EXAMINING THE INTERPLAY OF GUT MICROBIOME AND DIET-INDUCED RUMINAL ACIDOSIS IN DAIRY CATTLE

by

Suhair Khalaf Najm Al Janabi

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Suhair Khalaf Najm Al Janabi

Approved: _____

Shawn W. Polson, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

E. Fidelma Boyd, Ph.D.
Chair of the Department of Biological Sciences

Approved: _____

John Pelesko, Ph.D.
Interim Dean of the College of Arts and Science

Approved: _____

Douglas J. Doren, Ph.D.
Interim Vice Provost for the Office of Graduate and Professional Education

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ABSTRACT

Dairy cattle support an important industry worldwide and produce about 378 billion liters of milk per year. During industrial milk production, cows are switched from a diet rich in fiber to a diet rich in grain to support their increased energy output. Some cows respond to that diet in a healthy manner, while approximately 26 % develop metabolic diseases including ruminal acidosis, obesity, diabetes type 2, and laminitis, costing the dairy cattle industry $ 1 billion annually through decreased milk production and the culling of diseased animals from production. There are similarities at the mechanistic and characteristic levels between cattle metabolic disease and type 2 diabetes in humans. The current study investigates the rumen and intestinal microbiome of the dairy cattle to better understand the interaction between metabolic disease and gut microbiome due to a shift to a high starch diet. We propose that the rumen and intestinal microbiome distinctly shift among dairy cattle fed a high starch diet and exhibit ruminal acidosis. A total of 6 rumen-cannulated dairy cattle were randomly assigned to a control diet (high fiber) group or treatment diet (high starch) group (N=3 for each). The current study conducted a multiphase experimental trial which was divided into two phases based on the switch to a high starch diet and the subsequent addition of periodic corn starch infusions. During the diet phase, cows were switched from a standard non-lactation (dry) diet to an experimental diet. Cows in the control group were fed a total mixed ration (TMR) consisting of corn silage, alfalfa silage, by product feeds high in neutral detergent soluble fiber and soybean meal, whereas cows in the treatment group were fed ground barley (20 % on a dry matter basis) which was top dressed in TMR. During the infusion phase, cows continued to receive their corresponding diets and experienced three infusion cycles, each consisting of a control (water) or treatment (corn starch) infusion into the abomasum twice daily for one week followed by three weeks of recovery (no infusions). The infusion phase cycles were followed by a final 14 -day infusion prior to euthanasia. Rumen fluid and feces were collected throughout the study, and intestinal contents (digesta and tissue) samples from

two small intestine (jejunum and ileum) and large intestine (cecum and colon) locations were collected at euthanasia to evaluate the intestinal bacterial community using a high-throughput 16S rRNA bacterial gene amplification and barcoding pipeline for Illumina MiSeq sequencing. In a companion study, rumen fluid and fecal samples were collected to determine pH and the concentrations of volatile fatty acid (VFA) and lactate. Gene expression analysis of pro-inflammatory cytokines was determined using real time PCR. Immune cell phenotype was determined using flow cytometer.

Rumen fluid microbial analysis showed that the treatment diet impacted rumen fluid-associated microbial communities during the infusion phase. A high starch diet reduced ($p < 0.01$) the diversity (richness and evenness) and shifted the composition of rumen-fluid associated microbial communities as indicated by the Kruskal Wallis results of alpha diversity (Shannon index and Pielou's Evenness) and the PERMANOVA results of beta-diversity (Unweighted-Unifrac and Weighted-Unifrac) metrics. At the family level, a high starch diet and periodic corn starch infusion increased the relative abundance of some taxa including Bifidobacteriaceae and Erysipelotrichaceae and decreased the proportion of Lactobacillaceae. There was no treatment effect based on rumen pH and VFA concentrations.

Fecal microbial analysis results showed that corn starch infusion significantly reduced ($p < 0.01$) the richness and evenness of fecal associated microbial community, and shifted their composition as indicated by the Kruskal Wallis results of alpha diversity (Shannon index, OTU numbers, and Pielou's Evenness) and beta-diversity (Unweighted-Unifrac and Weighted-Unifrac) metrics. The principal coordinate analysis (PCoA) results showed that the fecal-associated bacterial communities of the treatment group significantly shifted in each infusion week then drifted back toward the control community during the subsequent three recovery weeks. At the family level, corn starch infusion increased the relative abundance of some taxa including Succinivibrionaceae, Erysipelotrichaceae, Lachnospiraceae, Clostridiaceae 1, Prevotellaceae, Bacteroidales S24 - 7 group, and Bifidobacteriaceae and decreased the proportion of Spirochaetaceae, p – 2534 - 18B5 gut group, and unknown Bacteroidetes. Fecal pH level decreased ($p <$

0.01) in treatment cows compared to the control during infusion and recovery week 1. The concentration of acetic acid increased ($p < 0.01$) in treatment recovery week 1 group compared to control group whereas the concentration of butyric acid increased ($p < 0.01$) in treatment infusion week group compared to control group. The concentrations of propionic acid and lactic acid were not affected by starch infusion.

Intestinal microbial analysis at the conclusion of the experiment showed that the digesta-associated bacterial community distinctly clustered by location (small intestine and large intestine) whereas no diet influence on tissue-associated bacterial community. Cytokine expression levels and immune cell populations were not affected by treatment (barley diet and periodic corn starch infusion).

Collectively, our study demonstrates that high starch diet (barley diet and periodic corn starch infusion) did not result in rumen acidosis. However, high starch diet can lower the diversity and change the ruminal bacterial community composition over time. Also, corn starch infusion can temporarily lower fecal pH, increase acetic and butyric acids, reduce the diversity and change the structure of fecal-associated bacterial community. That suggests that corn starch infusion is a selective force that might open the door for diseases. Also, the study demonstrated that high starch diet had no effect on the bacterial diversity among digesta and tissue, or on pro-inflammatory cytokine expression and immune cell population. That suggests that the homeostasis in the intestinal luminal and mucosal immune system resists microbiome disturbances associated with starch challenge. Thus, the distinct variation in how different part of the gastrointestinal tract respond to the dietary challenge may provide the basis to further explore the ways in which the gut microbiome contributes to animal health or disease.

Chapter 1

GENERAL INTRODUCTION

The ruminant gastrointestinal tract (GIT) provides a habitat to a complex and diverse ecosystem of microbes known as the microbiome. The ecosystem of the rumen and hindgut tract is populated by a diverse community of anaerobic microorganisms, including bacteria, fungi, protozoa and archaea. The ruminal microbial population works in a symbiotic and dynamic relationship with its host to provide important metabolic capabilities such as the degradation of cellulose-rich feedstuffs (Mao et al. 2015; Kinross, Darzi, and Nicholson 2011; Tajima et al. 1999). Following the degradation of feedstuffs, a number of different types of metabolites are produced and released, such as glucoses, ethanol, methylamines, and volatile fatty acids (VFA), which are absorbed either through the rumen epithelium or in the lower gastrointestinal tract, enter the blood circulation, and can be utilized by the host for maintaining host health and productivity (E. Khafipour et al. 2016; Weston and Hogan 1968). In general, the rumen function is a result of a symbiotic relationship between the host and its diverse microbiota, which is highly responsive to starch diet (Tajima et al. 1999). The symbiotic microbiome of the hindgut continues the fermentation process and provides the host with vitamin such as vitamins B and K and short chain-chain fatty acids (Burkholder and McVeigh 1942).

Dairy cattle are part of an important industry worldwide and produce about 378 billion liters of milk per year (Helen Marie Golder 2013). To support energy output in an industrial milk production system, cows are fed more concentrate and less forage

and are typically switched from a diet high in fiber to a diet high in starch (Arachchige et al. 2013; Soriano, Polan, and Miller 2000; Mitchell et al. 2016). Some cows respond to that diet in a healthy manner, while approximately 26 % develop nutritional and metabolic diseases including ruminal acidosis, obesity, diabetes type 2, laminitis, ruminitis, and liver abscesses (Nagaraja and Titgemeyer 2007).

Metabolic and nutritional diseases attributed to a high starch diet have impacts on feed intake, rumen microbiome, and digestion and may cause a pro-inflammation response (Ehsan Khafipour et al. 2009; Plaizier et al. 2008). Moreover, there are distinctive parallels at mechanistic and characteristic levels between cattle metabolic disease and diabetes type 2 in humans. For example, the physiological changes observed in unhealthy cows suffering from hoof laminitis, a tissue inflammation of the dermal layers in the hoof that can lead to lameness, are similar to the physiological changes observed in foot ulcerations in humans with diabetes type 2 (Nocek 1997). Ruminal acidosis, characterized by ruminal pH levels below 5.8 and the accumulation of organic acids, VFAs and lactate, results from the inability of the cow to adapt to a diet rich in rapidly fermentable carbohydrate (Nagaraja and Titgemeyer 2007; Li et al. 2012). Ruminal acidosis is the most important nutritional disease in dairy cattle because its impacts cost the US livestock industry $ 1 billion annually (Mitchell et al. 2016). In extreme cases, it may cause high acid levels leading to the disruption of the rumen microbiome and the release of excess endotoxins and acids into blood circulation, eventually causing shock or death to the animal (Enemark 2008; Krause and Oetzel 2005).

Understanding the interaction between gastrointestinal microbiota and its host is vital to control the impact of metabolic disease. In humans, many studies provided

evidence that the gut microbiome, as well as other factors such as changes in the human genome, nutritional habits or physical activity reduction, contributed to the development of obesity and related diseases. For instance, studies conducted on obesity in the mice model demonstrated that germ-free mice had 40 % less body fat than those with normal gut microbiota suggesting that changes in the intestinal microbial community can open the door to cause obesity (Ley et al. 2005). Also, studies conducted on obesity in the humans revealed that gut microbiome composition has a vital role in developing metabolic disease (DiBaise et al. 2008). Moreover, studies conducted on metabolic disease in the dairy cow model demonstrated that changes in the diversity of gut microbiota coupled with subacute ruminal acidosis activated the pro-inflammatory response (Gozho, Krause, and Plaizier 2007). Other dairy cattle studies suggested that feeding an excessive amount of fermentable carbohydrate caused the accumulation of lipopolysaccharides (LPS), an endotoxin released from the outer cell membrane of Gram-negative bacteria. Translocation of LPS specifically in the hindgut may result in gut barrier damage and the initiation of an inflammatory response associated with the onset of obesity (Li et al. 2012; Rietschel et al. 1994; Gozho, Krause, and Plaizier 2007).

The development of ruminal acidosis and its interaction with the complex ruminal bacterial community must be deeply addressed in order to limit the impacts of ruminal acidosis. The development of high throughput sequencing and metagenomic techniques advance the knowledge of the ruminal bacterial community diversity, richness, and structure, allowing for the exploration of the association between ruminal microbiota and other information from the host (McSweeney et al. 2007).

The overall objective of the current study was to investigate the interaction between the complex gastrointestinal microbial communities and its potential role in the

development of ruminal acidosis in a cow model, allowing for extrapolation to the microbiome-metabolic disease interaction in humans. This study used high-throughput sequencing to perform a 16S rRNA-based comparison of the rumen and intestinal bacterial community between a high fiber diet (control) and high starch diet (treatment) intended to cause ruminal acidosis. Periodic infusions of corn starch directly into the abomasum were added in an infusion phase to mimic the breakthrough of starch from the rumen, intended to contribute to ruminal acidosis. Microbial taxonomic profiles were evaluated alongside physiological data collected as part of a companion study, including ruminal and fecal pH, concentrations of VFA, and relative gene expression of pro-inflammatory cytokines and immune cell phenotype in the small and large intestine. We hypothesized that gastrointestinal bacterial communities would show distinct shifts with diet-induced ruminal acidosis in dairy cattle.

Chapter 2

EVALUATION OF RUMEN AND INTESTINAL BACTERIAL COMMUNITIES IN DAIRY CATTLE IN RESPONSE TO A SHIFT IN HIGH STARCH DIET

2.1 Introduction

The ecosystem of the ruminal microbiota and hindgut tract is populated by a diverse community of anaerobic microorganisms, including bacteria, fungi, protozoa and archaea, which works in a symbiotic and dynamic relationship with its host to degrade foodstuffs and convert them into energy such as volatile fatty acids VFA and other metabolites to be utilized by the host for growth and productivity (Mao et al. 2015; Kinross, Darzi, and Nicholson 2011; Tajima et al. 1999).

