

Email addresses:

LT: tomassini@vetmed.wsu.edu

CER: Charles.Robertson@Colorado.EDU

JKH: jonathan.harris@ucdenver.edu

SSL: stevel@uidaho.edu

WMS: wmsischo@vetmed.wsu.edu
ABSTRACT

While the bovine bacterial intestinal microbiota is assumed to have similar important immunological and metabolic functions to those studied in humans and mice, little is known of its structure and dynamics, and even less is known how management decisions affect its structure. We performed a longitudinal study investigating the community structure of fecal microbiota in preweaned dairy calves and measured the impact colostrum intake had on its structure. Rectal fecal samples were collected from untreated dairy calves housed on three dairy farms in the U.S. Pacific Northwest. These samples were collected at days 1, 3, 5, 10, 16 post-parturition. Total serum protein (TSP) values were determined for each calf at day 1, and calves categorized into two groups (≤ 5.0 or ≥ 5.2 g/dl). From the fecal samples, small subunit rRNA (16S rRNA) sequences using 454 pyrosequencing were obtained. The effects of farm, age, and TSP on microbiota structure were assessed using Principal Component Analysis, cluster analysis, and regression analysis. Fifty nine calves completed the study. A total of 297,715 16S rRNA gene sequences from 295 fecal samples were obtained. Across the study, we observed community succession, with bacterial microbiota transitioning from facultative anaerobes to strict anaerobes. Colostrum significantly impacted microbiota structure on days 3 and 5. Comparatively, calves with TSP ≤ 5.0 transitioned more rapidly to an anaerobic microbial community. These observations were consistent across all the study farms. This field-based investigation demonstrated significant but transient colostrum effect on intestinal microbiota in preweaned dairy calves.

Keywords: gut microbiota/calves/age/colostrum/pyrosequencing/statistical methodology
INTRODUCTION

The intestinal microbiota is both a metabolic and an immunologic organ within its host supporting immunological functions through microbiota and mucosal interactions (Taschuk and Griebel, 2012). These interactions trigger the development of mucosal immunity which subsequently modulates microbial composition, prevents microbe overgrowth, and impedes transit of bacteria across the intestinal wall (Taschuk and Griebel, 2012).

The fetal gut environment is sterile, but changes take place at parturition as the newborn comes in contact with maternal vaginal and fecal bacteria and environmental microbes (Penders et al., 2006; Cilieborg et al., 2012). These bacteria are ingested by the neonate and quickly colonize the intestine. At this time, the gut lumen has positive oxidation/reduction potential and facultative anaerobes colonize and utilize the available oxygen. When the oxygen is depleted, anaerobic bacteria dominate (Mackie et al., 1999; Penders et al., 2006).

Calves are born agammaglobulinemic and passively receive antibodies through colostrum intake (Cortese, 2009). This transfer needs to occur within the first hours following birth to be most effective. While its importance for calf health is well known, at least 20% of calves on U.S. farms do not receive adequate passive immunity through colostrum feeding (USDA, 2007; Beam et al., 2009). Besides the very important IgG and IgA components, colostrum is rich in nutrients and bioactive molecules that play an important role in an animal’s growth, intestinal epithelial maturation, defense from disease, and local immune system development (Cortese, 2009). These components likely create an ecological environment that is suitable for the physiological establishment of the intestinal microbiome. The importance of colostrum feeding in the establishment of effective calf’s passive immunity is well known; the impact of colostrum on
development of the bacterial microbiome is unexplored (Uyeno et al., 2010; Edrington et al., 2012; Oikonomou et al., 2013).

Culture independent methods based on sequencing 16S rRNA are commonly being used to characterize microbial communities (Arumugam et al., 2011; Oikonomou et al., 2013). While DNA sequence technology applied to 16S rRNA gene assessments has rapidly developed to be an inexpensive tool, the efficiency of the method to create large DNA libraries has created data analysis challenges. These include: high level dimensionality, the use of relative rather than actual values to measure abundance, and high level correlation between bacterial taxa. A number of approaches for handling these types of data are suggested in the literature (Yeung and Ruzzo, 2001; Lattin et al., 2003a), but their application in the analyses of community microbiota structure is still being evaluated.

Using culture-independent methods, based on 16S rRNA gene variability, our study objectives include: characterize the rectal microbial community in dairy calves from commercial dairy farms during the early pre-weaning period, determine the effect of farm, age and TSP on calf’s intestinal microbiota structure and dynamics during the first weeks of life, and utilize data transformation and multivariate statistics to describe microbiota dynamics.

MATERIALS AND METHODS

Farms, Animals, and Data Collection

The study was conducted between July and September 2011. The study included newborn Holstein calves housed on three Northwest U.S. dairy farms. Selected farms were a convenience sample based on recommendations from field veterinarians and prior collaborations. The 3 criteria for farm participation were: calves not exposed to prophylactic or metaphylactic antimicrobials, and availability of treatment records. Across the 3 study sites, we aimed to enroll
60 calves and to equally divide them into two risk groups: “adequate” total serum protein – TSP (≥5.2 g/dl) and “low” TSP (≤5.0 g/dl). In our study, TSP categories were indirect measures of receiving or not receiving colostrum. The sample size was determined using the difference in microbiota composition prevalence in each study group as the study outcome. The parameters for calculating sample size were: equal allocation to TSP groups, 35% difference in the prevalence of specific microbiota structure between the groups, Type I error=0.1, and Type II error=0.1 (SAS Power and Sample Size 9.3; SAS Institute Cary NC).

On each farm, calves were enrolled 24 hours after parturition. Calves included in the study were those with normal appetite, alert attitude, absence of systemic infection, and no history of antibiotic treatment. At enrollment, TSP levels were evaluated. From each potential study calf, 10 mls of blood were obtained by jugular venipuncture and collected in serum tubes (Becton Dickenson, NJ). The blood was left to clot at room temperature and centrifuged to collect serum. TSP was assessed using a temperature-compensating refractometer previously calibrated with distilled water. Based on serum protein results, calves were categorized into TSP groups. To avoid misclassifying a calf as ≤5.0 g/dl, calves with low TSP values were re-evaluated at 48 hours post parturition.

Starting at day 1 and continuing through day 16, study animals were monitored by study and farm personnel. Morning health assessments were performed and data recorded by study personnel (IACUC-04021-055). All animal care responsibilities (feeding, cleaning, and treatments) were managed by on-farm personnel. Records of antimicrobial treatments were maintained by on-farm personnel and available to study personnel. Enrolled animals that were treated with antibiotics or presented with clinical signs of systemic disease were dropped from the study. Five gm fecal samples were collected from study animals by rectal stimulation at days
1, 3, 5, 10, and 16. These samples were transported at 4°C to an on-site laboratory and stored short term at -20°C (two-three weeks) and long term at -80°C.

**DNA Extraction**

From the frozen fecal samples, DNA was extracted using a QIAamp DNA stool kit (Qiagen, Valencia, CA) using a modified version of the “Isolation of DNA from stool for pathogen detection” protocol found in the handbook. Specifically, 200 mg of frozen fecal sample was transferred into 2ml safe lock tubes (Eppendorf, San Diego, CA) containing 0.5g of 0.1mm zirconia silica beads (Research Products International, Mount Prospect, IL). Samples not immediately processed were stored back at -80°C until further processing. For DNA extraction, 140 ml of ASL buffer were mixed with 350 µl of Reagent DX (Qiagen, Valencia, CA) and 1.4 ml of this solution was added to each sample tube. The tube was left 10 minutes to thaw and put into the Qiagen TissueLyser at 30 Hz for 2 minutes. This suspension was heated at 95°C for 5 minutes, vortexed for 15 seconds, and centrifuged at 14,000 rpm for 2 minutes to pellet stool particles. A 1.2 ml aliquot of the supernatant was transferred into a new 2 ml microcentrifuge tube. One InhibitEX tablet was added to the sample and the tube put into the TissueLyser at 30 Hz for 30 seconds or until the tablet was completely suspended. The suspension was then incubated for 1 minute at room temperature and centrifuged at 14,000 rpm for 6 minutes to pellet inhibitors bound to InhibitEX matrix. At least 350 µl of supernatant was loaded into the QIAcube (Qiagen, Valencia, CA) and automated DNA extraction performed according to manufacturer’s instructions. The only modification to the automated DNA extraction protocol was at the final step DNA was eluted into buffer AE which had been previously diluted to 1:4 with PCR water.
Extracted DNA was checked for DNA quality and quantity. All samples were standardized to a DNA concentration of 5ng/µl, 25µl of volume frozen at -80°C and shipped to the Children's Hospital, CTRC Core Laboratory in Aurora (CO) for sequencing.

**16S rRNA PCRs, Sequencing, and Library Construction**

DNAs were amplified in three independent reactions using barcoded primers (27F-338R) (Hamady et al., 2008). Negative PCR controls for each primer were assayed in parallel and did not exhibit bands in agarose gels. The three independent reactions were pooled and amplicons were confirmed by agarose gel electrophoresis. Pooled DNA content was normalized using the SequalPrep Normalization plate (Life Technologies), and equal amounts mixed to construct the amplicon pool (Harris et al., 2010). The amplicon pool was concentrated by evaporation, size-selected by electrophoresis on a 1.5% agarose gel (Tris-acetate EDTA buffer) and gel purified by Montage kit (Millipore) prior to sequencing. Pyrosequencing was conducted per manufacturers’ protocols using Roche 454 titanium chemistry.

**Sequence Analysis**

Raw pyrosequences were quality filtered and sorted into their respective barcoded libraries with BARTAB (Frank, 2009). Filtering pyrosequence data removed nucleotides with mean Q < 20 at 5’ and 3’ ends and over a 10 nt window; sequences with >1 ambiguous base were discarded; and all sequences of length < 200 nt were discarded. Infernal (Nawrocki et al., 2009) and ChimeraSlayer (Haas et al., 2011) were used to confirm match to the bacterial rRNA secondary structure model, and to screen chimeric sequences, respectively. Taxonomic assignment of sequences was done by the Ribosomal Database Project (RDP) Classifier (Wang et al., 2007). The taxonomic information provided by the RDP Classifier was used to construct sequence groups with identical taxonomic rank.
Ecological Analysis

To assess the adequacy of our sequencing depth, species richness (number of unique taxa identified) based on Chao1 richness calculations (Magurran, 2004) was estimated using sample-based rarefaction (Gotelli and Colwell, 2001). A rarefaction curve and unconditional confidence intervals were computed using a bootstrap size of 100 and 295 knots (Colwell, 2000). Moreover, Good’s coverage estimator per DNA library was calculated using 1,000 bootstraps and the average of the estimates and 95% CI were reported (Good, 1953; Robertson et al., 2013).

We assessed our sample’s biological diversity by measuring species richness (total number of species) and evenness (variability in abundance). We adopted an approach described previously (Mather et al., 2012; Afema et al., 2014). Briefly, four diversity indices that weight the importance of species richness and evenness differently were calculated for age and TSP groups separately, and compared. The diversity indices were: species richness ($R$), Shannon’s index ($H'$), Simpson’s diversity index ($SD$) and Berger-Parker ($BP$), which cover the range of weightings for richness and evenness (Renyi, 1961). These are related to Hill’s number ($N_\alpha$) (Hill, 1973) as follows: $N_0 = R$, $N_1 = \exp(H')$, $N_2 = 1/SD$ and $N_\infty = 1/BP$. $N_0$ assesses diversity as the total count of taxa present irrespective of abundance, $N_1$ and $N_2$ assess diversity weighted by common taxa and $N_\infty$ assesses diversity weighted by the predominant taxa. As diversity measures are greatly influenced by sample size, we compared genera diversity within and between ages and TSP by sub-sampling the larger dataset 10,000 times without replacement to the size of the smaller dataset. We then calculated the mean and 95% confidence intervals (CI) of the subsamples.
**Statistical Analyses**

Community structure of the rectal microbiota was initially described at a phyla taxonomic level and phyla relative abundance investigated at each age category. Multiple pairwise comparisons of phyla abundance at each age level were made and Tukey’s HSD (honestly significant difference) test used to correct p-values for multiple comparisons (Daniel, 2005a). We used Proc Mixed from SAS 9.4 including the ‘Repeated’ statement to control for ‘calf’ as a repeat.

Sequence counts within a library were standardized to proportions and subsequently transformed by principal components analysis (PCA) (Lattin et al., 2003c). Number of principal components to be retained for downstream analysis was chosen based on eigenvalues >1 and evaluation of supportive scree plot, by which additional principal components contributing less than 1% of overall dataset variability would be discarded (Lattin et al., 2003d). The selected principal components were used to group the data into clusters using a two-step process. Initial clustering used “partitioning around medoids” or k-medoid partitioning to obtain 20 clusters (pam function, R 3.0.1) (Kaufman and Rousseeuw, 1990). The medoids of the clusters were used as starting seeds for k-means clustering to produce 20 new clusters (Proc Fastclus, SAS Version 9.4) (Lattin et al., 2003b). These clusters were subsequently reduced by selecting high frequency clusters and retaining those cluster means as seeds to create a parsimonious cluster set. Criteria for final clusters design were: maximizing the value of Pseudo F, minimizing small membership clusters, minimizing within cluster variation, and targeting cluster design to align with the natural co-variate structure associated with the sampled age groups. DNA libraries were assigned to the final clusters and membership was cross-tabulated against study co-variates of age and TSP category.
To evaluate interaction of TSP and age, cluster membership was cross tabulated against age and then stratified by TSP categories; the statistical significance of this interaction was evaluated using exact $\chi^2$ tests (Daniel, 2005b). Multinomial logistic regression models were developed using cluster membership as the dependent variable and TSP level as the main effect. Farm and age were assessed as a potential confounding variables or interactions (Proc Logistic SAS 9.4) (Kleinbaum et al., 1998).

RESULTS

Sixty calves from three dairy farms were enrolled in the study. One calf was lost to follow leaving 59 calves completing the study. Across all farms, the enrolled calves were approximately equally divided into two TSP categories: ≤5.0 g/dl (n=30) and ≥5.2 g/dl (n=29) (Table 1). Fecal samples were collected from each calf at days 1, 3, 5, 10, and 16 for a total of 295 fecal samples for subsequent microbiome assessment.

16S rRNA Gene Libraries.

Each fecal sample was used to obtain a 16S rRNA library. A total of 297,715 sequences were initially obtained, trimmed, and screened for quality. This yielded 237,284 sequences with an average of 800 sequences per library (range 16-2150 sequences/library) for microbiota analysis.

Did We Adequately Describe the Microbiota Diversity?

We identified 133 genera. Ninety nine percent of the total sequence was covered by 40 genera (Figure 1). A rarefaction curve based on Chao 1 calculations describing genera richness estimated that our sample contained 154 genera (C.I.=141-190), and that additional sampling would discover few unidentified genera and that we adequately described fecal microbiota.
genera richness in our samples (Figure 2). Confirming our sequencing depth was adequate, Good’s coverage was 95% (CI: 90-99%).

**Does Fecal Microbiota Genus Diversity Change with Age and TSP and Is the Impact of TSP Conditional on Age?**

Diversity analyses showed a decrease in richness from day 1 to day 3 and subsequent increase as calves became older (Table 2). Higher evenness at day 3 compared to day 1 was noted, suggesting that the transition from facultative anaerobes to strict anaerobes is characterized by co-dominance at this age. We also evaluated TSP effect on microbiota diversity and observed lower diversity in low TSP calves from the Hill’s measures weighting all degrees of taxa abundance (Table 3). When stratifying by age, day 3 and day 16 calves with low TSP had less diversity across all measures (richness and evenness) compared to calves with adequate TSP (Table 7).

**Does Fecal Microbiota Phyla Change with Age?**

From the 16S rRNA libraries, 133 genera were classified into 8 bacterial phyla though 5 contained the great majority of the 16S rRNA sequence: Bacteroidetes, Firmicutes, Fusobacteria, Actinobacteria, and Proteobacteria. The remaining 3 phyla, Spirochaetes, Verrucomicrobia, and Tenericutes, contained relatively few sequences and were not further evaluated. Phyla composition changed with age though this was variable across calves (Figures 3 and 4). The two phyla with the greatest change across ages were Bacteroidetes (increasing with age) and Proteobacteria (decreasing with age). Although we identified 133 bacterial genera, across calf ages each phylum was dominated by a single genus. Actinobacteria was dominated by *Corynebacterium* at day 1 and *Collinsella* at d 3, 5, 10, and 16. Bacteroidetes was dominated by *Bacteroides* across all ages. Firmicutes was dominated by *Clostridium* at d 1, *Butyrificoccus* at d
3, *Faecalibacterium* at d 5, and *Lactobacillus* at days 10 and 16. Fusobacteria was dominated by *Cetobacterium* across all ages. Proteobacteria was dominated by *Escherichia* at days 1, 3, 5, and 10 and by *Sutterella* on d 16.

**Clustering Analysis and Inferential Statistics.**

We used the first 33 components from PCA (accounting for 60% of PCA component variability) to create 5 k-means clusters describing microbial community in our sample. Microbial structure representing 90% of sequences per cluster is shown (Table 4).

Based on dominant genera, cluster A was equally represented by *Escherichia, Citrobacter* and *Colostridium* (facultative anaerobes) and 7 additional genera to account for 90% of the cluster sequence. Cluster B was represented primarily by *Butyricicoccus* and *Escherichia* and secondarily by *Bacteroides*, and *Roseburia* with 5 additional genera for 90% of sequence.

Cluster C was primarily represented by *Bacteroides* and to a lesser extent by *Faecalibacterium* and *Lactobacillus* with 9 additional genera. Cluster D had two primary genera, *Bacteroides* and *Cetobacterium* and secondarily *Prevotella* and 9 additional genera, while cluster E primary genus was *Bacteroides* (strictly anaerobe) with a large series of genera (n=18) to account for 90% of the cluster’s sequence.

By cross-tabulating clusters against age categories we found significant shifts in cluster membership across ages (*P*<0.001) (Table 5). The change in microbiota structure across ages was generally characterized by microbial community succession from facultative anaerobe bacteria to strictly anaerobes. When clusters were cross-tabulated against age categories by TSP category, there were significant differences (*P*=0.0057) in the distribution of cluster membership between TSP categories, particularly at days 3 and 5, i.e. the effect of TSP on microbiota structure was conditional on age (Table 6).
Multinomial logistic regression was used to evaluate the strength of TSP effect on cluster membership and to account for potential farm effects as a confounding variable. Because there was an interaction between TSP and age on cluster membership, separate models by age were evaluated. For age categories days 3 and 5, there were few observations at these ages in clusters A, D, and E and only membership distribution in clusters B and C were used as outcomes. The day 3 logistic regression model indicated that calves with adequate TSP were 2.7 times more likely to belong to cluster B vs cluster C compared to calves with low TSP (90% CI=1.02-7.2). The day 5 logistic regression model indicated a stronger effect of TSP on cluster membership: calves with adequate TSP were 6.8 times more likely to belong to cluster B vs cluster C compared to calves with low TSP (90% CI=1.08-42.6). TSP category had no effect on cluster membership for days 1, 10 and 16. Farm was not a significant confounder in our multivariate models.

**DISCUSSION**

The objective of this study was to evaluate the effect of age and TSP on calves’ fecal microbiota during first two weeks of life. We found marked microbiota succession across the first 16 days of life and a transient effect of TSP status on microbiota succession at days 3 and 5. These effects were not confounded by farm, i.e. the age dependent structure and the influence of TSP was consistent across farms.

A number of factors could influence shifts in fecal microbiota, particularly diet. In our study, calves were being fed milk with some exposure to forage and grain and this was consistent across the study period. Therefore age dependent changes we observed likely reflect physiological microbiota succession rather than external factors such as diet. In a previous study evaluating rumen microbiota, bacterial community changes were observed between 6 months
and 2 years old even though the rumen is fully developed by 6 months of age. This successional change was also not associated with diet as it remained unchanged across that period of time (Jami et al., 2013).

In calves with adequate TSP, a physiological bacterial succession occurred as facultative anaerobe coliforms (Enterobacteriaceae) dominated at day 1 and 3 with later appearance of Clostridia and Lactobacillus by day 3 and 5, followed by the strictly anaerobes Bacteroides and Prevotella. In comparison, calves with low TSP transitioned more rapidly to a strictly anaerobic structure. This transition is driven by early colonizing bacteria from the calves’ environment that produce substances that conditionally support or inhibit the establishment of allochthonous bacteria passing through the gut tract (Thompson-Chagoyan et al., 2007). Pioneer bacteria modulate and “educate” gut immune development and create a gut environment favorable to themselves and their own growth and unfavorable to other non-resident bacteria (Thorbecke and Benacerraf, 1959; Neish et al., 2000; Thompson-Chagoyan et al., 2007); the gut immunity develops tolerance towards the established bacteria and therefore the initial bacteria settlers can influence bacterial composition at later stages and contribute to the establishment of the final adult microbiota composition (Thompson-Chagoyan et al., 2007).