During the standard milk production cycle in dairy cattle, energy output is greater than energy consumption. To compensate, cows are switched from a diet rich in fiber to a diet rich in starch to support milk production, maximize energy intake and achieve body weight gain (Ye et al. 2016). However, studies showed that not all cows respond to that diet in a healthy manner, and that approximately 26 % of dairy cows develop nutritional diseases such as ruminal acidosis which impact negatively cattle productivity (Nagaraja and Titgemeyer 2007; Li et al. 2012). Ruminal acidosis is a metabolic disease characterized by ruminal pH levels below 5.8 and the accumulation of organic acids (Nagaraja and Titgemeyer 2007; Li et al. 2012). It causes a number of related metabolic disorders such as laminitis, ruminitis, and liver abscesses (Nagaraja and Titgemeyer 2007). These diseases affect feed intake, rumen microbiome, and digestion and may cause a pro-inflammation response (Fukuda et al. 2011; Ehsan

Khafipour et al. 2009; Plaizier et al. 2008). Experimental evidence indicated that rumen microbiota composition had a positive impact on host energy homeostasis through functions such as carbohydrate and amino acid metabolism (Kinross, Darzi, and Nicholson 2011). Hence, dairy cattle are at risk of developing ruminal acidosis without a diverse microbial community or a sufficient amount of fibrous materials to sustain that community. The consumption of a high starch (grain) diet can disrupt rumen function through increasing fermentation process leading to accumulation of organic acids, low pH level and eventually may initiate the risk of ruminal acidosis (Tao et al. 2014). Also, a high amount of a readily fermentable carbohydrate can flow into the hindgut from the rumen resulting in reduced pH and increased concentrations of VFA in the hindgut, disrupting the intestinal bacterial community (R. M. Petri et al. 2013; Mao et al. 2012). Studies conducted on ruminants demonstrated that feeding an excessive amount of fermentable carbohydrate caused the accumulation of endotoxin lipopolysaccharides (LPS) released from the lysis of the outer cell membrane of Gram-negative bacteria in the gastrointestinal tract. High levels of LPS specifically in the hindgut can increase gut permeability leading to the translocation of LPS from the GIT into blood circulation, which may result in gut barrier damage and the initiation of an inflammatory response associated with the onset of obesity and insulin resistance (Li et al. 2012; Rietschel et al. 1994; Gozho, Krause, and Plaizier 2007).

Advances in high throughput sequencing and metagenomic techniques increase the knowledge of the diversity, richness, and structure of rumen and intestinal microbiota, providing the means to interpret the association between gastrointestinal microbiota and other information from the host (McSweeney et al. 2007). The objective of the current study was to investigate the interaction between the gastrointestinal

microbial community and their potential role in the development of ruminal acidosis due to a rapidly fermentable carbohydrate diet. We hypothesized that rumen and intestinal bacterial communities would show distinct shifts with diet-induced ruminal acidosis in dairy cattle. This study characterized the rumen and intestinal bacterial community resulting in a multiple phase acidosis challenge study and measured ruminal and fecal pH level, concentrations of VFA, and relative gene expression of pro-inflammatory cytokines and immune cell phenotype, eventually correlating microbial taxonomic profiles with biomarkers of metabolic disease progression in dairy cattle.

2.2 Material and methods

2.2.1 Animals

The multiple phase experimental study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Delaware (Newark, DE). The animal experiment was performed at the University of Delaware dairy farm of the College of Agriculture and Natural Resources from December 21, 2016 to May 26, 2017. A total of four primiparous and two multiparous ruminally cannulated Holstein cows were used in this experiment. The experiment was conducted using a complete randomized block design in which cows were blocked by parity, body weight and body condition score, were randomly assigned to two groups (control and treatment) each containing three animals and were housed in individual pens. All cows had free access to clean water. Using the Cornell-Penn-Miner (CPM-dairy) nutrition model, the rations were restricted and fed to an average of 3 % of body weight for cows to achieve weight gains of approximately 45 kg over the course of the experiment. Prior to the start of the experiment, cows were confirmed non-pregnant.

2.2.2 Experimental Design

The multiple phase experimental study was divided into two phases (Fig. 1)

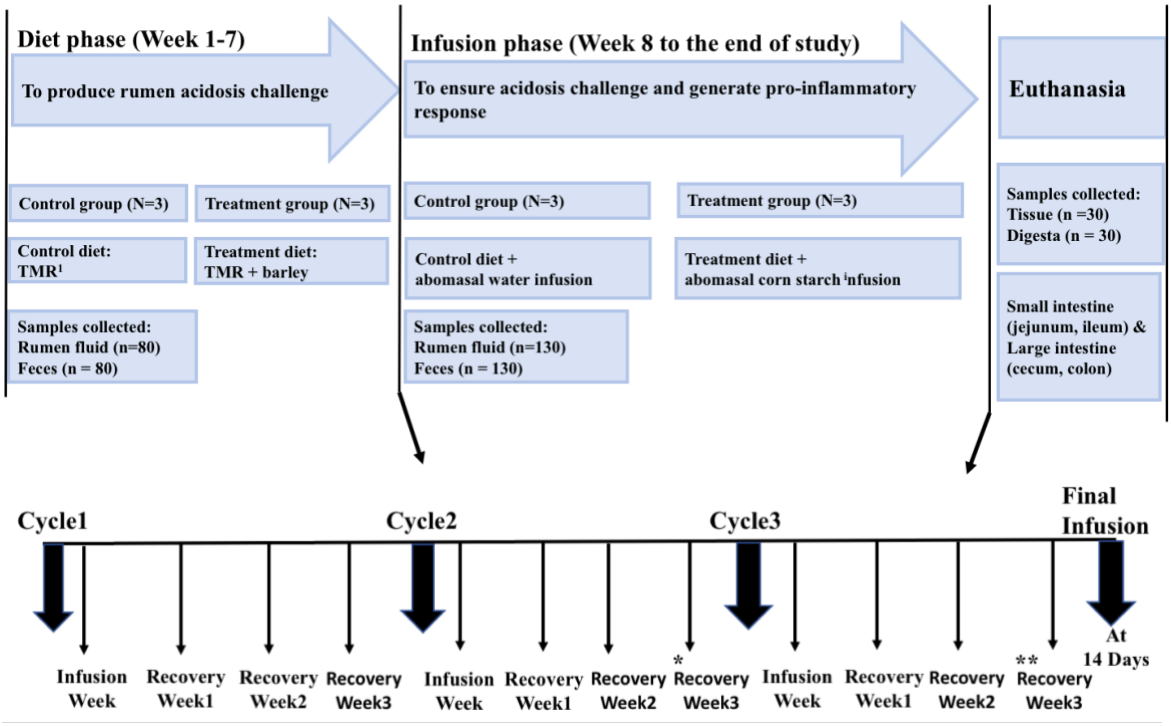

Figure 1: The multiple phase experimental study. In diet phase, control and treatment groups of dairy cattle fed control diet composed of total mixed ration (TMR) and treatment diet composed of 20 % ground barley on a dry matter basis which was top dressed in TMR, respectively. Cows fed once a day for 7 weeks. The infusion phase consisted of 3 cycles, each cycle composed of a one-week infusion period and a three-week recovery period, followed by a final 14 - day infusion period with twice daily infusions into the abomasum (the fourth stomach of a cow). Control and treatment groups continued their corresponding diet plus they infused directly into the abomasum with water (control) or corn starch (treatment), respectively. The amount of starch administrated was initially 1.5 g/ kg BW per day but this was increased during the experiment to 4 g/ kg BW per day. *: In the recovery week 3 of cycle 2, one of the treatment cows was lost due to accident. **: The recovery week 3 of cycle 3 is not one week because final infusion started at different day for cows. N: number of animals. n: number of samples.

2.2.2.1 Pre-Study

During the pre-study period, all cows were fed a dry diet containing fresh forage and hay in order to establish a baseline gut microbial community composition. During the one week prior to experimental start, fecal, rumen fluid and blood samples were collected three times approximately 6 hours after feeding.

2.2.2.2 Diet Phase Experimental Ration Study

The diet phase was conducted for seven weeks immediately following the pre-study preparation period. The cows were switched from the dry diet to an experimental control or treatment ration (Table 1). The control group was fed a diet composed of a total mixed ration (TMR) with no supplementation. The treatment group was fed a diet composed of 20 % ground barley on a dry matter basis which was top dressed in TMR. The treatment diet was expected to cause a low-grade inflammatory response in the rumen and increased carbohydrate flow into the hindgut. The animals were fed equal portions once a day at 0800.

The health of the cows was monitored throughout the phase and their body weight was measured on the first day of every week before feeding. Fecal, rumen fluid and blood samples were collected three times weekly during weeks 1 and 2, and once weekly during weeks 3 through 7.

Table 1: composition of the experimental control and treatment diet.

Ingredient	% ration DM[1](control)	% ration DM (treatment)
Corn silage	53.37	42.69
Alfalfa silage	20.74	16.32
Ground barley	-	20.00
Protected soybean meal[2]	8.44	6.75
Canola meal	5.88	4.70
Ground corn	3.98	3.18
Porcine blood meal	1.50	1.20
Sugar byproduct	1.42	1.13
Calcium carbonate	0.69	0.55
Sodium bicarbonate	0.66	0.52
Rumen bypass fat	0.53	0.42
Corn gluten meal	0.53	0.42
Sodium Chloride	0.51	0.40
Trace mineral and vitamin mix	0.49	0.39
Monensin[3]	0.40	0.32
Rumen protected methionine	0.10	0.08
Urea 281 CP	0.07	0.05
Vitamin E	0.06	0.04
AjiPro-L[4]	0.06	0.04
MagOx 56 %	0.01	0.008

[1]Percent dry matter basis.
[2]Extruded and expelled soybean meal.
[3]Rumensin 90 (Elanco, Greenfield, IN).
[4]AjiPro-L Generation 2 (Ajinomoto Heartland, Inc., Chicago, IL).

2.2.2.3 Infusion phase Starch Infusion Study

The infusion phase consisted of weeks 8 through study end (week 21 - 22; study length was different for each study animal). The infusion phase consisted of three cycles, each composed of a one-week infusion period in which cows were infused directly into the abomasum with water (control) or corn starch (treatment) twice daily for one week (infusion week), and a three - week recovery period (recovery week 1, recovery week 2, recovery week 3), followed by a final 14 - day infusion period with

twice daily infusions into the abomasum. To accommodate extensive sampling at study end (euthanasia), each cow experienced a different recovery period length in the infusion phase cycle 3 as the consistent 14 - day final infusion began on a different day per cow. The amount of corn starch administrated was initially 1.5 g/ kg body weight (BW) per day but this was increased during the experiment to 4 g/ kg BW per day when lower doses failed to change fecal consistency or decrease fecal pH level. Specifically, cows received 1.5 g/ kg BW of starch on days 1 - 2 of week 8, 2 g/ kg BW on days 3 - 7 of week 8, and 3 g/ kg BW on day 1 of week 12. For infusions beginning day 2 of week 12 through the end of the experiment, corn-starch was administered at 4 g/ kg BW. Corn-starch was suspended in 8 L of tap water per day. Control cows received the same volume of tap water as corn-starch infused cows.

The health of the cows was monitored throughout the phase and their body weight was measured on the first day of every week before feeding. Rumen fluid and fecal samples were collected approximately 6 hrs after feeding three times during the infusion week and one-week post-infusion (recovery week 1), one time weekly during the last two recovery weeks (recovery weeks 2 and 3), and five to seven times during the final infusion.

Abomasal infusion lines were placed in each cow through the ruminal cannula during week 7 and the lines remained in place for the remainder of the experiment. Abomasal infusion lines were constructed and inserted as previously described (Gressley et al, 2006). Approximately 5 feet of flexible tubing were attached to a flexible plastic disc, which was folded into an insertion device made of PVC pipe. The PVC pipe with disc was placed through the rumen and inserted into the abomasum. A second PVC pipe was used to push the disc into the abomasum. Once in the abomasum, the disc

unfolded to hold the infusion line in place. The other end of the infusion line was threaded through a hole in the rumen cannula plug and held in place with clamps. Lines were checked weekly to verify placement within the abomasum and were removed by hand before cow's euthanasia.

After the 14 - day final infusion, 100 ml of pentobarbital was administered through a jugular catheter prior to transport to the University of Pennsylvania New Bolton Center (Chester, PA) post-mortem room. Cows were transported on different dates for euthanasia and intestinal contents sampling. Intestinal tissue (unwashed and washed with ice-cold phosphate buffered saline, PBS), intestinal digesta and adipose samples from two small intestine (jejunum and ileum) and large intestine (cecum and colon) locations were collected.

2.2.3 Sample Collection

Location of sampling showed in (Fig. 2).