We analyzed diversity across ages and across TSP levels stratified by age. Except for day 3, microbial richness tended to increase with age and the evenness values fluctuated across ages. This finding could be due to microbial community succession and transitioning towards an adult microbiota structure. Moreover low TSP calves tended to have lower microbial biodiversity suggesting presence of microbial imbalance. Since lower biodiversity has been frequently associated with microbial imbalance and host’s disease (Manichanh et al., 2006; Cho et al.,
low colostrum calves may have gut microbiota characterized by decreased resistance to environmental damage or change, and may consequently be at higher risk of disease. The interaction of TSP and age on cluster membership at days 3 and 5 suggests that colostrum intake has a transient effect on the maturation of the rectal fecal microbiota with low TSP calves transitioning to a more mature anaerobic community faster than calves with adequate colostrum intake. Low TSP calves began transitioning towards a day 10 microbiota observed in adequate TSP calves beginning at day 3. This was a community dominated by cluster C genera (anaerobes represented by dominance of *Bacteroides*). By day 10, the TSP effect was no longer evident. There are no previous studies assessing colostrum effect on microbiota in calves or mammals.

An interaction of colostrum with the gut is well known. For example, the normal intestinal dynamics with ingested colostrum is the absorption of IgGs (most effectively during the first 24 hours of life) and then subsequent secretion into the intestinal lumen from blood (Besser et al., 1988b). This passive immunity also affects local GI immunity (Besser et al., 1988a). While the focus on the significance of colostrum is deservedly on the transfer of passive immunity, our data suggest that bovine colostrum also impacts intestinal microbial community development. Colostrum is nutrient dense with carbohydrates, lipids, proteins, minerals, vitamins, nonspecific antimicrobial factors (lactoferrin, lysozyme, lactoperoxidase), other immunoglobulins (IgA and IgM) and bio active factors important for the animal’s homeostasis (hormones, growth factors, cytokines, enzymes, polyamines, nucleotides, lymphocytes and neutrophils) (Blum and Hammondate, 2000). Colostrum components may also affect microbiota directly for establishment of normal commensal microbiota by having bactericidal activity (colostrum contains lysozyme and lactoferrin) (Arnold et al., 1980; Blum and Hammondate,
(Nattress and Baker, 2003), and by acting as prebiotics (colostrum contains oligosaccharides) (Gyorgy et al., 1974). It appears therefore that the action of bovine colostrum at the gut level covers a wider range of tasks beyond supporting passive immunity. It also appears that the effect it has on microbiome composition is short-lived (undetectable by day 10) although covers an important transition period for calves.

Our rarefaction curve and Good’s coverage calculation showed that our sequence sampling was adequate. We used 454 pyrosequencing, and despite availability of sequencing methods that allow obtaining higher number of sequences per sample, we were able to have sufficient coverage to fulfill the aims of our study. Our results demonstrate that although sequencing methodologies allow deeper sequencing at reasonable costs, additional sequence depth of calf microbiome will not likely improve our understanding of the role microbiomes have in supporting calf health.

To assess community structure we used several analytical approaches for data analysis. Our goal was not to simply describe gut bacteria as independent entities but to observe microbiota structure and assess factors determining that structure. We created a data analysis pipeline characterized by several steps. Our initial dataset was characterized by 295 libraries (observations) and 133 bacteria (attributes or dimensions). Our goal was to retain as much information as possible from the bacterial composition but reduce the amount of information needed to characterize microbiota structure. For this we used PCA to reduce dimensions and correlation in our initial dataset. This reduced dataset was used in a clustering analysis to create 5 clusters that best described the intestinal bacterial community structure recovered from our calves. Logistic regression methods were used to identify relationships between cluster membership and age, TSP, and farm effects.
There are no previous animal or human studies assessing colostrum effect on host’s gut microbiota though there are studies from the human literature that demonstrate a difference in gut microbiota structure between infants fed formula or breast milk and an effect of single components from colostrum (Penders et al., 2006; Fan et al., 2014). Significant to our results is that human health effects (such as allergies and autoimmune diseases) have been linked to whether an infant receives breast or formula milk as related to early intestinal microbiota composition (Johansson et al., 2011). In humans, the gut microbiota structure has been shown to have an immune function as it interferes with adhesion of pathogens to epithelial cells (Zachar and Savage, 1979; Blomberg et al., 1993; Bernet et al., 1994), increases the expression of genes involved in building the cross-bridging junctions at the epithelial level and consequently strengthens the intestinal barrier (Hooper et al., 2001), produces bacteriocins harmful for competitors (Mossie et al., 1981), and secretes fermentation end-products that may change the intestinal pH such that the gut environment may become less favorable for pathogens (Sinha, 1986; Ogawa et al., 2001). Our study only included calves that remained healthy throughout the study period, but the impact of colostrum intake on age-related microbiota structure occurred at a critical risk period for neonatal calf disease. Our study suggests that there may be mitigating health benefits for colostrum beyond its important role in providing calves passive immunity via its impact on microbiota development, and subsequent studies should explore these relationships.
ACKNOWLEDGEMENTS

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Conflict of Interest Statement

The authors declare no conflict of interest
REFERENCES


Table 1 Distribution of calves by farm and total serum protein (TSP) category enrolled in a study of the impact of colostrum fed at birth on the rectal fecal microbiota during the first 16 days of life

<table>
<thead>
<tr>
<th>Farm</th>
<th>TSP ≤ 5.0 g/dl</th>
<th>TSP ≥ 5.2 g/dl</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>TOTAL</td>
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<td>29</td>
<td>59</td>
</tr>
</tbody>
</table>
Table 2 Diversity of rectal fecal microbial community detected by use of 16S rRNA sequences from preweaned dairy calves (n=59) across ages

<table>
<thead>
<tr>
<th>Diversity</th>
<th>Day old</th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (n=63260)</td>
<td>3 (n=47443)</td>
<td>5 (n=44934)</td>
<td>10 (n=43957)</td>
<td>16 (n=38050)</td>
</tr>
<tr>
<td>(N_0)</td>
<td>72.40 (67.68-77.12)</td>
<td>62.51 (59.65-65.36)</td>
<td>73.36 (70.32-76.40)</td>
<td>79.50 (77.20-81.80)</td>
<td>86</td>
</tr>
<tr>
<td>(N_1)</td>
<td>9.35 (9.28-9.43)</td>
<td>10.10 (10.04-10.15)</td>
<td>8.01 (7.97-8.06)</td>
<td>9.65 (9.60-9.70)</td>
<td>10.42</td>
</tr>
<tr>
<td>(N_2)</td>
<td>6.54 (6.49-6.59)</td>
<td>7.35 (7.31-7.38)</td>
<td>5.09 (5.06-5.12)</td>
<td>5.46 (5.43-5.50)</td>
<td>5.31</td>
</tr>
<tr>
<td>(N_\infty)</td>
<td>5.16 (5.12-5.20)</td>
<td>5.81 (5.78-5.85)</td>
<td>3.61 (3.59-3.64)</td>
<td>3.63 (3.61-3.65)</td>
<td>3.33</td>
</tr>
</tbody>
</table>

a: to compare microbial diversity across ages each DNA sequences’ sample size (which corresponds to an age category) was subsampled x10000 to the size of the smallest sample size (16 day old).

n: number of DNA sequences; \(N_0\), \(N_1\), \(N_2\), and \(N_\infty\): Hill’s numbers assessing diversity (from counts of all genera present to counts of the most predominant genera).
Table 3 Comparison between two TSP levels (Adequate ≥5.2 g/dl; Low ≤5.0 g/dl) of rectal fecal microbial community diversity detected by use of 16S rRNA sequences from preweaned dairy calves (n=59)

<table>
<thead>
<tr>
<th>Diversity</th>
<th>Adequate (n=112932)(^a)</th>
<th>Low (n=124352)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N_0)</td>
<td>111</td>
<td>115.78 (112.40-119.20)</td>
</tr>
<tr>
<td>(N_1)</td>
<td>15.08</td>
<td>13.03 (12.99-13.06)</td>
</tr>
<tr>
<td>(N_2)</td>
<td>9.79</td>
<td>7.98 (7.95-8.00)</td>
</tr>
<tr>
<td>(N_\infty)</td>
<td>6.47</td>
<td>4.97 (4.95-4.98)</td>
</tr>
</tbody>
</table>

\(^a\): to compare microbial diversity across TSP levels the bigger DNA sequences’ sample size (Low TSP) was subsampled x10000 to the size of the smaller sample size (Adequate TSP).

\(n\): number of DNA sequences; \(N_0, N_1, N_2\) and \(N_\infty\): Hill’s numbers assessing diversity (from counts of all genera present to counts of the most predominant genera).
Table 4 Means of rectal fecal bacterial genera present in k-means clusters representing microbiota from newborn dairy calves. The listed genera contain 90% of the sequence within that cluster.

<table>
<thead>
<tr>
<th>Genera</th>
<th>Cluster A</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Cluster B</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Cluster C</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Cluster D</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Cluster E</th>
<th>Mean</th>
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</thead>
<tbody>
<tr>
<td>Escherichia</td>
<td>0.25</td>
<td>0.16</td>
<td></td>
<td>Butyricoccus</td>
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<td>0.20</td>
<td></td>
<td>Bacteroides</td>
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<td>0.16</td>
<td></td>
<td>Cetobacterium</td>
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<td>0.22</td>
<td></td>
<td>Prevotella</td>
<td>0.11</td>
<td>0.19</td>
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<td></td>
<td></td>
<td>Alistipes</td>
<td>0.01</td>
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</tr>
<tr>
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<td>Roseburia</td>
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</tbody>
</table>
Table 5 Rectal fecal samples from preweaned dairy calves were used to identify microbiota 16S rRNA sequences. Microbiota composition data were used in partitioning clustering analysis and clusters’ frequencies were cross tabulated against age levels. (Chi-square test; $P<0.001$)

<table>
<thead>
<tr>
<th>Day old</th>
<th>Cluster A</th>
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<th>Cluster C</th>
<th>Cluster D</th>
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Table 6 Rectal fecal samples from preweaned dairy calves were used to identify microbiota 16S rRNA sequences. Microbiota composition data were used in partitioning clustering analysis and clusters’ frequencies were cross tabulated against age levels and stratified by TSP. (Cochran-Mantel-Haenszel test; \( P=0.006 \))

### a) Adequate TSP

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<tr>
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</thead>
<tbody>
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<td>1</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
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<tr>
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<tr>
<td><strong>Total</strong></td>
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### b) Low TSP

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<tr>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>19</strong></td>
<td><strong>22</strong></td>
</tr>
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</table>
Table 7 Comparison between two TSP levels (Adequate ≥5.2 g/dl; Low ≤5.0 g/dl) stratified by age of rectal fecal microbial community diversity detected by use of 16S rRNA sequences from preweaned dairy calves (n=59)