2.2.3.1 Rumen fluid samples

Rumen fluid samples were collected by inserting a 50 ml sterile conical centrifuge tube (Thermo Fisher Scientific, Waltham, MA, USA) with a sleeve-gloved hand through the ruminal cannula towards the center of the rumen. Rumen fluid samples were filtered through four layers of sterile cheesecloth to remove large particles and collected into duplicate 5 ml sterile cryovials (Thermo Fisher Scientific) clearly labelled with animal identification and date. Samples were transferred immediately to the lab for snap freezing in liquid nitrogen and stored at – 80 ° C.

2.2.3.2 Fecal samples

Fecal material was collected directly from the rectum of each cow with a sleeve-gloved hand and loaded into a 50 ml sterile beaker (Thermo Fisher Scientific). Duplicate samples were collected with a sterile spatula into 5 ml cryovials (Thermo Fisher Scientific) clearly labelled with animal identification and date. Samples were transferred immediately to the lab for snap freezing in liquid nitrogen and stored at - 80 ° C.

2.2.3.3 Intestinal samples

Intestinal contents (digesta and tissue) samples from two small intestine (jejunum and ileum) and large intestine (cecum, colon) locations were collected from each euthanized cow using sterile blades. A single digesta sample was collected into a 15 ml wide-mouthed cryovial (Thermo Fisher Scientific). The remaining intestinal tissue was divided into two 2 x 4cm sections. One section (unwashed tissue) was placed into a 15 ml wide-mouthed cyrovial (Thermo Fisher Scientific), and the second section (washed tissue) was washed with ice-cold PBS and placed into a 15 ml wide - mouthed cyrovial. Samples were immediately snap frozen in liquid nitrogen and transferred to the lab for storage at – 80 ° C.

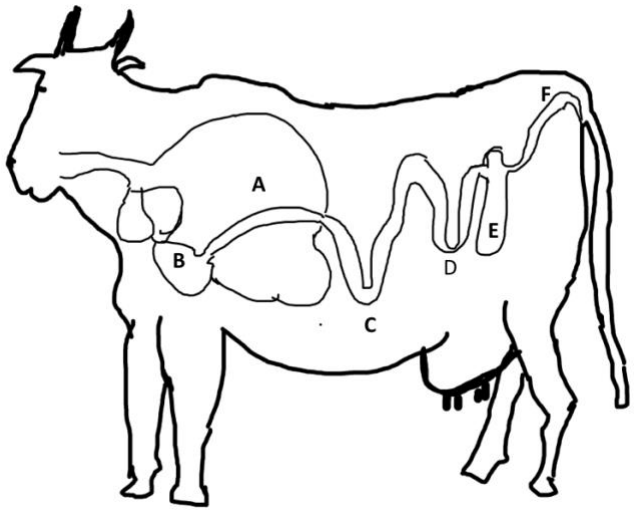

Figure 2: Showed abomasum location (B) and sampling locations among the gastrointestinal tracts in dairy cattle: (A): rumen; (C): jejunum; (D): ileum; (E): cecum; (F): colon.

2.2.4 Physiological parameters measurements

Rumen fluid and fecal pH were measured by a portable pH - meter (Yorba Linda, CA, USA). The levels of VFA and lactate levels were measured by high - performance liquid chromatography (HPLC) (Shimadzu Scientific Instruments, Columbia, MD, USA) with an Aminex HPX - 87H column (Bio-Rad Laboratories, Hercules, CA, USA). Marker gene expression analysis of pro-inflammatory cytokines was determined by real time PCR (Thermo Fisher Scientific). Immune cell phenotype was determined by flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA).

2.2.5 Microbial Community Processing

Approximately 1 ml fluid samples (rumen), 0.15 g of solid samples (fecal and digesta) or 0.015 g of unwashed tissue was used for DNA extraction. The process was

performed using a MoBio PowerMag Microbiome RNA/ DNA Isolation Kit (Qiagen, Hilden, Germany) using WeLLevator (Qiagen) protocol modifications and then quantified with a Quant-iT^{TM} dsDNA High-Sensitivity Assay Kit (Invitrogen, Carlsbad, CA, United States). Solid samples (fecal or tissue) were placed in a 0.1 mm Glass Bead Tube (Qiagen) with 350 ul of warmed lysis buffer and homogenized using a Tissue-Lyzer II (Qiagen) for 20 mins. The tubes were centrifuged at 10,000 x g for 1 min at ambient temperature and the supernatant was transferred to a Deep Well Plate to follow the rest of the procedures. Fluid samples (rumen fluid, fecal, digesta, and tissue supernatant) were centrifuged at 10,000 x g for 10 min at ambient temperature, the supernatant was discarded, and the remaining pellet was re-suspended in 650 ul of lysis solution before being transferred into a Deep Well Plate to follow the rest of the procedures.

The WeLLevator protocol (https://mobio.com/media/wysiwyg/pdfs/protocols/11970.pdf) was used to process 450 uL of the remaining solution.

2.2.6 PCR Amplification and Illumina MiSeq Sequencing

The primers used to target the V3 - V4 region of the bacterial 16S ribosomal RNA (16S rRNA) gene amplification were 16S Amplicon PCR Illumina Forward Primer 341 (5’TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 16S Amplicon PCR Illumina Reverse Primer 806 (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) with an amplicon length of approximately 465 base pairs (bp). The full primer (oligo) structure included the gene targeting, overhang, and Ilumina adapter regions. The amplification process included initial denaturation at 95 ° C for 3 min, 25 cycles of denaturation at 95 ° C for 30 s, annealing at 55 ° C for 30 s, elongation at

72 ° C for 30 s, and final extension at 72 ° C for 5 min. The 2ul of PCR products were electrophoresed on 1.3 % agarose gels to confirm target production amplification. Purification of the remaining PCR product was carried out utilizing Agencourt AMPure XP beads (Brea, CA, USA). The quantification of amplicons was performed using the Quant-iT^{TM} dsDNA High-Sensitivity Assay Kit. The amplicons from all samples were utilized to construct multiple sequencing libraries with Illumina Nextera XT Index Kits (Illumina, San Diego, CA United States). Paired-end 2 x 351 bp sequencing was performed to sequence all libraries on an Illumina MiSeq instrument (Caporaso et al. 2012).

The raw sequence data from the Illumina MiSeq platform were analyzed using Quantitative Insights into Microbial Ecology (QIIME2; version 2018.2.0) software package (Caporaso et al. 2010). Based on 8 bp sample-specific barcodes, the 16S rRNA reads were demultiplexed (through dividing the sequence reads into separate fastq files for each barcode/ sample required for downstream analysis) and high-quality sequences were chosen. The Quality filters is utilized to choose sequences with about 465 bp length. The sequences were clustered by 100 % sequence identity into unique sequence variants using dada2 which includes quality control method designed for detecting and filtering chimeric sequences and any phiX 174 reads used for MiSeq calibration that are identified in the sequencing reads. Trim and truncate parameters used through dada2 to perform the quality filtering (Callahan et al. 2016). All the representative sequences put in the artifact to be analyzed. Taxonomy was assigned to each cluster based on homology of the representative sequence to the Silva-132 (Quast et al. 2013) reference database. FastTree (Price, Dehal, and Arkin 2010) was utilized to generate a rooted phylogenetic tree of the representative sequences for phylogenetic diversity analyses.

The statistical significance of biodiversity differences was analyzed by PERMANOVA or Kruskal-Wallis for non-parametric data. Alpha-diversity metrics including the Shannon index, Pielou's Evenness, and observed OTUs, were utilized to estimate the richness, evenness and the number of OTUs of each community (Kruskal and Wallis 1952). Beta-diversity metrics including unweighted Unifrac and weighted Unifrac were utilized to compare the abundance and structure of the microbial community (Swenson 2011). A principal coordinate analysis (PCoA) based- unweighted Unifrac and weighted Unifrac plots were utilized to visualize the dissimilarities among the samples. Taxonomic composition of the samples was assigned using Silva-based 16S classifier. Deferential abundance test was run through R-studio using DEseq2 packages to detect differentially abundance of OTUs. Log2 Fold Change cutoff of 1/ -1 was a criteria which was used for greater/lesser abundance of taxa, meaning the taxa with Log2 Fold Change above 1 is present significantly in treatment group whereas taxa with Log2 Fold Change below -1 is present significantly in control group. One-way ANOVA with pairwise comparison (Orosa 2011) in VassarStat website or the two - tailed t test was run to perform statistical computation on data of pH value and concentration of VFAs between control and treatment groups. Multiple correlation analyses were assessed by Spearmen correlation test through QIIME2. Results were considered statistically significant when $p < 0.01$.

2.3 Results

To investigate the effects of a barley diet and periodic corn starch infusion challenge on rumen and intestinal microbial community of dairy cattle, a multiple phase experimental trial was conducted comparing the rumen and fecal microbial communities of a high fiber diet (control) and high starch diet (treatment) to promote

ruminal acidosis. The initial 7 - week diet phase was followed by an 11- to 12 - week infusion phase consisting of three 4 - week cycles (Fig. 1). Each cycle included an infusion week in which cows were periodically infused into abomasum with water (control) and corn starch (treatment) to promote the breakthrough of starch into the intestinal tract and three recovery weeks. To accommodate the extensive sampling at euthanasia, recovery in cycle 3 varied among cows. A staggered - start 14 - day final infusion preceded study end (euthanasia).

Rumen and fecal samples were collected one to three times weekly during the experiment. Intestinal tissue and digesta samples were collected at euthanasia. The microbial community of rumen fluid, fecal, and intestinal tissue and digesta was assessed through 16S rRNA gene V3-V4 region amplicons sequenced on the Ilumina MiSeq system. Because of the distinct microbial community of each sample type, rumen fluid, fecal, intestinal tissue, and intestinal digesta samples were analyzed separately through QIIME2 pipeline for identifying the microbial community. Rumen fluid and fecal pH level, rumen-fluid and fecal VFA and lactic acid concentrations, and gene expression of pro-inflammatory cytokines and immune cell phenotype in the small and large intestine of tissue were measured by companion study. An experimental anomaly of note occurred during the study as cow 190 in the treatment group died of a broken leg during the second half of the study (infusion week of cycle 3). As it was impossible to replace this cow, we continued with 5 cows (3 cows in control group and 2 cows in the treatment group) from that point forward. Also, of note, is that cow 188 in the control group went out of her pen for one night (week 20) and ate 16 lbs of barley. We kept it during the study for keeping the size number as high as possible, months of data of this cow were collected and we ended up analyzing data as a pool of control cows and a pool

of treated cows. There was not any obvious shift in the rumen or fecal microbial community of cow 188 after consuming barley based on principal coordinate analysis (PCoA) plots based on unweighted Unifrac and weighted Unifrac metrics and through the results of PERMANOVA tests.

Rarefaction curves, used to estimate the depth of coverage of diversity of bacterial communities, showed that observed OTUs in rumen, fecal, and intestinal contents samples approached a plateau, suggesting that sequencing depth was sufficient to detect the majority of bacteria present in each sample. Figure 3 showed one of the rarefaction plot representatives for the rumen fluid-associated microbiota across during diet phase.

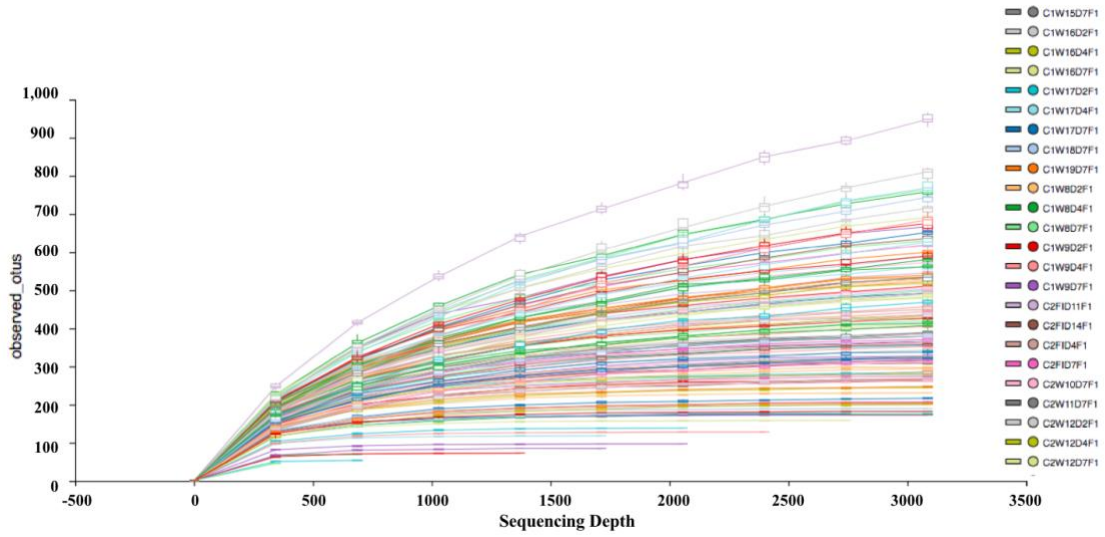

Figure 3: The rarefaction curves of observed OTUs in the rumen fluid-associated microbiota across the control and treatment groups during the diet phase showed that the rarefaction curves approached a plateau, suggesting that sequencing depth was sufficient to detect the majority of bacteria present in each sample.

2.3.1 Rumen Fluid-Associated Bacterial Community during diet and infusion phases

The beta diversity of rumen fluid-associated bacterial communities was significantly ($p < 0.01$) different between the diet and infusion phases, as shown in the principal coordinate analysis (PCoA) plots based on unweighted Unifrac (Fig. 4) and weighted Unifrac metrics and through the results of PERMANOVA tests. The diversity (richness and evenness) of the bacterial community was significantly ($p < 0.01$) lower in the diet phase than in the infusion phase for both the control and treatment based on the Kruskal Wallis results of alpha diversity metrics including the Shannon index (Fig. 5) and Pielou's Evenness.

Since there was a difference between phases, the diet phase and infusion phase were analyzed separately to understand whether the high grain diet (barley diet and periodic corn starch infusion) had an effect on the ruminal bacterial community.

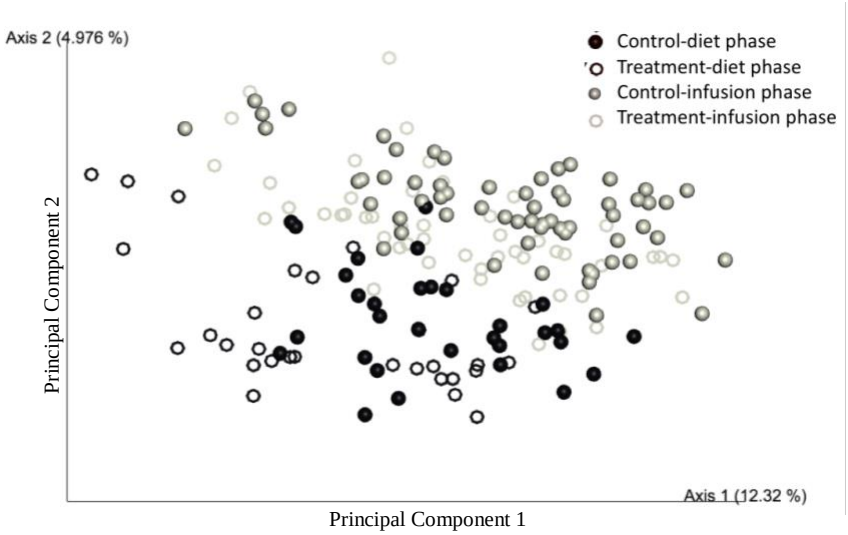

Figure 4: Principal coordinate analysis (PCoA) plot based on Unweighted UniFrac metric showed the beta diversity of microbial community from rumen fluid samples between the diet phase and infusion phase. There was a separation between the diet and infusion phases along principal component 2 which describes 4.976 % of the variability in the bacterial community.

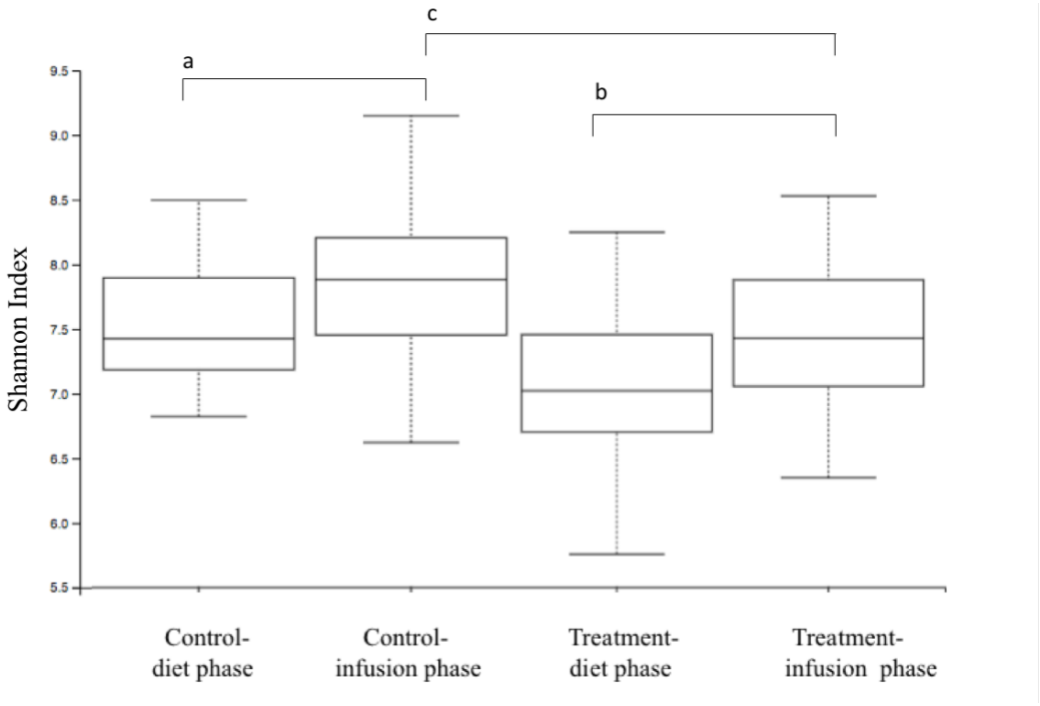

Figure 5: Shannon index metric boxplot showed the alpha-diversity of microbial community from rumen fluid samples between the diet phase and infusion phase. The diversity (richness and evenness) of bacterial community was significantly ($p < 0.01$) lower between the (a) control group of diet phase and control group of the infusion phase, (b) treatment group of diet phase and treatment group of infusion phase, and (c) treatment and control groups in the infusion phase based on the Kruskal Wallis test.

2.3.2 Rumen Fluid-Associated Bacterial Community during diet phase

The beta diversity of rumen fluid-associated bacterial communities during the diet phase showed that there was not a separation or a significant difference between control and treatment groups, as shown in PCoA plots based on unweighted Unifrac metric and weighted Unifrac metric (Fig. 6) and through the results of PERMANOVA tests. Also, there was no significant difference in richness, evenness, and number of OTUs of bacterial communities between the control and the treatment groups' during

the diet phase as indicated by the Kruskal Wallis results of alpha diversity metrics including the Shannon index, Pielou's Evenness, and OTUs.

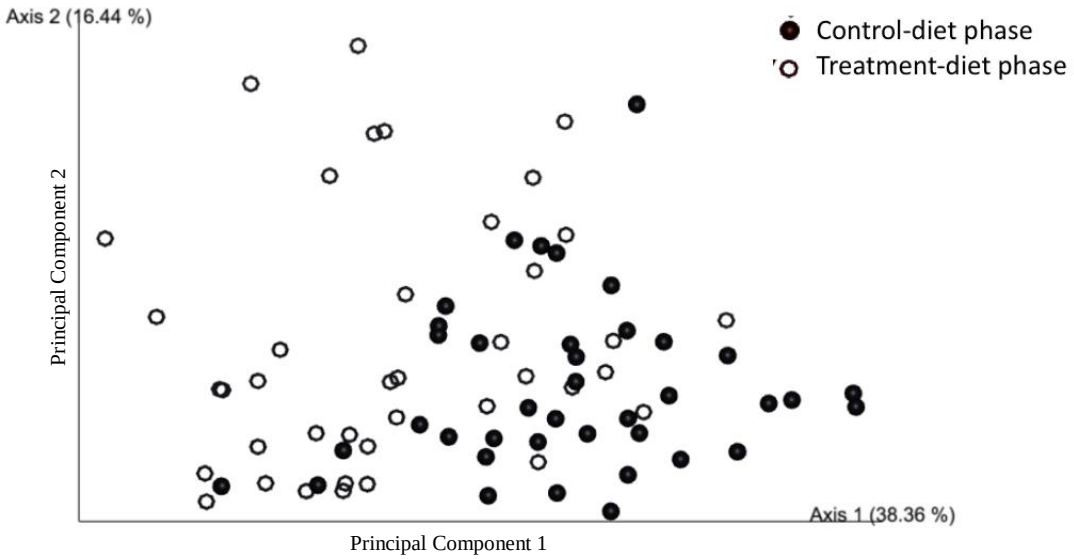

Figure 6: Principal coordinate analysis (PCoA) plot based on Weighted UniFrac metric showed the beta diversity of microbial community from rumen fluid samples between the control and treatment groups during the diet phase. There was no separation between groups.

2.3.3 Rumen Fluid-Associated Bacterial Community during infusion phase

The rumen fluid-associated bacterial communities between the control and treatment groups were tested during the infusion phase. The beta diversity of rumen fluid-associated bacterial communities showed a clear separation, as shown in PCoA plots based on unweighted Unifrac (Fig. 7) and weighted Unifrac metrics. There was a significant difference ($p < 0.01$) in the composition and abundance of bacterial community between the control and treatment groups, as shown through the PERMANOVA tests of unweighted Unifrac (Fig. 8) and weighted Unifrac metrics.

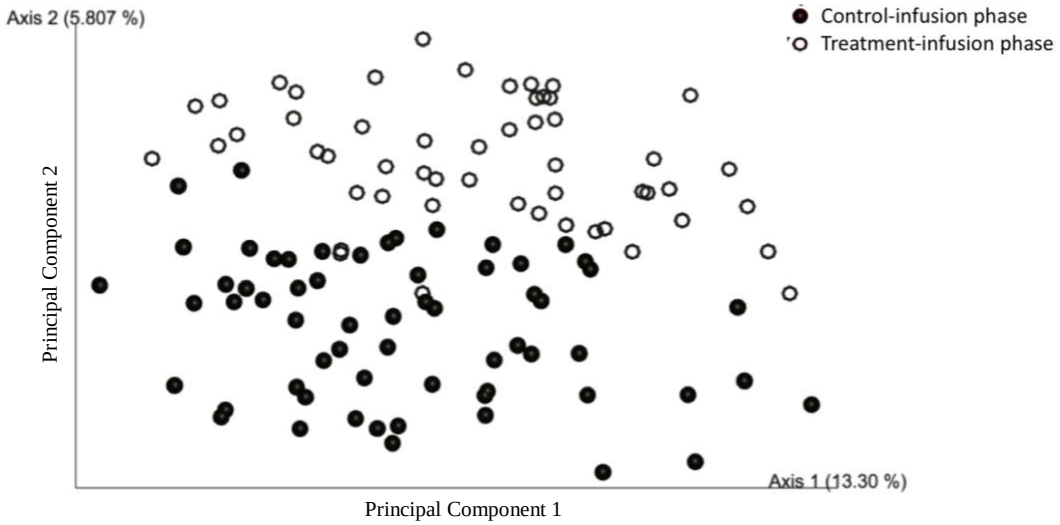

Figure 7: Principal coordinate analysis (PCoA) plot based on Unweighted UniFrac metric showed the beta-diversity of rumen fluid-associated bacterial communities between the control and treatment groups during the infusion phase. There was a separation between groups of the infusion phase along principal component 2 which describes 5.807 % of the variability in the bacterial community.

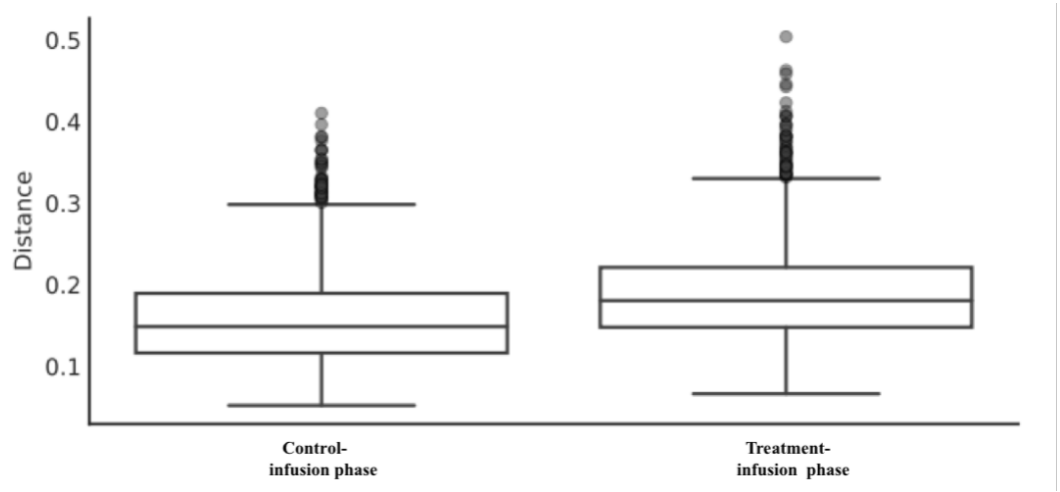

Figure 8: Weighted Unifrac metric boxplot showed beta-diversity of rumen fluid associated-microbial community between the control and treatment groups during the infusion phase. There was a significant difference ($p < 0.01$) in the abundance of bacterial communities between the control and treatment groups during the infusion phase based on the PERMANOVA test.