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th>3 Day old</th>
<th></th>
<th></th>
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<th>16 Day old</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TSP</td>
<td>Adequate (n=31196)(^a)</td>
<td>Low (n=32064)</td>
<td>Adequate (n=22720)(^a)</td>
<td>Low (n=24723)</td>
<td>Adequate (n=19586)(^a)</td>
<td>Low (n=25348)</td>
<td>Adequate (n=21097)(^a)</td>
<td>Low (n=22500)</td>
<td>Adequate (n=18333)(^a)</td>
<td>Low (n=19717)</td>
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<td>Low (n=32064)</td>
<td>Adequate (n=22720)(^a)</td>
<td>Low (n=24723)</td>
<td>Adequate (n=19586)(^a)</td>
<td>Low (n=25348)</td>
<td>Adequate (n=21097)(^a)</td>
<td>Low (n=22500)</td>
<td>Adequate (n=18333)(^a)</td>
<td>Low (n=19717)</td>
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<td></td>
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<td>N(_0)</td>
<td>64</td>
<td>62.59 (61.36-63.83)</td>
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<td>57</td>
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<td>71.36 (69.78-72.93)</td>
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<td>N(_2)</td>
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<td>6.80 (6.79-6.82)</td>
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<td>7.86</td>
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<td>4.96 (4.92-5.01)</td>
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<td>3.64</td>
<td>3.61 (3.59-3.63)</td>
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</tr>
</tbody>
</table>

\(^a\) Numbers are mean and 95% CI
a: to compare microbial diversity across TSP levels the bigger DNA sequences’ sample size (Low TSP) was subsampled \times 10000 to the size of the smaller sample size (Adequate TSP).

n: number of DNA sequences; \(N_0, N_1, N_2\) and \(N_\infty\): Hill’s numbers assessing diversity (from counts of all genera present to counts of the most predominant genera).
Figure 1 Proportions of microbial genera (n=133) found in rectal fecal samples from newborn dairy calves. Forty bacteria (displayed in the histogram) represented 99% of the available DNA sequences.
Figure 2 Rarefaction curve estimating genera richness of rectal microbial community in preweaned dairy calves (first 16 days of life). Richness estimates and 95% confidence limits are based on Chao1 predictor sample-based methods (n=295) and 237,284 SSU-rRNA gene sequences.
Figure 3 Phyla composition stratified by age of rectal fecal microbiota from new-born dairy calves.
Figure 4 Box plots across five calf age categories depicting the proportion of total 16S rRNA microbiota found in the five major phyla recovered from dairy calf rectal fecal samples. Ages with different superscript letters are significantly different at $P<0.1$ using Tukey’s HSD test.
CHAPTER THREE

Evaluation of gut microbiota as risk factor for neonatal diarrhea and antibiotic treatment in preweaned dairy calves.

Letizia Tomassini*, Jonathan K. Harris‡, Charles E. Robertson§, Stephen S. Lee#, William M. Sischo*1

*Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman WA 99164

‡Department of Pediatrics, Division of Pulmonology, University of Colorado Denver, School of Medicine, Aurora, CO 80045

§ Division of Infectious Diseases, University of Colorado, School of Medicine, Aurora, CO 80045

#Department of Statistics, College of Science, University of Idaho, Moscow, ID 83844

1William M. Sischo, College of Veterinary Medicine, PO Box 646610, Pullman, WA 99164-6610 Telephone: 509-335-7495 • Fax: 509-335-0880 wmsischo@vetmed.wsu.edu

Email addresses:

LT: tomassini@vetmed.wsu.edu

CER: Charles.Robertson@Colorado.EDU

JKH: jonathan.harris@ucdenver.edu

SSL: stevel@uidaho.edu

WMS: wmsischo@vetmed.wsu.edu
ABSTRACT

Investigation on the link between gut microbiota and human health constitutes an ongoing important research area. With neonatal diarrhea being a major challenge in calves’ raising, gut microbiota may play a crucial role in neonatal calf health. However, the link between gut microbiota and calf neonatal diarrhea is unknown. The goal of this study was to explore fecal rectal microbiota as risk factor for diarrhea and antibiotic treatment in calves. Across three farms, we collected rectal fecal samples from 1 and 3 day old calves. Calves were followed up during the first two weeks of life. Diarrhea level was recorded by assigning a diarrhea score to each calf on daily morning visits, and antibiotic treatments were recorded by communications from farm personnel. DNA from samples was extracted, 16S rRNA gene PCR amplified, and 454 sequencing performed. Microbial taxonomy was obtained by use of Ribosomal Database Project classifier. Microbiota as risk factor for outcomes of interest was evaluated by using principal component analysis, clustering, regression analysis, and cox proportional hazard model. A total of 141 fecal samples were collected, and a total of 78,225 16S rRNA gene sequences at day 1 and a total of 58,260 16S rRNA gene sequences at day 3 were obtained. Calves were divided into three diarrhea levels categories: “little/no diarrhea”, “moderate diarrhea”, and “severe diarrhea”. Microbiota clusters from 1 day old calves significantly predicted severe diarrhea and microbiota clusters from 3 day old calves significantly predicted severe and moderate diarrhea. Moreover, microbiota clusters from 3 day old calves significantly predicted treatment and was significantly linked to treatment incidence rate. In this study we show that a certain microbiota may play a role as risk factor for diarrhea and treatment in neonatal calves. However, the microbiota associated with “little/ nodiarrhea” level was the most likely to receive antibiotic treatment. Our study may help understand the role gut microbiota has in host defense, and begin to describe alternatives that would affect antimicrobial use on farms.
INTRODUCTION

One of the major challenges for gut microbiota research is to define the roles that bacteria and bacterial communities play in human and animal health (Sekirov et al., 2010). There are studies that identify generalized health functions. In vitro and mice studies have shown that commensal bacteria play a role in host defense and disease prevention by out-competing with pathogens for resources, and contributing to a host’s immune system development and response modulation (Thorbecke and Benacerraf, 1959; Blomberg et al., 1993; Neish et al., 2000). Based on human studies, microbiota participate in resolving gut disease such as diarrhea (Wolvers et al., 2010; Borody and Campbell, 2012).

Relatively little is known of how change in microbiota structure may influence the appearance of a disease state. Gut microbiota dysbiosis triggered by antibiotic treatments may lead to antibiotic-associated diarrhea (Beaugerie et al., 2003). Not studied is whether a non-antibiotic associated dysbiosis may increase disease risks (Whelan et al., 2009; de La Cochetiere et al., 2010).

Neonatal calf diarrhea is a multifactorial disease with high morbidity and a major cause of calf death on calf ranches and dairy farms (Ok et al., 2009; USDA-NAHMS, 2011; Windeyer et al., 2014). In addition to the animal health costs of neonatal diarrhea, there are economic losses associated which include: decreased weight gains; increased labor costs for managing sick calves; material expense for antibiotics and other supportive care (Donovan et al., 1998; Anderson et al., 2003; Berge et al., 2009). The use of antibiotics in food production also may have implications for public and animal health and discussions about appropriate use to affect antimicrobial resistance are ongoing (Ajiboye et al., 2009; World Health Organization, 2012).
Our goals in this study were to describe the fecal microbiota community at post parturient days 1 and 3 in calves and evaluate these communities as risk factor for diarrhea and antimicrobial treatment.

MATERIALS AND METHODS

Farms, Animals, and Sample Collection

Study Enrollment Procedures--A convenience sample of 3 Northwest US dairy farms were identified to participate in the study. These farms were selected based on recommendations from collaborating field veterinarians or prior collaborations. Seventy-one calves from three dairy farms were enrolled in the study. Criteria for including calves in our cohort study were: calves had no signs of disease; calves were not exposed to antibiotics in the feed or milk; and availability of on-farm treatment records. Calves were enrolled 24 hours post parturition and serum collected at enrollment to assess TSP, a proxy for colostrum intake. Enrollment began July 2011 and was completed by September 2011.

From enrolled calves, fecal samples were collected at days 1 and 3 post-parturition and calves were followed daily through day 16. Each morning, study personnel assessed calves for fecal consistency based on a previously described 5-point scoring system (0=Formed, 1=Semi-formed/Soft, 2=Runny, 3=Watery, 4=Runny/watery with blood) (Berge et al., 2009). A score ≥2 was considered diarrhea. At this time, calf health was assessed for respiratory signs, attitude, appetite, and body temperature. Fever was recorded only when an animal was treated or lacked appetite. During the follow up period, all antibiotic and not-antibiotic treatments were recorded. Farm personnel were responsible for all aspects of animal care and antibiotic and non-antibiotic therapy decisions and their administration.
**TSP Assessment**--From each study calf, 10 mls of blood were obtained by jugular venipuncture and collected in serum tubes (Becton Dickenson, NJ). The blood was left to clot at room temperature and centrifuged to collect serum. TSP was assessed using a temperature-compensating refractometer previously calibrated with distilled water. Based on serum protein results, calves were categorized into TSP groups: ≤5.0 g/dl for low colostrum intake and ≥5.2 g/dl for high colostrum intake. To avoid misclassifying a low colostrum calf, animals with ≤5.0 g/dl were re-evaluated for TSP at 48 hours after birth (IACUC-04021-055).

**Fecal Sample Collection and Handling**--Five grams of fecal material were collected from each calf by rectal stimulation at day 1 and day 3. Samples were transported at 4°C to an on-site laboratory and stored short term at -20°C (≤ 3 weeks) or > 3 weeks at -80°C.

**Metagenomic Assessment**
DNA extraction, Small subunit rDNA (16S rRNA) PCR, sequencing, and library construction, and 16S-rRNA sequence analysis were performed according to methods previously described (Tomassini, 2015).

**Ecological Analyses**--To assess depth of sequencing, taxa richness (number of unique taxa identified) based on Chao1 richness estimator (Magurran, 2004) was estimated using sample-based rarefaction (Gotelli and Colwell, 2001). A rarefaction curve and unconditional confidence intervals were computed using a bootstrap size of 100 (Colwell, 2000). In addition, Good’s coverage estimator per DNA library was calculated using 1,000 bootstraps and the average of the estimates and 95% CI were reported (Good, 1953; Robertson et al., 2013).

**Diarrhea Categories**
Calves were stratified into 3 diarrhea categories levels groups. For each calf, the number of days a diarrhea score was ≥2 was determined, and all ≥2 observed scores were summed into a
single diarrhea severity score, which was then reassigned to each calf. Overall, calves were comparatively ranked based on severity score distribution into terciles and labeled as: “little of/no diarrhea”, “moderate diarrhea”, and “severe diarrhea”.

**Statistical Analyses**

**Summarizing Taxa Abundance and Diversity**--The 16S rRNA rectal microbiota on day 1 and 3 calf samples were described at both phylum and genus taxonomic levels. Specific microbiome community structure was assessed at the genus taxa level only by standardizing counts of specific taxa (based on RDP classifications) within a sample to a proportion of the recovered taxa. Taxa that comprised 99% of the available sequence were selected and transformed by principal components analysis (PCA) to reduce the taxa dimensions for further analyses (Lattin et al., 2003).

**Cluster Analyses to Describe Sample-based 16S-rRNA Community Structure**--Clusters were designed according to methodology previously described (Tomassini, 2015).

Logistic regression was used to test microbiota effect as risk factor for the outcomes of diarrhea and antibiotic treatment; specifically, since diarrhea has three categories (“little/no diarrhea”, “moderate diarrhea”, “severe diarrhea”), we used multinomial logistic regression to test for microbiota effect on diarrhea outcome; farm and TSP were controlled as possible confounders. We also assessed diarrhea as risk factor for treatment (SAS Enterprise Guide 6.1).

Days to diarrhea and treatment events conditional on microbiota structure were assessed by Kaplan-Meier and Cox proportional hazard models (Hosmer and Lemeshow, 1999). Proc lifetest and proc phreg were used (SAS Enterprise Guide 6.1).
RESULTS

Seventy-one calves from three dairy farms were enrolled in the study. A day 1 sample was not analyzed which left 70 calves for the final day 1 analyses. Across all farms, the enrolled calves were divided into two TSP categories: ≤5.0 g/dl (n=32) and ≥5.2 g/dl (n=39) (Table 1). Diarrhea and treatment episodes clustered between days 5-12 (Figures 1 and 2). Median value from Kaplan Meier analysis for first day of diarrhea was day 7 (95% C.I. =day 6-7) and for first day of treatment was day 7 (95% C.I. =day 6-9). Ninety percent of study calves were observed with at least one diarrhea episode (score ≥2) across the study period, but only 28% were classified as severe. In contrast, 17% of study calves were treated with an antibiotic. Fever and lack of appetite were occasionally present, and no other major symptoms of illness were noted. Antibiotics that were administered from farm personnel to calves were in the classes of cephalosporin, penicillin, sulfonamide and trimethoprim. A combination of sulfonamide and trimethoprim was used by all farms in our study; in addition to this drugs combination, farm “DR” used cephalosporin, and farm “S” used penicillin. According to farm management based policy, these treatment protocols were meant for scours treatment.

16S rRNA Libraries

Each fecal sample (n=141) contributed a complete 16S-rRNA sequence library. From the day 1 samples (n=70), a total of 107,023 sequences were initially obtained, trimmed, and screened for quality. This yielded 78,225 sequences with an average of 1029 sequences per sample (range 16-2086 sequences/sample) for microbiota analysis. From the day 3 samples (n=71) a total of 74,560 sequences were initially obtained, trimmed, and screened for quality. This yielded 58,260 sequences with an average of 757 sequences per sample (range 276-1844 sequences/sample) for microbiota analysis.
**Phyla Composition**

Across all samples, 11 bacterial phyla were identified: Bacteroidetes, Firmicutes, Fusobacteria, Actinobacteria, and Proteobacteria were most abundant; Spirochaetes, Verrucomicrobia, Tenericutes, Cyanobacteria, Deinococcus-Thermus, and TM7 were detected by presence of relatively very few sequences. Phyla composition changed from day 1 to day 3. At day 1 Bacteroidetes was found at 6% relative abundance, Firmicutes at 37.5%, Fusobacteria at 7.5%, Actinobacteria at 0.03%, and Proteobacteria at 49%. At day 3 Bacteroidetes was found at 22.9%, Firmicutes at 49.7%, Fusobacteria at 1%, Actinobacteria at 0.1%, and Proteobacteria at 26.4%.

**Genera Composition**

At day 1 we identified 129 taxa at the genus level. Ninety nine percent of the total sequence was represented by 27 taxa (Figure 3). Chao1 analysis estimated 184 genera at rarefaction point (95% C.I. =154-251) with Goods coverage of 98% (C.I. =95-100%).

At day 3 we identified 68 taxa. Ninety nine percent of the total sequence was covered by 26 taxa (Figure 4). Chao1 analysis estimated a richness of 103 genera (95% C.I. =78-195) for our study calves and Good’s coverage of 99% (C.I. =98-100%).

**Cluster Analysis and Inferential Statistics**

Using genera as variables, we performed clustering analysis keeping the data from our two age levels distinct: we grouped our calves into four clusters at day 1, and into four other clusters at day 3.

**Day 1 Microbiota Clusters**—The day 1 microbiome community from 70 calves grouped into 4 k-means clusters (Table 2). Each of our four clusters was dominated by a different genus:
Cluster 1 was dominated by *Escherichia*, Cluster 2 by *Bacteroides*, Cluster 3 by *Butyricicoccus*, and Cluster 4 by *Clostridium*.

**Day 1 Association between Microbiota and Diarrhea Severity and Antibiotic Treatment**--Using the *Clostridium* dominated cluster and low/no diarrhea level as references, at day 1 we found that the *Escherichia* and *Butyricicoccus* dominated clusters was 6.43 and 18.38 times more likely to be associated with calves classified with severe diarrhea, respectively (Table 3). There were no associations between microbiome clusters and antibiotic treatment.

**Day 3 Microbiota Communities**--The day 3 microbiome community from 71 calves grouped into 4 k-means clusters (Table 4). Each cluster was dominated by a different bacterial genus: Cluster 1 was dominated by *Bacteroides*, Cluster 2 by *Escherichia*, Cluster 3 by *Butyricicoccus*, and Cluster 4 by *Roseburia*.

**Day 3 Association between Microbiota and Diarrhea Severity and Antibiotic Treatment**--Using the *Roseburia* dominated cluster and low/no diarrhea level as references, at day 3 we found that the *Bacteroides* dominated cluster was 12.85 times more likely to be in the severe diarrhea category group and 204.27 times more likely to be in the moderate diarrhea group, and the *Escherichia* dominated cluster was 21.70 times more likely to be in the moderate diarrhea group (Table 5).

In both day 1 and day 3 models we found the presence of farm effect, consequence of less diarrhea in farm S compared to all other farms in the study.

We found a link between microbiota at day 3 and antibiotic treatment using the *Bacteroides* dominated cluster and non-treated animals as references we found that the *Roseburia* cluster was 18.96 times more likely to be treated and *Butyricicoccus* cluster 14.09 times more likely to be treated (Table 6). We also found that at day 3 microbiota is not linked to
diarrhea incidence rate but microbiota structure is linked to treatment incidence rate: using the *Bacteroides* dominated cluster and non-treated animals as references we found that the *Roseburia* cluster was 11.79 times at higher risk of being treated and the *Butyricicoccus* cluster 13.12 times at higher risk of being treated at any point in time.

*Assess the Link between TSP, Diarrhea and Treatment*

No relationships between TSP and any diarrhea level, and between treatment and any diarrhea level were found.

**DISCUSSION**

This study investigated gut microbiota in 1 and 3 day old calves by studying the significance of rectal fecal microbiota structure as risk factor for neonatal diarrhea and farm management based treatment. The phyla and genera we found were very similar to those found in previous studies on preweaned calves’ microbiota (Oikonomou et al., 2013; Xie et al., 2013; Tomassini, 2015). We found a larger number of taxa at day 1 compared to day 3. This is consistent with diversity analysis results observed previously where richness is higher at day 1 compared to day 3 (Tomassini, 2015). Because of the presence of facultative anaerobes at day 1, microbiota at this stage might be a close representation of the environmental microbiota the calf comes in contact with at time of delivery (Julien et al., 2008; Quigley et al., 2013). The day 3 microbiota is characterized by the presence of strict anaerobes, consistent with establishment of anaerobic conditions in the gut.

Interestingly, the microbial community at day 1 was predictive of severe diarrhea and day 3 microbiota was predictive of both moderate and severe diarrhea.

For simplicity purposes we refer to each cluster by mentioning the dominant bacterial genus per cluster and describe possible association of this cluster’s dominant with health or
disease as has been found in previous studies. However, association of microbiota to health might be explained not only by the action of a single bacterial group, but by the contemporary collaborative action of several genera present within the microbial community. By using clustering analysis, we developed an approach to investigate microbiota as a community and test its potential role in animal health while conducting an epidemiologic study. At day 1 the Escherichia dominated cluster was associated with severe diarrhea, and the clostridium dominated cluster was associated with low/no diarrhea. In previous studies, Clostridia have been found in farm environments (Julien et al., 2008) and strains of clostridia have been associated with health. For example, a group of Clostridia strains lacking toxins and virulence factors can act as a community to promote production of anti-inflammatory molecules and immune homeostasis in the host (Atarashi et al., 2013). Moreover, Escherichia is commonly found in the farm environment (Pradhan et al., 2009) and is considered indigenous among calf gut microbiota (Berge et al., 2005; Pereira et al., 2014). Escherichia can also be pathogenic including strains considered among the main causes of neonatal diarrhea in calves (Foster and Smith, 2009).

At day 3, the Bacteroides dominated cluster was associated with severe and moderate diarrhea and the Escherichia dominated cluster was associated with moderate diarrhea. The Butyricicoccus and Roseburia dominated clusters were associated with low/no diarrhea. Interestingly, Bacteroides that belonged to a health-associated cluster at day 1 shifted to a diarrhea-associated cluster at day 3 and Butyricicoccus that belonged to a diarrhea-associated cluster at day 1 shifted to a health-associated cluster at day 3. It has been reported that anaerobic bacteria that are indigenous in gut microbiota can become infectious under particular host circumstances. Bacteroides is strictly anaerobe and is commonly found among gut microbiota dominants (Oikonomou et al., 2013; Xie et al., 2013). However, species of Bacteroides genus
contain a gene encoding for an enterotoxin that can cause secretory diarrhea for which diarrhea in young children and calves can be found (Border et al., 1985; Sack et al., 1994; Almeida et al., 2007). Moreover, *Bacteroides* can shift from commensal to pathogenic bacterium under particular host circumstances (Wexler, 2007). *Butyricicoccus* and *Roseburia* have been associated with health. Both are butyrate producers (Louis and Flint, 2009; Geirnaert et al., 2013). Butyrate is a metabolite important for colonic health because it represents an energy source for intestinal mucosa (Donohoe et al., 2011). Particularly *Butyricicoccus* has been evaluated for probiotic use (Eeckhaut et al., 2013) and *Roseburia* has been described for beneficial actions at gut level (Louis and Flint, 2009; Van den Abbeele et al., 2013; Xie et al., 2013).