The bacterial communities in the treatment group distributed less evenly ($p < 0.01$) than those in the control group as well as the diversity (richness and evenness) of bacterial community in the treatment group was significantly lower ($p < 0.01$) than those in the control group based on the results of Kruskal Wallis tests of alpha diversity metrics including Pielou's Evenness (Fig. 9) and Shannon index, respectively.

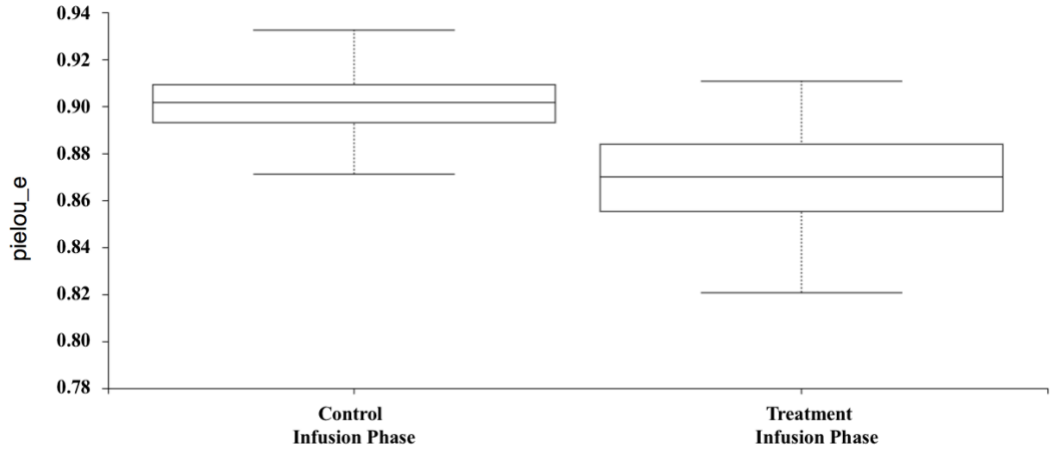

Figure 9: Pielou's Evenness metric boxplot showed the alpha-diversity of rumen fluid associated-microbial community between the control and treatment groups during the infusion phase. Evenness of bacterial community was distributed significantly lower ($p < 0.01$) among treatment group compared to that in the control group during infusion phase based on the Kruskal Wallis test.

Additionally, the rumen fluid-associated bacterial communities between the groups (control and treatment) were tested by cycle number during the infusion phase. The beta-diversity of rumen fluid-associated bacterial communities in the treatment group had varied by cycle number. The bacterial community had the same exact pattern that was in the treatment groups across the control groups during the infusion phase as shown in PCoA plots based on unweighted Unifrac metric (Fig. 10) and weighted

Unifrac metric. However, statistical significance was found in the differences in cycle number 1, cycle number 3, and in the final infusion of the control group. Similarly, statistical significance was found in the differences in cycle number 1, cycle number 3, and in the final infusion of the treatment group. These statistical significances were indicated by the PERMANOVA results of beta diversity metrics including unweighted Unifrac and weighted Unifrac as well as by the Kruskal Wallis results of alpha diversity metrics including the Shannon index and Pielou's Evenness.

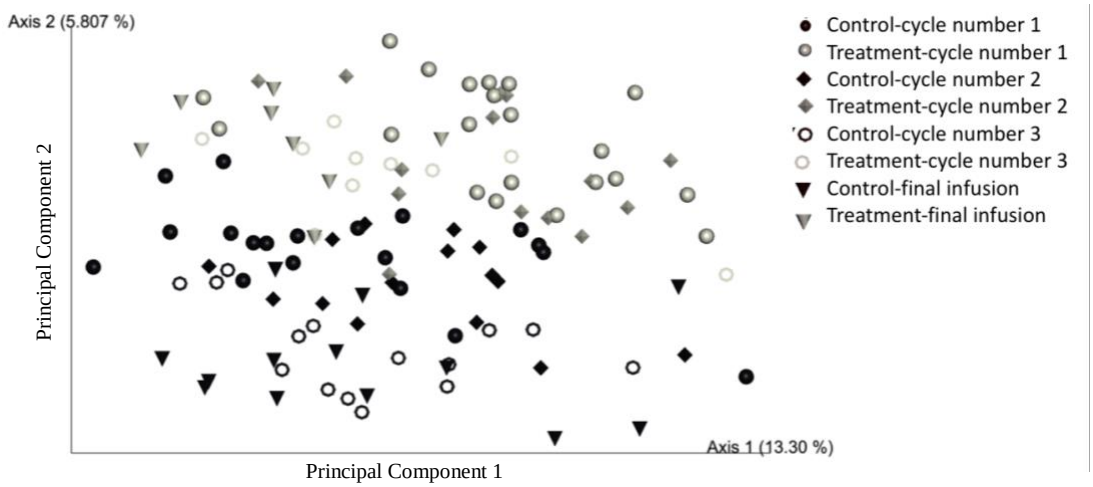

Figure 10: Principal coordinate analysis (PCoA) plot based on Unweighted UniFrac metric showed the beta-diversity of rumen fluid-associated bacterial communities across control and treatment groups of cycle number during infusion phase. The bacterial communities differed by cycle number across the treatment groups as well as the bacterial communities varied by cycle number across the control groups during the infusion phase along principal component 2 which describes 5.807 % of the variability in the bacterial community.

To understand how microbial communities between the control and treatment groups during the infusion phase were affected over time, the number of OTUs among the control and treatment groups was evaluated. Out of the 9220 OTUs identified in

the rumen fluid-associated bacterial communities during the infusion phase, a relative abundance (ratio of OTUs abundance to the total OTUs abundance) cutoff of 1 % was used for a robust comparison between the groups. Of the 157 OTUs that passed the 1 % cutoff, 118 (75.2 %) OTUs were common among all the bacterial communities, 23 (14.6 %) OTUs belonged only to the control group, 16 (10.2 %) OTUs belonged only to the treatment group (Fig. 11).

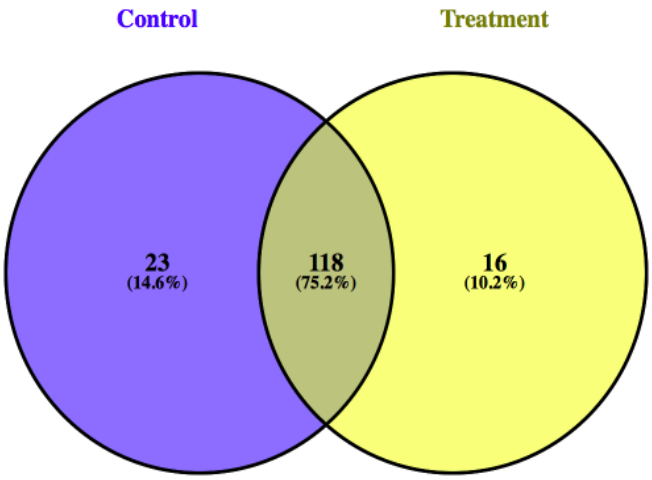

Figure 11: Venn diagram showing the number of observed operational taxonomic units (OTU) shared across the rumen fluid-associated bacterial communities' control and treatment groups during the infusion phase. The majority of OTU were shared among the two groups.

2.3.4 Composition of Rumen-Associated Bacterial Community during Infusion Phase

In the current study, a total of 2,224,664 quality 16S rRNA gene sequences were produced from 130 rumen fluid samples across the control and the treatment groups of the infusion phase with a mean sequence number of 17,112 per sample.

At the family level, 18 taxa with a relative abundance ≥ 1 % in at least one group between the control and treatment groups during the infusion phase were detected as the common families. The barley diet and periodic corn starch infusion resulted in a log2 fold change in the abundance of 3 families between the control and treatment groups (Fig. 12). Of them, Erysipelotrichaceae and Bifidobacteriaceae were more abundant in the treatment group, whereas Lactobacillaceae was more abundant in the control group during the infusion phase.

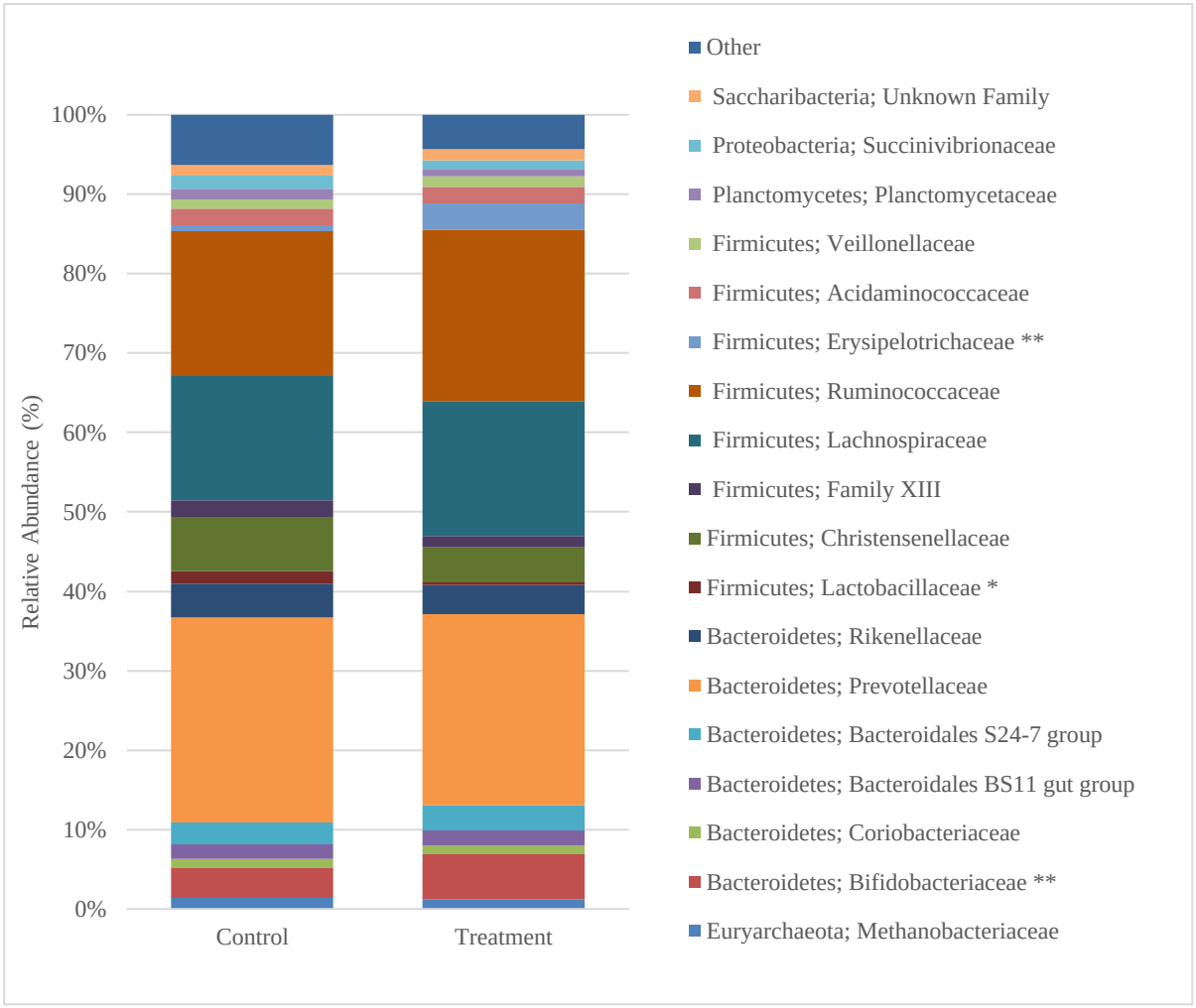

Figure 12: Distribution of rumen fluid bacterial taxonomy. Average relative abundance based on distribution of bacterial family as a percentage of the total number of classified 16S rRNA sequences. Samples are grouped based on the control group and treatment groups during the infusion phase. families with ≥ 1 % in at least one group were shown. Families with < 1 % were aggregated as "other". (**) indicates the families that present more abundantly among treatment group, whereas (*) indicates the families that present more abundantly among control group during infusion phase based on Log2 Fold Change test. The cutoff of 1/ -1 was a criteria which was used for greater/ lesser abundance of families.

2.3.5 pH level and Concentrations of VFA and Lactate in the Rumen Fluid Samples during diet and infusion phases

The results of rumen fluid pH level showed that there was not a significant difference between the control and treatment groups during the diet phase or infusion phase. The results of rumen fluid concentration of VFA showed that there was not a difference between the control and treatment groups during the diet phase. However, the concentrations of butyric acid and lactic acid tended to be higher ($p = 0.08$, $p = 0.02$), respectively, in the treatment group compared to those in the control group during the infusion phase. The concentrations of acetic acid and propionic acid were not affected by starch infusion (Table 2).

Table 2: pH level and concentrations of volatile fatty acid (VFA) in the rumen fluid samples between the control and treatment groups during the diet phase and infusion phase.

	Diet phase			Infusion phase		
	Control	Treatment	p - value[a]	Control	Treatment	p - value
pH	6.06	6.13	0.31	6.21	6.15	0.33
VFA (mmol/L)	Control	Treatment	p-value	Control	Treatment	p-value
Acetic acid	31.09	28.80	0.53	40.71	42.58	0.51
Butyric acid	8.52	7.37	0.92	8.84	10.12	0.08
Propionic acid	10.38	8.86	0.86	11.52	10.83	0.40
Lactic acid	0.002	0.004	0.32	0.05	0.19	0.02

[a] p - values generated with t - test; significant differences when $p < 0.01$.

2.3.6 Fecal-Associated Bacterial Community during Diet and Infusion Phases

There was a separation with some overlapping between the beta diversity of fecal-associated bacterial communities of the diet and the infusion phases, as shown in

PCoA plots based on unweighted Unifrac (Fig. 13) and weighted Unifrac metrics. There was a significant ($p < 0.01$) difference between the beta diversity of fecal-associated bacterial communities of the diet and the infusion phases, as indicated by PERMANOVA tests of unweighted Unifrac (Fig. 14) and weighted Unifrac metrics. The diversity (richness, evenness, and number of OTUs) of the microbial community was significantly ($p < 0.01$) lower in the infusion phase than that of the diet phase for the treatment group based on the Kruskal Wallis results of alpha diversity metrics including the Shannon index (Fig. 15), Pielou's Evenness, and OTUs.

Since there was a separation between the infusion phase and diet phase with some overlapping between phases as well as a significant difference in the diversity, each phase was analyzed separately in order to understand how microbial communities respond to high starch diet in both phases and to figure out the overlapping trend that occurred between phases.

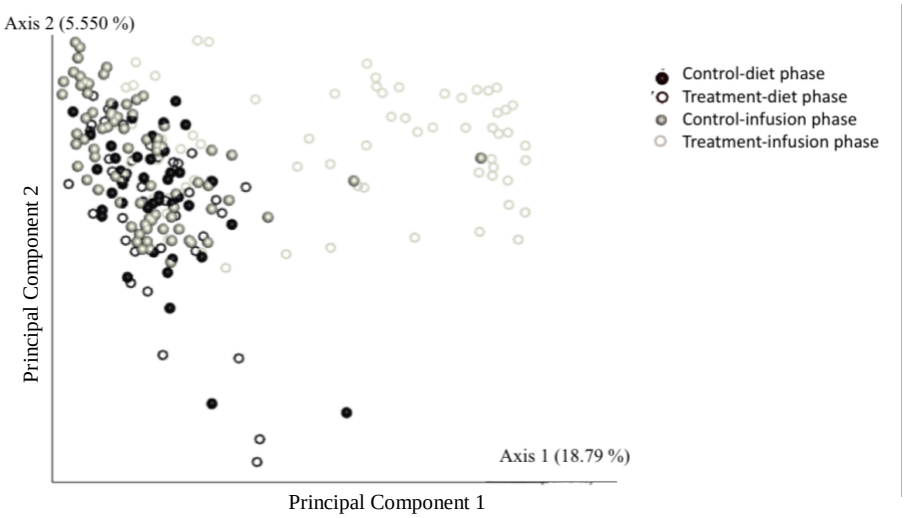

Figure 13: Principal coordinate analysis (PCoA) plot based on Unweighted UniFrac metric showed the beta diversity of microbial community from fecal samples between the diet phase and infusion phase. There was a separation with some overlapping between bacterial community of diet and infusion phases along principal component 1 which describes 18.79 % of the variability in the bacterial community.

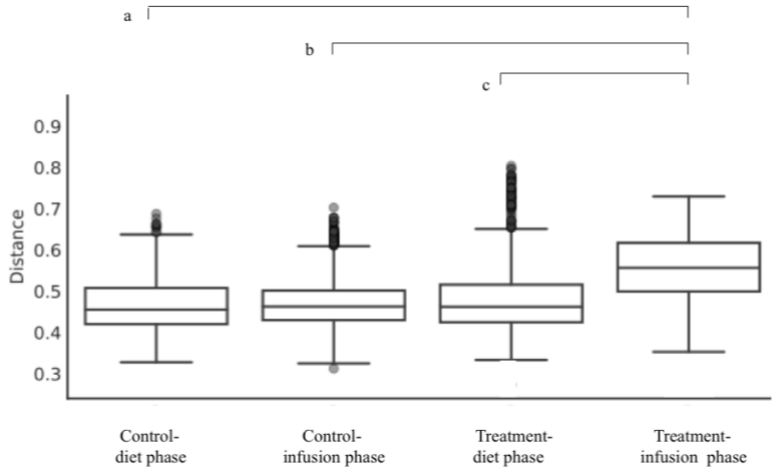

Figure 14: Unweighted Unifrac metric boxplot showed the beta - diversity of fecal associated - microbial community across groups (control and treatment) of the diet phase and infusion phase. The composition of bacterial community in treatment group of infusion phase was significantly ($p < 0.01$) different than that in (a) control group of diet phase, (b) control of infusion phase, and (c) treatment of diet phase based on the PERMANOVA test.

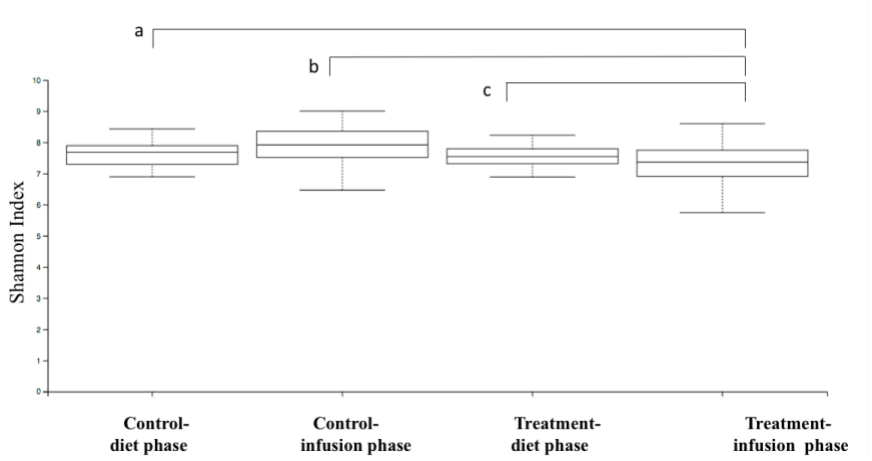

Figure 15: Shannon index metric boxplot showed the alpha-diversity of fecal associated - microbial community across groups (control and treatment) of the diet phase and infusion phase. The richness and evenness of bacterial community was significantly ($p < 0.01$) lower in (a) treatment group of infusion phase than that in control group of diet phase, (b) treatment of infusion phase than that in control of infusion phase, and (c) treatment of infusion phase than that in control of diet phase based on the Kruskal Wallis test.

2.3.7 Fecal-Associated Bacterial Community during Diet Phase

There was neither a separation nor a significant difference between the beta diversity of fecal-associated bacterial communities of the control and treatment groups during the diet phase as shown in PCoA plots based on unweighted Unifrac metric (Fig. 16) and weighted Unfrac metric and through PERMANOVA tests. Also, there was not a significant difference in richness, evenness, and number of OTUs of bacterial communities between the control and treatment groups during the diet phase as indicated by the Kruskal Wallis results of alpha diversity metrics including the Shannon index, Pielou's Evenness, and OTUs.

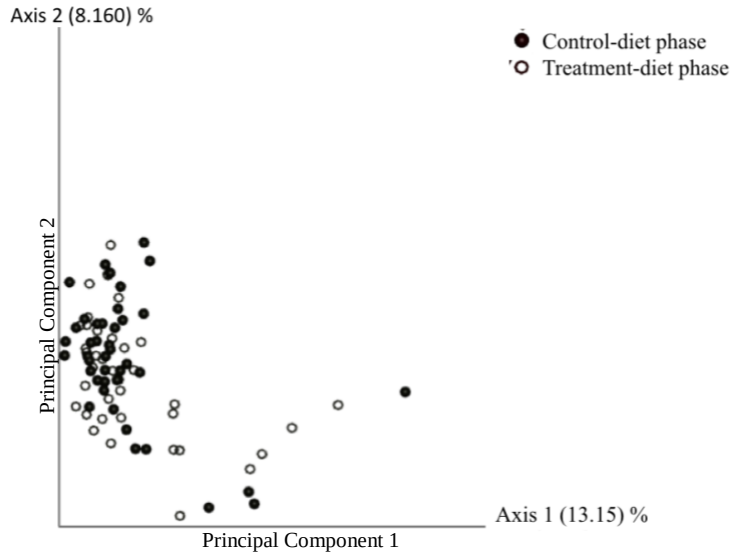

Figure 16: Principal coordinate analysis (PCoA) plot based on Unweighted UniFrac metric showed the beta diversity of microbial community from fecal samples between control and treatment groups during the diet phase. Both groups were aggregated together.

2.3.8 Fecal-Associated Bacterial Community during Infusion Phase

The fecal-associated bacterial communities separated with some overlapping between control and treatment groups in terms of cycle week during the infusion phase, as shown in PCoA plots based on unweighted Unifrac (Fig. 17) and weighted Unifrac metrics.

The bacterial communities of the control groups, including control infusion week (C - I), control recovery week 1 (C - R1), control recovery week 2 (C - R2), and control recovery week 3 (C - R3) were clustered together. The bacterial communities of the treatment infusion week (T - I) independently clustered and then they drifted back toward the control community during recovery week 1 (T - R1), whereas they returned to reflect the control community during treatment recovery week 2 (T - R2) and treatment recovery weeks 3 (T - R3).

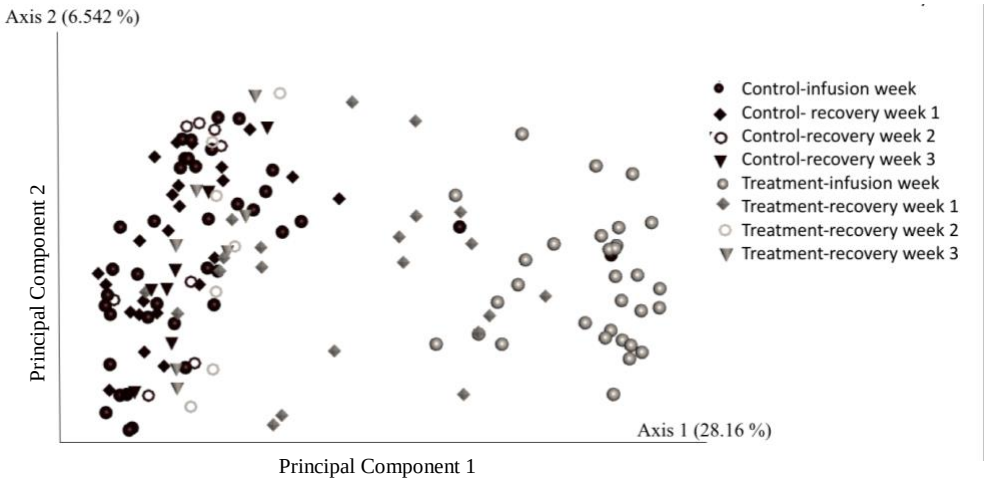

Figure 17: Principal coordinate analysis (PCoA) plot based on Unweighted UniFrac metric showed the beta-diversity of fecal - associated bacterial communities between treatment and control groups in terms of cycle week during the infusion phase. The bacterial communities of the control group clustered together. The bacterial communities of the treatment infusion week (T - I) independently clustered. Then they drifted back toward the control community during treatment recovery week 1 (T - R1), then they returned to the control community during treatment recovery week 2 (T - R2) and treatment recovery weeks 3 (T - R3) along principal component 1 which describes 28.16 % of the variability in the bacterial community.