Discussions with farm management indicated that the decision to treat calves with antibiotics was typically made on the basis of presence of scours (diarrhea). Interestingly, our analysis found no correlation between any diarrhea level and the decision to treat with antibiotics. Microbiota at day 3 was predictive of treatment: the low/no diarrhea-associated microbiota was more likely to undergo treatment in following days. It is possible, then, that farm management based antibiotics administration may impede the establishment of a gut microbiota beneficial to the calf. These findings (lack of correlation between diarrhea and treatment, and correlation between “healthy microbiota” and treatment) raise concerns regarding antibiotics use on farm that are consistent with previous studies reporting that treatment on dairy farms is applied often without use of established protocols and close veterinarian supervision (Sawant et al., 2005; Raymond et al., 2006). Moreover, several scientists and public health organizations/authorities have stressed the importance of prudence in antibiotics use to preserve animal and public health (Berge et al., 2009; World Health Organization, 2012).
CONCLUSIONS

In our study we found that microbiota can represent a risk factor for diarrhea. Association of bacteria to diarrhea might not be explained by a single dominant bacterium only at a single point in time, but also by a combination of bacteria undergoing succession or by keystone bacteria that may be found in less abundance within the microbial community. Moreover, farm management based decisions on administering antibiotics were not correlated with diarrhea but were associated with “healthy” microbiota. Further investigations are warranted to understand the link between gut microbiota and health, and to improve management of antibiotics on dairy farms.
REFERENCES


USDA-NAHMS. (2011). Diary Heifer Raizer. USDA APHIS


Table 1 Baseline data of calves enrolled in a follow up study investigating rectal fecal microbiota as risk factor for diarrhea and treatment during first two weeks of life

<table>
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<td>23</td>
<td>21</td>
<td>44</td>
</tr>
<tr>
<td>S</td>
<td>7</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>V</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>TOTAL</td>
<td>32</td>
<td>39</td>
<td>71*</td>
</tr>
</tbody>
</table>

*One sample was lost from our day 1 analyses leaving 70 samples total
Table 2 Means of rectal bacterial genera present in k-means clusters for 1 day old dairy calves. The genera listed here explain 90% of the sequence information within each cluster.

<table>
<thead>
<tr>
<th>Genera</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Mean</th>
<th>Std Dev</th>
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<tbody>
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<td>0.29</td>
<td>Butyricoccus</td>
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<td>Clostridium</td>
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</tr>
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<td>Citrobacter</td>
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<td>0.17</td>
<td>Cetobacterium</td>
<td>0.21</td>
<td>0.23</td>
<td>Roseburia</td>
<td>0.14</td>
<td>0.12</td>
<td>Escherichia</td>
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<td>Escherichia</td>
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<td>Citrobacter</td>
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<td>Bacteroides</td>
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<td>Sporacetigenium</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Comamonas</td>
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</table>
Table 3 Multinomial logistic regression model evaluating rectal fecal microbiota from 1 day old dairy calves as risk factor for diarrhea during first 16 days of life. Calves were grouped into 4 clusters according to their microbiota composition. (Model $P=0.009$; farm $P=0.011$; cluster $P=0.403$)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Variable level</th>
<th>Diarrhea level</th>
<th>Odds ratio</th>
<th>Lower</th>
<th>Upper</th>
<th>P value</th>
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</tr>
<tr>
<td></td>
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<td>2</td>
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<td>50.19</td>
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</tr>
<tr>
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<tr>
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<td>0.01</td>
</tr>
<tr>
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<td>0.02</td>
<td>0.32</td>
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</tr>
<tr>
<td></td>
<td>V</td>
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<td>0.22</td>
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<td>0.96</td>
</tr>
<tr>
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<td>Reference</td>
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</tr>
</tbody>
</table>
Table 4 Means of rectal bacterial genera present in k-means clusters representing microbiota from 3 day old dairy calves. The listed genera explain 90% of the sequence information within each cluster.

<table>
<thead>
<tr>
<th>Genera</th>
<th>Cluster 1</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Cluster 2</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Cluster 3</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Cluster 4</th>
<th>Mean</th>
<th>Std Dev</th>
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<tbody>
<tr>
<td>Bacteroides</td>
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<td>0.38</td>
<td>0.21</td>
<td>Escherichia</td>
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<td>Butyricoccus</td>
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<td>Roseburia</td>
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<td>0.14</td>
</tr>
<tr>
<td>Escherichia</td>
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<td>0.17</td>
<td>0.14</td>
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<td>0.10</td>
<td>Bacteroides</td>
<td></td>
<td>0.15</td>
<td>0.23</td>
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<td>Butyricoccus</td>
<td>0.17</td>
<td>0.14</td>
</tr>
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<td>0.12</td>
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<td>0.07</td>
<td>Faecalibacterium</td>
<td></td>
<td>0.13</td>
<td>0.19</td>
<td></td>
<td>Escherichia</td>
<td>0.14</td>
<td>0.07</td>
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<td>Escherichia</td>
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<td>0.07</td>
<td></td>
<td>Faecalibacterium</td>
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<td>0.11</td>
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<td>0.07</td>
<td>Roseburia</td>
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<td>0.08</td>
<td>0.06</td>
<td>Roseburia</td>
<td></td>
<td>0.08</td>
<td>0.07</td>
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<td>0.14</td>
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<td>Citrobacter</td>
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<td>0.03</td>
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<td>0.08</td>
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<td>0.04</td>
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<td>Enterobacter</td>
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<td>0.02</td>
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<tr>
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<td></td>
<td>0.02</td>
<td>0.03</td>
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<tr>
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<td></td>
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<td>0.03</td>
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<td></td>
</tr>
</tbody>
</table>
Table 5 Multinomial logistic regression model evaluating risk of 3 day old rectal fecal microbiota on dairy calves’ diarrhea during the first 16 days of life. Calves were grouped into 4 clusters according to their microbiota composition. (Model $P<0.001$; farm $P=0.008$; cluster $P=0.036$)

<table>
<thead>
<tr>
<th>Variable level</th>
<th>Variable level</th>
<th>Diarrhea level</th>
<th>Odds ratios</th>
<th>Lower</th>
<th>Upper</th>
<th>P value</th>
</tr>
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<td>&gt;999.999</td>
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<td>1</td>
<td>1.97</td>
<td>0.37</td>
<td>10.46</td>
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</tr>
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<td>6.03</td>
<td>0.62</td>
<td>58.52</td>
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<tr>
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<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.02</td>
<td>0.00</td>
<td>0.20</td>
<td>0.01</td>
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<tr>
<td></td>
<td>0.02</td>
<td>0.00</td>
<td>0.13</td>
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<tr>
<td>Farm V</td>
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<tr>
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<td>Reference</td>
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</table>
Table 6 Logistic regression model evaluating rectal fecal microbiota from 3 day old dairy calves as risk factor for antibiotic treatment during first 16 days of life. Calves were grouped into 4 clusters according to their microbiota composition. (Model $P=0.003$; cluster $P=0.051$; farm $P=0.100$; TSP $P=0.1558$)

<table>
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<th>P value</th>
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<td>Upper</td>
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<td></td>
</tr>
</tbody>
</table>

*TSP: ≤5.0 g/dl for low colostrum intake and ≥5.2 g/dl for high colostrum intake

**Non-treated animals were used as reference
Figure 1  Survival analysis showing incidence of neonatal diarrhea in dairy calves
Figure 2 Univariate Kaplan-Meier analysis evaluating effect of rectal fecal microbiota structures from 3 day old calves on treatment rates (Wilcoxon test, \( P=0.008 \)). Calves were grouped into four clusters based on their microbiota composition.
Figure 3 Microbial genera from rectal fecal samples of 1 day old dairy calves. Bacteria displayed in the histogram represent 99% of the available DNA sequences.*

Figure 4 Microbial genera from rectal fecal samples of 3 day old dairy calves. Bacteria displayed in the histogram represent 99% of the available DNA sequences.*

* Bacteria that represented 1% of DNA sequences: Klebsiella, Salmonella, Veillonella, Fusobacterium, Megasphaera, Subdoligranulum, Collinsella, Lactococcus, Comamonas, Anaerofilum, Lactonifactor, Dialister, Raoultella, Eubacterium, Hespellia, Oscillibacter, Actinobacillus, Prevotella, Paraprevotella, Lachnobaeterium, Gallibacterium, Anaerostipes, Anaerovibrio, Parasutterella, Dickeya, Odoribacter, Pasteurella, Citricoccus, Eggerthella, Chryseobacterium, Leuconostoc, Caldalkalibacillus, Ruminococcus, Sharpea, Cupriavidus, Acidovorax, Microvirgula, Neisseria, Proteus, Mannheimia, Acinetobacter, Stenotrophomonas.
CHAPTER FOUR

Antimicrobial treatment impact on fecal rectal microbiota from preweaned dairy calves and evaluation of microbiota resilience following treatment

Letizia Tomassini*, Jonathan K. Harris‡, Charles E. Robertson§, Stephen S. Lee#, William M. Sischo*1

*Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman WA 99164

‡Department of Pediatrics, Division of Pulmonology, University of Colorado Denver, School of Medicine, Aurora, CO 80045

§ Division of Infectious Diseases, University of Colorado, School of Medicine, Aurora, CO 80045

#Department of Statistics, College of Science, University of Idaho, Moscow, ID 83844

1William M. Sischo, College of Veterinary Medicine, PO Box 646610, Pullman, WA 99164-6610 Telephone: 509-335-7495 • Fax: 509-335-0880 wmsischo@vetmed.wsu.edu

Email addresses:

LT: tomassini@vetmed.wsu.edu

CER: Charles.Robertson@Colorado.EDU

JKH: jonathan.harris@ucdenver.edu

SSL: stevel@uidaho.edu

WMS: wmsischo@vetmed.wsu.edu
ABSTRACT

Antibiotics are among the most important factors causing changes on microbiota composition and biodiversity. Microbiota dysbiosis due to antibiotics can be detected immediately after antimicrobial administration and may last for a variable length of time. Nothing is known on the effect of antibiotic treatment on preweaned calves’ gut microbiota. The goal of this study was to assess the effect of antibiotic treatment on fecal rectal microbiota in preweaned dairy calves and explore calves’ microbiota resilience following antibiotic treatment. Rectal fecal samples were collected from preweaned Holstein calves across four Northwest US dairy farms. Sampling days were ‘day before treatment’, ‘day of treatment’, ‘day after treatment’, and ‘day 14 after last day of treatment’. DNA was extracted from fecal samples, and 16S rRNA gene PRC amplified and sequenced by 454 pyrosequencing. Taxonomical information was obtained through Ribosomal Database Project classifier. Microbiota biodiversity was assessed through use of Hill’s numbers. Principal Component Analysis, clustering, and regression were used to test for antibiotic effect on microbiota structure and microbiota resilience after treatment. Thirty animals were sampled for a total of 120 collected samples. Pyrosequencing yielded a total of 76,413 sequences. We found a decrease in microbiota biodiversity caused by antibiotics. Moreover, antibiotics significantly impacted microbiota structure immediately after treatment but did not have lasting effect. In fact, microbiota recovered a profile similar to control animals by 14 days after last day of treatment. We conducted an epidemiological study exploring the impact of antibiotic treatment on calves’ fecal microbiota biodiversity and structure. In our study, antibiotic treatment was associated with decreased microbial biodiversity, and had a short term effect on microbiota composition. Future studies are warranted to evaluate the impact of antibiotics on the presence of antibiotic resistance in the calves’ gut microbial community.
INTRODUCTION

Gut microbiota commensalism covers a wide range of functions beneficial to host’s health and homeostasis. Microbiota facilitated immunomodulation and use of ecological niches may decrease hosts’ susceptibility to infections and diseases (Neish et al., 2000; Ferreira et al., 2011; Kabat et al., 2014). Microbiota’s breakdown of certain molecules increases bioavailability of nutrients contributing to acquisition of energy for the host (Mirande et al., 2010). Especially in the early stages of life, microbiota contributes to development of host innate and adaptive immunity and general growth (Thorbecke and Benacerraf, 1959; Blottiere et al., 2003; Gloux et al., 2007; Hrncir et al., 2008).

Antibiotics are among the most important factors causing changes in microbiota composition and biodiversity (Penders et al., 2006; Modi et al., 2014). Degree of impact on microbiota may depend on the interaction of several factors such as class of antibiotic, duration of treatment, dose concentration, route of administration, structure and functions of microbial community, and presence of resistance genes in the microbial community (Jernberg et al., 2010; Tannock et al., 2013; Perez-Cobas et al., 2013; Greenwood et al., 2014). Microbiota change due to antibiotics can be detected immediately after antimicrobial administration and may last for a length of time (de La Cochetiere et al., 2005). After disruption, microbiota commonly tends to return to its original stages but the time necessary for community recovery to normal may vary and complete recovery may never be detected (Dethlefsen et al., 2008; Suchodolski et al., 2009).

Microbiota dysbiosis has been linked to increased susceptibility to diarrheagenic pathogens and to a wide range of disease types (Beaugerie et al., 2003; de La Cochetiere et al., 2008; Keeney et al., 2014). Moreover, importance of striving for prudent antimicrobial use is related not only to the importance of preserving and improving host’s health, but to contain
antimicrobial resistance spread in pathogens of public health concern. In fact, antibiotic use may increase the load of antimicrobial resistance genes within the microbial community with consequent horizontal transfer of resistance genes to pathogenic bacteria (Dantas et al., 2008; Lee et al., 2010; Stecher et al., 2012).

Antibiotic use in dairy industry has gained pivot role in calf disease management. Antibiotics at sub-therapeutic doses are used to prevent disease and at treatment doses to treat disease (Berge et al., 2005; Walker et al., 2012). Animals affected by neonatal diarrhea are most likely to be treated with antibiotics and neonatal diarrhea represents the main reason for antibiotic treatment in U.S. dairy calves (USDA-NAHMS, 2011; Walker et al., 2012).

Controversial theories have been postulated regarding the role of antibiotics in treating and resolving calf diarrhea, and the actual benefits of antimicrobial use on calves’ health. Antibiotic treatment may be necessary in case of systemic disease and may resolve a disease condition (including neonatal diarrhea) when the antibiotic class of choice effectively targets the disease causing pathogen (Radostits, 1975; Tennant et al., 1978; Bakheit and Greene, 1981; Constable, 2009). However, a distinction has been made between animals that truly need to receive antibiotics for diarrhea and animals that may recover by administering electrolytes only (Berge et al., 2009; Constable, 2009). Also, some antibiotics that are commonly used for treating diarrhea lack scientific peer reviewed studies supporting their effectiveness.

The hypotheses of this study were that antibiotic treatment has an effect on fecal rectal microbiota in preweaned dairy calves and calves’ microbiota recover a profile similar to non-treated calves following treatment.
MATERIALS AND METHODS

Farms, Animals, and Sample Collection

Fecal samples were collected between July and September 2011 from preweaned Holstein calves from 4 Northwest US dairy farms. We performed convenience sampling based on recommendations from field veterinarians or prior collaborations. Farm’s criteria for calf enrollment included: farm did not use antibiotics in calves’ feed or milk, and farms had treatment records available and were willing to share the treatment information. To be included in the study, calves at day 1 were required to have normal appetite, alert attitude, and absence of systemic infection. Starting at 1 day old, calves were monitored according to a previously published protocol for health assessment routine (Berge et al., 2009) and a fecal sample was collected every day. Morning health assessments were performed by study personnel, and farm personnel was responsible for all aspects of animal care and antibiotics administration (IACUC-04021-055). Working in collaboration and communicating with farm personnel, treatments episodes during follow up were recorded.

Fecal samples that were considered for microbiota analysis corresponded to: day before treatment; day of treatment; day after treatment; and day 14 after last day of treatment. In selecting controls for our study we focused on matching cases (treated animals) and controls (non-treated animals) by farm and age.

Five gm of fecal sample was collected every day from each calf by rectal stimulation. Samples were transported at 4°C to an on-site laboratory and stored short term at -20°C (two-three weeks) and long term at -80°C. At the end of our follow-up study we selected among the frozen samples the ones of interest to study microbiota at established time points: day before treatment, day of treatment, day after treatment, and 14 days after last day of treatment.
**Metagenomic Assessment**

DNA extraction, small subunit rRNA PCRs, sequencing, library construction, sequence analysis, and ecological analysis were performed according to methods previously described (Tomassini, 2015).

**Statistical Analysis**

Statistical analysis were performed according to methods previously described (Tomassini, 2015).

Our representing microbiota structure clusters were inserted in a logistic regression model as multilevel categorical dependent variable to test for treatment effect and return of microbiota structure to a healthy profile after treatment. The model was assessed for the presence of possible confounders (SAS Enterprise Guide 6.1, Proc Logistic).

**RESULTS**

Thirty animals from four dairy farms were enrolled in the study. Sampling of animals was performed with the aim to match cases and controls by farm and age (Table 1). Fecal samples were collected from each calf at day before treatment, day of treatment, day after treatment, and 14 days after last day of treatment. This yielded a total of 120 samples for subsequent microbiome assessment. Fecal sample collected on day of treatment was collected few hours before treatment episode: our study personnel collected fecal samples at mid-morning while antibiotic treatments would be administered in the early afternoon. Therefore, we refer to the day after treatment to evaluate antibiotic effect on microbiota.

There were some differences in treatment protocols across farms: farm “B” mostly used a combination of sulfamethoxazole and trimethoprim, and on one animal only used an aminoglycoside; farm “DR” used a combination of ceftiofur, sulfamethoxazole and
trimethoprim; farm “V” used a combination of sulfamethoxazole and trimethoprim; farm “S” used a combination of ampicillin, sulfamethoxazole and trimethoprim. Following farm management based policies, all these treatments were meant for scours and number of treatment days varied. Ampicillin, sulfamethoxazole and trimethoprim were used via oral route at the dose of 20mg/Kg, while ceftiofur was injected in the muscle and used at the dose of 5mg/Kg.

From our daily health assessments we observed scours and not any other symptoms related to other possible diseases. Also we observed that diarrhea levels as recorded by study personnel were not different between antibiotic treated and non-treated calves.

**16S rRNA Gene Libraries and Sampling Coverage**

Each fecal sample was used to obtain a 16S rRNA library. A total of 90,304 sequences were initially obtained, trimmed, and screened for quality. This yielded 76,413 sequences with an average of 637 sequences per sample (range 33-1692 sequences/sample) for microbiota analysis.

With a Good’s coverage of 95% (C.I. = 90-99%) the sampling appeared to provide extensive cover of the microbiota community.

**Phyla and Genera**

From the 16S rRNA gene libraries we identified 103 genera of which 40 genera explained 99% of information within the dataset (Figure 1). Genera were classified into 8 bacterial phyla though 5 contained the great majority of the 16S rRNA gene sequence and were found at variable relative abundance: Bacteroidetes at 44.04%, Firmicutes at 39.92%, Proteobacteria at 11.76%, Actinobacteria at 3.29%, Fusobacteria at 0.93%. The remaining 3 phyla, Spirochaetes, Verrucomicrobia, and Tenericutes, were detected by presence of relatively very few sequences.
**Does Fecal Microbiota Genus Diversity Change with Treatment?**

Treated animals had higher biodiversity compared to controls across all time points (Table 2). Particularly, diversity analysis comparing cases and controls on day after treatment showed higher richness and lower diversity of remaining Hills’ numbers in control animals. Diversity at 14 days after last day of treatment showed higher biodiversity across all Hill’s numbers in cases compared to controls.

When comparing day of treatment vs day after treatment within the same study group, we found decreased biodiversity at day after treatment in treated calves and an opposite trend in controls (Table 2).

**Clustering Analysis and Inferential Statistics**

We used 13 components from PCA (accounting for 60% of variability in the dataset) to create 4 k-means clusters describing microbial community in our sample. Microbial structure representing 90% of sequences per cluster is shown (Table 3). Based on dominant genera, cluster 1 was mostly represented by *Lactobacillus* followed by *Bacteroides* in second place, and *Escherichia* and *Collinsella* in third place. *Faecalibacterium* was found in fourth place in this particular cluster. Additional 10 genera completed representation of 90% of information in cluster 1.

Cluster 2 was dominated by *Bacteroides* which was followed at distance by *Faecalibacterium* and *Prevotella* present in similar relative abundance; 12 additional genera explained for the remaining 90% of information from this cluster. Cluster 3 was dominated by *Faecalibacterium* followed at distance by *Bacteroides*; the striking feature of cluster 3 was that 90% of cluster’s information was distributed among fewer number of bacteria compared to other clusters: only 6 more genera were present after *Faecalibacterium* and *Bacteroides*. Cluster 4 had
Bacteroides as primary genus, followed at distance by Parasutterella and Lactobacillus; 8 additional genera were present. In addition, Lactobacillus and Escherichia seemed to be representative genera preferably of clusters 1 and 3 compared to cluster 2.

Logistic regression was used to test for treatment effect on microbiota structure and microbiota recovery after 14 days from treatment. We found that cluster microbial membership was affected by treatment administration (table 4). Separate models were evaluated at day before treatment, day of treatment, day after treatment, and 14 days after last day of treatment. The day after treatment logistic regression model indicated that treated calves were 12.8 times more likely to belong to cluster 1 (90% CI=2.3-70.2) and 22 times more likely to belong to cluster 3 vs cluster 2 (90% CI=2.4-204.9) compared to non-treated calves. Microbiota across the remaining evaluated models was not different between study groups, meaning that microbiota had recovered structure 14 days after treatment.

**DISCUSSION**

We conducted an epidemiological study on the impact of antibiotic treatment on biodiversity and structure of fecal rectal microbial community from dairy calves.

By running an analysis comparing biodiversity within study groups between day of treatment and day after treatment, we observed that biodiversity decreased in case animals and increased in controls. These results are consistent with previous findings showing microbiota biodiversity decrease due to antibiotics, and increasing microbiota biodiversity as correlated with increasing age during time of rapid host growth (Koenig et al., 2011; Panda et al., 2014).