There was no significant difference in the composition and abundance of the bacterial community across the cycle weeks of the control groups based on the results of the PERMANOVA tests of beta diversity metrics, including unweighted Unifrac and weighted Unifrac metrics. Also, there was no significant difference in the richness, evenness, number of OTUs, of bacterial communities across the cycle weeks of the control group as indicated by the Kruskal Wallis results of alpha diversity metrics including the Shannon index, Pielou's Evenness, and OTUs. Therefore, all control samples were pooled for subsequent analysis.

The composition and abundance of fecal-associated bacterial community were significantly dissimilar ($p < 0.01$) between the following groups: the control group and the treatment infusion week (T -I), the control group and treatment recovery week 1 (T - R1), the treatment infusion week (T - I) and the treatment recovery week 1 (T- R1), the treatment infusion week (T - I) and the treatment recovery week 2 (T - R2), the treatment infusion week (T - I) and the treatment recovery week 3 (T - R3). However, there is no significant difference in the composition of the bacterial community between treatment recovery week 2 (T - R2) and treatment recovery week 3 (T - R3) as indicated by the PERMANOVA results of beta diversity metrics including unweighted Unifrac (Fig. 18) and weighted Unifrac.

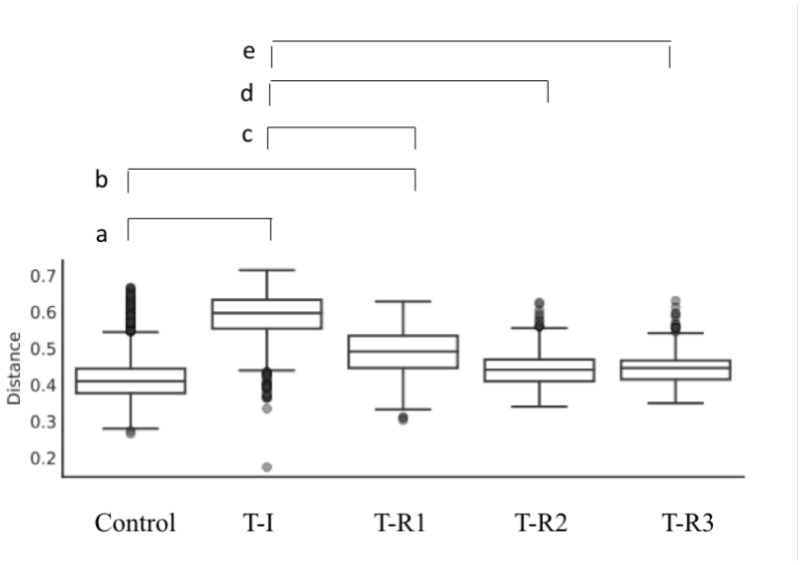

Figure 18: Unweighted Unifrac metric boxplot showed the beta - diversity of fecal associated - microbial community across control and treatment groups during the infusion phase. Composition of bacterial communities was significantly ($p < 0.01$) different between (a) the control group and treatment infusion week (T - I), (b) the control group and treatment recovery week 1 (T - R1), (c) the treatment infusion week (T - I) and treatment recovery weeks 1 (T - R1), (d) treatment infusion week (T - I) and treatment recovery weeks 2 (T - R2), (e) the treatment infusion week (T - I) and treatment recovery weeks 3 (T - R3) based on the PERMANOVA test.

The richness, evenness, and OTU numbers of bacterial community were significantly lower ($p < 0.01$) between the following: the treatment infusion week (T - I) and the control group, the treatment recovery week 1 (T - R1) and the control group, the treatment infusion week (T - I) and the treatment recovery week 1 (T - R1), the treatment infusion week (T - I) and the treatment recovery week 2 (T - R2), the treatment infusion week (T - I) and the treatment recovery week 3 (T - R3) as indicated by the Kruskal Wallis results of the Shannon index (Fig. 19), Pielou's Evenness, and OTUs metrics.

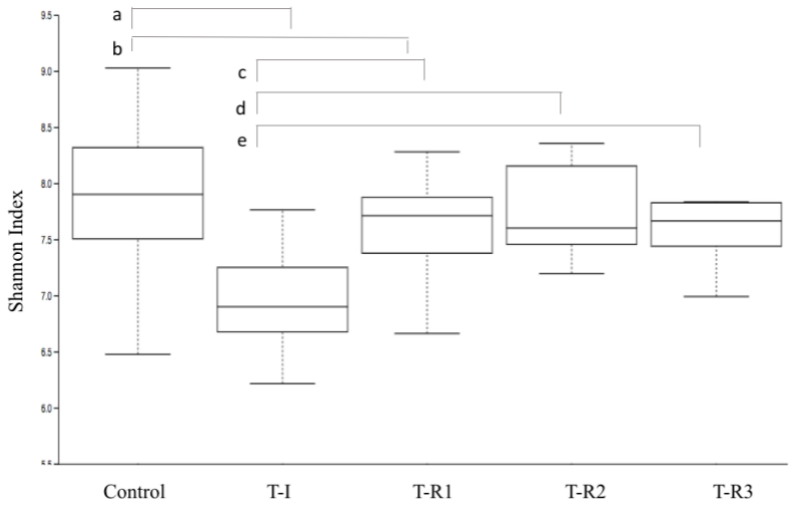

Figure 19: Shannon index metric boxplot showed the alpha - diversity of fecal associated - microbial community between the control and treatment groups during the infusion phase. Diversity (richness and evenness) of bacterial community was significantly lower ($p < 0.01$) between (a) treatment infusion week (T - I) and control group, (b) treatment recovery week 1 (T - R1) and control group, (c) treatment infusion week (T - I) and treatment recovery week 1 (T - R1), (d) treatment infusion week (T - I) and treatment recovery week 2 (T - R2), (e) treatment infusion week (T - I) and treatment recovery week 3 (T - R3) based on the Kruskal Wallis test.

Since the bacterial communities between treatment recovery weeks 2 (T - R2) and treatment recovery weeks 3 (T - R3) were no different from each other in terms of composition, abundance, richness, evenness, and number of OTUs, they all were pooled for subsequent analysis.

To understand how the microbial communities were affected by corn starch infusion, the number of OTUs among the following groups were evaluated during infusion phase: the control group, treatment infusion week (T - I), treatment recovery week 1 (T - R1), treatment recovery week 2 (T - R2), and treatment recovery week 3 (T - R3). Out of the 9,465 OTUs identified in the fecal-associated bacterial communities of the infusion phase, a relative abundance (ratio of an OTU's abundance to the total abundance) cutoff of 1 % was used for a robust comparison between the groups. Of the 123 OTUs that passed the 1 % cutoff, 55 (48.7 %) OTUs were common among all of the bacterial communities, 15 (13.3 %) OTUs belonged only to the control group, 6 (5.8 %) OTUs belonged only to the treatment infusion week (T - I) group, 4 (3.5 %) OTUs belonged only to the treatment recovery week 1(T - R1) group, and 3 (2.7 %) OTUs belonged only to the treatment recovery week 3 and 4 group (Fig. 20).

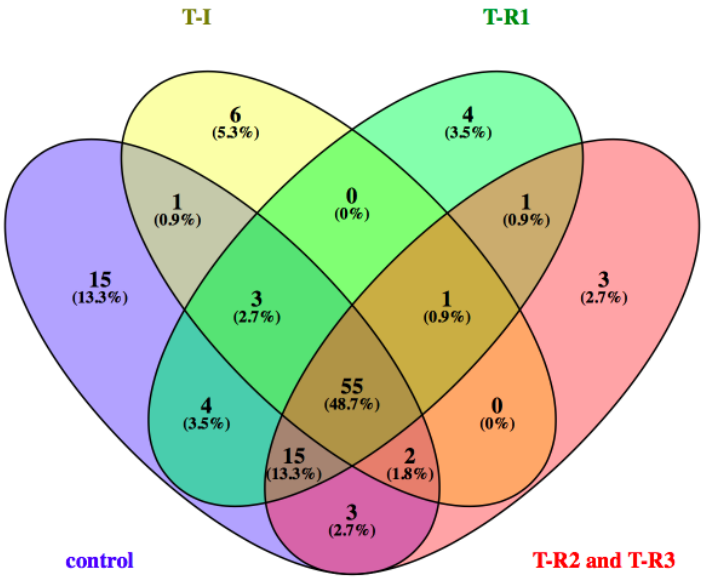

Figure 20: Venn diagram showed the number of observed operational taxonomic units (OTU) shared across groups of control, treatment infusion week (T - I), treatment recovery week 1 (T - R1), treatment recovery week 2 (T - R2) and treatment recovery week 3 (T - R3) during the infusion phase. only 55 OTU were shared among the four groups.

2.3.9 Composition of Fecal-Associated Bacterial Community during Infusion Phase

In the current study, a total of 1,685,502 quality 16S rRNA gene sequences were produced from 130 fecal samples across the control and treatment groups during the infusion phase with a mean sequence number of 12,965 per sample. At the family level, 20 taxa with a relative abundance ≥ 1 % in at least one group across the cycle weeks of the infusion phase were detected as the common families. Corn starch infusion resulted in a log2 fold change in the abundance of 10 families across the treatment infusion week and control groups (Fig. 21). Of them, Succinivibrionaceae, Erysipelotrichaceae, Lachnospiraceae, Clostridiaceae 1, Prevotellaceae, Bacteroidales S24 - 7 group, and Bifidobacteriaceae were more abundant in the treatment infusion weeks' group whereas

Spirochaetaceae, p – 2534 - 18B5 gut group, and unknown Bacteroidetes were more abundant in the control group during the infusion phase.

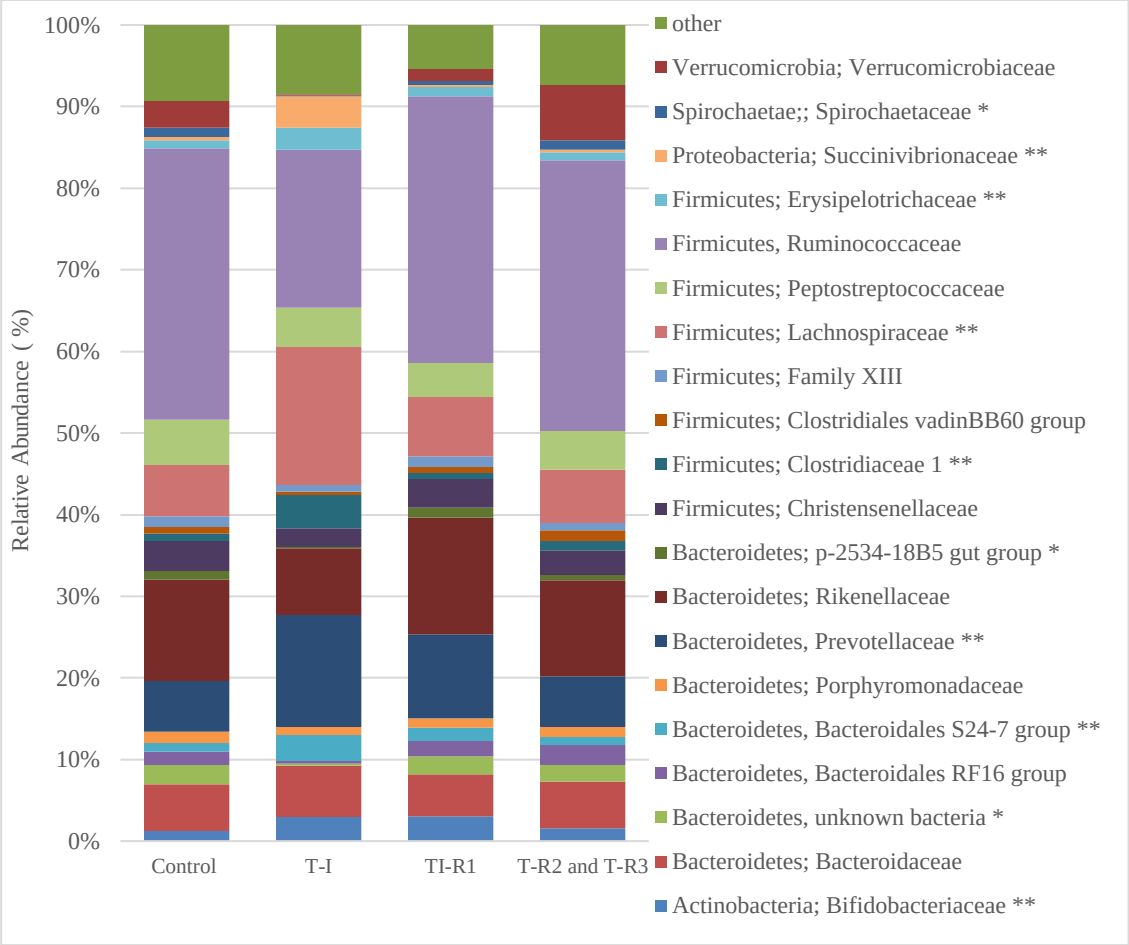

Figure 21: Distribution of fecal bacterial taxonomy. Average relative abundance based on distribution of bacterial family as a percentage of the total number of classified 16S rRNA sequences. Samples are grouped based on the control group and treatment groups across cycle weeks (treatment infusion weeks T - I, treatment recovery weeks1 T - R1, treatment recovery weeks 2 T - R2 treatment recovery weeks 3 T - R3) during infusion phase. Taxa with ≥ 1 % in at least one group were shown. Taxa with < 1 % were aggregated as "other". (**) indicates the taxa significantly present more abundantly among treatment infusion weeks (T - I) whereas (*) indicates the taxa that significantly present more abundantly among control group during the infusion phase based on Log2 Fold Change test and the cutoff of 1/ -1 was a criteria which was used for greater/ lesser abundance of taxa.