Results in our study showed antibiotic impact on microbiota structure at 24 hours after antibiotic administration. This is consistent with previous results reporting microbiota dysbiosis immediately after antibiotic administration (de La Cochetiere et al., 2005; Videnska et al., 2013).
This change impacts the microbial community during an important transition time of the microbial community within calf life (Koenig et al., 2011).

We described the shifts and changes of microbiota affected by treatment evaluating the microbial community as a whole entity. Within the clusters that resulted different between study groups, we observed shifts in dominating genera, and shifts in genera relative abundance at lower clusters’ ranks positions. Particularly, *Bacteroides* was reduced and *Escherichia* and *Lactobacillus* proliferated in treated animals. Future studies should evaluate the biological significance of these shifts, and evaluate the presence of antibiotic resistance in the microbial community which may favor survival of some genera versus the others.

We also observed microbiota structure recovery at 14 days after end of treatment. The resilience of microbiota and its tendency to recover its original profile in a short period of time after treatment has been observed before (Robinson and Young, 2010). However, most studies show that complete recovery is not observed until months from treatment; and most times only partial recovery is observed within the time span involved in the research project (Dethlefsen et al., 2008; Suchodolski et al., 2009; Puhl et al., 2012).

In this study, we evaluated impact of antibiotics on microbiota biodiversity and structure. Antibiotics decreased biodiversity in preweaned dairy calves and had an impact on microbiota composition that was detected shortly after treatment administration. Recovery of calf microbiota was observed by 14 days from end of treatment. Future studies should further assess antibiotic impact on antibiotic resistance level within the microbial community.
REFERENCES


USDA-NAHMS. (2011). Diary Heifer Raiser. USDA APHIS


Table 1 Distribution of calves by farm and day old sampling time enrolled in a study on the impact of antibiotic treatment on calves’ rectal fecal microbiota at pre-weaning time

a) Farm=B

<table>
<thead>
<tr>
<th>Animals</th>
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<tbody>
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<td>12</td>
</tr>
<tr>
<td>Control</td>
<td>0 1 3 3 2 0 0 0 0 0 1 1 0 0 0 1 0 0 0</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
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</thead>
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</tr>
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</tr>
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c) Farm=S

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<td>12</td>
</tr>
<tr>
<td>Control</td>
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d) Farm=V

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</tr>
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<td>Total</td>
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Table 2 Diversity of rectal fecal microbial community detected by use of 16S rRNA sequences from preweaned dairy calves (n=30) across time points from time of antimicrobial treatment

<table>
<thead>
<tr>
<th>Diversity</th>
<th>'Day Bef Trt</th>
<th>Day of Trt</th>
<th>Day Aft Trt</th>
<th>Day 14 Aft Trt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (n=8765)</td>
<td>Controls (n=10457)</td>
<td>Cases (n=8734)</td>
<td>Controls (n=10224)</td>
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<tr>
<td>N₀</td>
<td>47.68 (44.89-50.48)</td>
<td>55</td>
<td>56</td>
<td>54.85 (44.52-46.80)</td>
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<tr>
<td>N₁</td>
<td>8.85 (8.75-8.96)</td>
<td>7.81</td>
<td>14.4</td>
<td>7.46 (7.36-7.55)</td>
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<tr>
<td>N₂</td>
<td>5.91 (5.83-5.98)</td>
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<td>9.91</td>
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<td>N∞</td>
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b) Cases Day of Trt vs Day Aft

<table>
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<th>Day Aft (n=11580)</th>
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</thead>
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<td>37.66 (36.52-38.79)</td>
</tr>
<tr>
<td>N₁</td>
<td>14.40</td>
<td>10.21 (10.14-10.27)</td>
</tr>
<tr>
<td>N₂</td>
<td>9.91</td>
<td>7.08 (7.02-7.13)</td>
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<td>7.01</td>
<td>5.22 (5.17-5.27)</td>
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Controls Day of Trt vs Day Aft

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<th>Day Aft (n=11148)</th>
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<td>N₀</td>
<td>56</td>
<td>58.66 (57.52-59.80)</td>
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<tr>
<td>N₁</td>
<td>7.46</td>
<td>9.94 (9.88-9.99)</td>
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<td>N₂</td>
<td>3.90</td>
<td>5.10 (5.07-5.14)</td>
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<tr>
<td>N∞</td>
<td>2.60</td>
<td>3.17 (3.15-3.18)</td>
</tr>
</tbody>
</table>

a: to compare microbial diversity across ages each DNA sequences’ sample size was subsampled x10000 to the size of the smallest sample size.

N: number of DNA sequences; N₀, N₁, N₂ and N∞: Hill’s numbers assessing diversity (from counts of all genera present to counts of the most predominant genera).

*Bef= before; Trt= treatment; Aft= after.
Table 3 Means of rectal fecal bacterial genera present in k-means clusters representing microbiota from newborn dairy calves. The listed genera contain 90% of the sequence within that cluster.

<table>
<thead>
<tr>
<th>Genera</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Genera</th>
<th>Mean</th>
<th>Std Dev</th>
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</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td>0.29</td>
<td>0.19</td>
<td>Bacteroides</td>
<td>0.44</td>
<td>0.18</td>
<td>Faecalibacterium</td>
<td>0.42</td>
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<td>Bacteroides</td>
<td>0.36</td>
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<tr>
<td>Bacteroides</td>
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<td>0.15</td>
<td>Faecalibacterium</td>
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<td>0.09</td>
<td>Bacteroides</td>
<td>0.23</td>
<td>0.16</td>
<td>Parasutterella</td>
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<td>0.10</td>
</tr>
<tr>
<td>Escherichia</td>
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<td>0.14</td>
<td>Prevotella</td>
<td>0.08</td>
<td>0.15</td>
<td>Butyricicoccus</td>
<td>0.07</td>
<td>0.08</td>
<td>Lactobacillus</td>
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<tr>
<td>Collinsella</td>
<td>0.08</td>
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<td>Paraprevotella</td>
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<td>0.13</td>
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<td>Paraprevotella</td>
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<td>Oscillibacter</td>
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<td>Xylanibacter</td>
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<td>0.04</td>
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</table>
Table 4 Rectal fecal samples from preweaned dairy calves were used to identify microbiota 16S rRNA sequences. Microbiota composition data were used in partitioning clustering analysis; clusters’ frequencies were cross tabulated against time points from time of antimicrobial treatment and stratified by study groups.

a) Treated animals

<table>
<thead>
<tr>
<th>Sampling time from treatment</th>
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<th>Cluster 2</th>
<th>Cluster 3</th>
<th>Cluster 4</th>
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<td>4</td>
<td>2</td>
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b) Control animals

<table>
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<th>Sampling time from treatment</th>
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<th>Cluster 2</th>
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<tr>
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<td>1</td>
<td>4</td>
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<td>11</td>
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<td>1</td>
<td>13</td>
<td>0</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>38</td>
<td>5</td>
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Figure 1 Proportions of microbial genera (n=103) found in rectal fecal samples from newborn dairy calves. Forty bacteria (displayed in the histogram) represented 99% of the available DNA sequences.

GENERAL CONCLUSIONS

Microbiota has been long recognized for its importance to host’s health. With the development of molecular techniques and sequencing technologies starting in 1970s’ with Sanger sequencing and since five years with Next Generation Sequencing (NGS), our ability to study microbial communities has increased exponentially. NGS allows assessing microbial biodiversity and taxonomical composition, and establishing the link between microbiota and host-associated covariates of interest. The aim in the microbiome research area is to discover the biological significance of microbes and address the cause-effect relationship between microbiota and a wide range of diseases. Unfortunately, there has not been a similar development rate of statistical data analysis techniques allowing to effectively using the information delivered by NGS technology for microbial communities’ investigation. Structure of microbiota data is compositional, multidimensional, and overdispersed for the presence of many zeros. The challenge is to extract useful information on microbiota from such complex datasets and study its relationships to covariates of interest.

The goal of this PhD research was to study fecal rectal microbiota from preweaned dairy calves and assess microbiota dynamics depending on colostrum intake, presence of diarrhea, and antibiotic treatment. We performed a cross-sectional study on dairy calves during their first month of life to detect the dominant rectal bacterial taxa in preweaning time. We found an age-dependent succession in the bacterial community composition. The main goal of the study was to detect the dominant taxa and to develop a data analysis pipeline suitable to explore the link between microbiota communities and covariates of interest. We found principal component analysis was a useful tool to present community structure collected as relative abundance (proportion of taxa in a sample) to subsequent multivariate analyses. The method reduces taxa
dimensionality, removes correlation between taxa, and removes the problem introduced by the numeric constraint of relative abundance (range 0-1).

We then studied colostrum effect on fecal rectal microbiota structure and biodiversity in the first two weeks of calves’ life. We found calves that did not receive adequate colostrum had lower microbial biodiversity compared to calves that received adequate colostrum. Moreover, colostrum affected 3 and 5 day old calves’ microbiota structure and the effect was no longer detectable by day 10. Microbiota dysbiosis conditional on colostrum intake happens in a sensitive time of calf’s life, because risk for developing neonatal diarrhea is higher between the first and the second week of life. Also, the short term colostrum impact detected on fecal microbiota may have long term consequences on gut microbiota development, gut immunity and susceptibility to diseases of the host, and subsequent studies should explore these relationships. Our study adds to the knowledge on the importance of colostrum in calf raising. Dairy operations should strive to better their management practices in order to ensure adequate transfer of passive immunity to calves. In addition, design of compounds (probiotics, prebiotics, fecal transplants) to compensate for lack of colostrum administration might be useful in supporting calf raising.

In macrobiota research area, of primary importance is to assess and understand cause-effect dynamics between microbial communities and host’s susceptibility to disease. Aiming to explore the relationship of microbiota with calf health, we evaluated microbiota structures as risk factors for calf diarrhea and antibiotic treatment. We found 1 day old calves’ microbiota predicts diarrhea, and 1 and 3 day old calves’ microbiota predicts diarrhea and treatment. Diarrhea pathogenesis might originate by the action of a single bacterium, or a combination of bacteria, or a variety of factors involved in microbiota development dynamics. This study may help to better understand the pathogenic processes involved in calves’ neonatal diarrhea development. Further
studies should address the interaction between bacteria and the calf gut immune system during this early stage of life. Moreover, because of the significance we found on microbiota structure predicting antibiotic treatment, further investigations are warranted to understand the link between gut microbiota and health, and improve the target of antibiotic treatments on dairy farms.

One of the main factors affecting gut microbiota is the antibiotics use. We evaluated the impact of antibiotics on microbiota biodiversity and structure. We found that antibiotics may decrease biodiversity in preweaned dairy calves and antibiotics impact microbiota composition shortly after treatment administration. Recovery of calf microbiota was observed by 14 days from the end of treatment. Future studies should further assess antibiotic impact on microbiota by exploring development of antibiotic resistance within the microbial community.