2.3.10 pH level and Concentrations of VFA and Lactate in the Fecal Samples during Diet Phase and Infusion Phase

The results of fecal pH level showed that there was not a difference between the control and treatment groups during the diet phase, but the results of fecal pH level showed that fecal pH level decreased ($p < 0.01$) among the treatment infusion group compared to that of the control group, as well as compare to that of the treatment recovery week 1 group. The concentration of acetic acid increased ($p < 0.01$) in the treatment recovery week 1 group compared to the control group, whereas the concentration of butyric acid increased ($p < 0.0$) in the treatment infusion week group compared to the control group. The concentrations of propionic acid and lactic acid were not affected by starch infusion (Table 3).

Table 3: Fecal pH level and concentrations of volatile fatty acid (VFA) based on the control group and treatment groups in terms of cycle week during the infusion phase.

	Control	Treatment infusion	Treatment recovery week1	Treatment recovery week2 and 3	p-value
Fecal pH	[a] 7.47	[a,b] 6.89	[b] 7.36	7.27	< 0.01
VFA (mmol/ L)	Control	T - I	T - R1	T - R2, T - R3	p - value
Acetic acid	[c] 14.62	22.45	[c] 24.59	22.91	< 0.01
Butyric acid	[d] 1.04	[d] 3.71	2.46	2.26	< 0.01
Propionic acid	3.13	4.95	5.01	5.04	> 0.01
Lactic acid	0.03	0.58	0.16	0.18	> 0.01

The fecal pH level of treatment infusion group was significantly lower ($p < 0.01$) than (a) the control group and (b) treatment recovery week 1. The concentration of acetic acid was significantly lower ($p < 0.01$) in (c) the control group than in treatment recovery week1. The concentration of butyric acid was significantly lower ($p < 0.01$) in (d) the control group than in treatment infusion group based on one-way ANOVA test.

2.3.11 Tissue-Associated Bacterial Community

The tissue-associated bacterial communities showed that there was neither a separation between control and treatment groups, a clustering by location among tissue - associated microbiota, nor a significant difference, as shown in PCoA plots based on weighted Unifrac (Fig. 22) and unweighted Unifrac metrics and through the PERMANOVA tests.

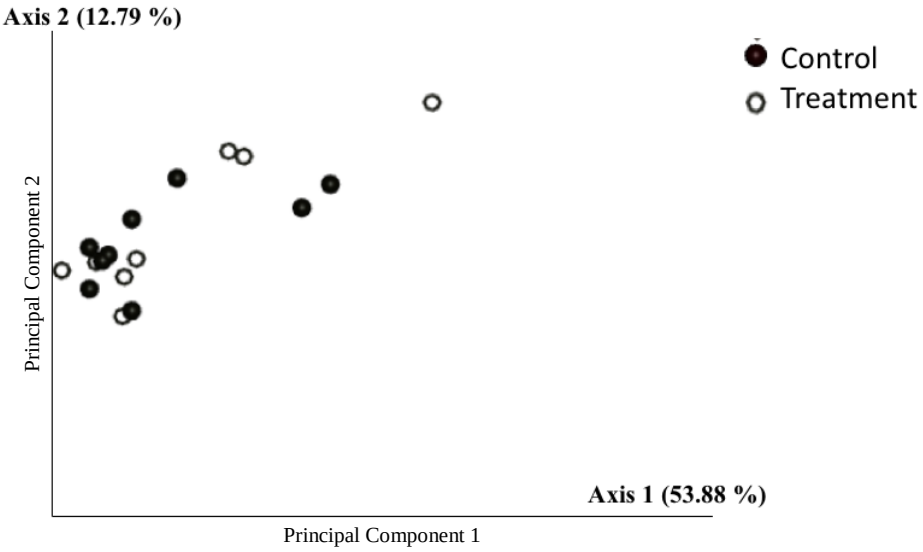

Figure 22: Principal coordinate analysis (PCoA) plot based on Weighted UniFrac metric showed the beta diversity of microbial community from tissue samples between the control and treatment groups. The control and treatment groups included samples from two locations, small intestine (jejunum and ileum) and large intestine (cecum and colon). There was no separation between the control and treatment groups.

Also, there was no difference in the richness, evenness, and number of OTUs of tissue-associated microbiota between the control and treatment groups as indicated by the Kruskal Wallis results of alpha diversity metrics including the Shannon index, Pielou's Evenness, and OTUs.

2.3.12 Digesta-Associated Bacterial Community

The digesta-associated bacterial communities showed that there was neither separation nor significant difference between control and treatment groups. However, they distinctly clustered by location (small intestine and large intestine) as shown in PCoA plots based on weighted Unifrac (Fig. 23) and unweighted Unifrac metrics and through the PERMANOVA tests.

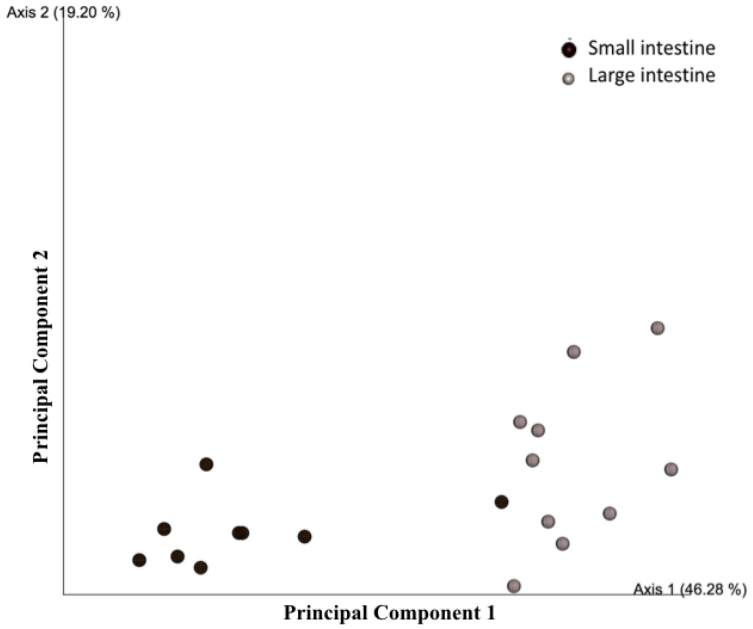

Figure 23: Principal coordinate analysis (PCoA) plot based on Weighted UniFrac metric showed the beta diversity of microbial communities from digesta samples. They were clustered by location (small and large intestine) along principal component 1 which describes 46.28 % of the variability in the bacterial community.

Also, there was no difference in the richness, evenness, and number of OTUs of digesta-associated microbiota among control and treatment groups according to the Kruskal Wallis results of alpha diversity metrics including the Shannon index, Pielou's Evenness, and OTUs.

Since there was a distinct cluster by location (small intestine and large intestine), the taxonomy analysis was tested to look at the taxa among small and large intestines. At the family level, 16 taxa with a relative abundance ≥ 1 % in at least one group of the small intestine or large intestine were detected as the predominant taxa (Table 4). The results showed that certain bacterial groups exhibited differences in the relative abundance between small and large intestines in the digesta.

Table 4: Average relative abundance of taxa at family level (% of total sequences) in small and large intestine of digesta.

Family	Small Intestine	Large Intestine
Bacteroidaceae	2.1	3.6
Bacteroidales S24-7 group	1.4	5.0
Bifidobacteriaceae	7.5	3.7
Christensenellaceae	2.2	2.7
Clostridiaceae 1	8.5	4.5
Clostridiales Family XIII	1.6	1.0
Coriobacteriaceae	1.8	0.8
Erysipelotrichaceae	5.6	4.6
Lachnospiraceae	14.6	12.5
Peptostreptococcaceae	26.8	8.5
Porphyromonadaceae	0.7	1.4
Prevotellaceae	2.3	8.0
Rikenellaceae	1.6	8.0
Ruminococcaceae	15.4	22.0
Unknown Saccharibacteria	1.4	0.4
Other	< 0.01	< 0.01

2.3.13 Correlation Analysis

A correlation analysis was carried out to determine whether there were any relationships between the microbiota diversity and pH value or between the microbial diversity and concentrations of VFA among control and treatment groups during the infusion phase. The results of the Spearmen correlation test revealed that there was not a correlation between the rumen fluid-associated bacterial community and pH level or between the rumen fluid-associated bacterial community and the concentrations of VFA. Also, the results of the Spearmen correlation test revealed a positive association between the diversity of microbial community and fecal pH level as indicated by the Shannon Index, Pielou's Evenness, unweighted, and weighted metrics. There was a positive association between the diversity of bacterial community and acetic acid concentrations as indicated by Pielou's Evenness and unweighted metrics. There was a positive association between the diversity of bacterial community and butyric acid concentrations as indicated by Pielou's Evenness, unweighted, and weighted metrics (Table 5).

Table 5: The correlation of pH level and concentrations of volatile fatty acid (VFA) and diversity of the fecal samples between the control and treatment groups of cycle weeks during the infusion phase.

Alpha diversity metric	pH	Acetic acid	Butyric acid	Propionic acid	Lactic acid
Shannon Index	0.30 *	0.15	-0.30	-0.13	-0.02
Pielou's Evenness	0.36 *	-0.36 *	-0.43 *	-0.19	-0.22
OTUs	0.23	-0.08	-0.22	-0.10	0.007
Beta diversity metric	pH	Acetic acid	Butyric acid	Propionic acid	Lactic acid
Unweighted	0.18 *	0.23 *	0.45 *	0.12	0.27
Weighted	0.23 *	0.26	0.45 *	0.11	0.37

(*) indicates the significant correlation (< 0.01) between alpha or beta diversity metrics and pH level or concentrations of VFA based on Spearman test.

2.3.14 Relative mRNA Gene Expression and Immune Cell Population in the Small and Large Intestine Tissue

No significant differences ($p > 0.05$) were detected in the gene expression of pro-inflammatory cytokines (TNF - alpha, IL - 1 beta and IL - 6) and in the immune cell phenotype, including T cell markers (CD4 and CD8) and dendritic cell activation markers (CD40, CD80), among the control and treatment groups in the small and large intestine of tissue based on Student T - test.

2.4 Discussion

The purpose of this study is to understand the link between the host-gut microbiome interaction in the dairy cattle acidosis model. Current research proposes that a difference in the resilience of the bacterial communities and their ability to recover might occur due to a high starch diet (barley diet and periodic corn starch infusion).

Dynamic Shifts in the Rumen Fluid-Associated Bacterial Community During Diet and Infusion Phases

The ruminal acidosis challenge model in the current study showed that a one week periodic corn starch infusion did not result in ruminal acidosis. Both concentrations of ruminal pH and fermentation VFAs remained constant in all treatment groups compared with control groups. The barley diet that was fed and the periodic corn starch infused to cows in this study, may not be as high as expected or as high as that of fructose or histidine used in previous studies (H. M. Golder et al. 2013) to result in ruminal acidosis. Also, the lack of effect of a barley diet and periodic corn starch infusion on VFA production suggests that fermentation end products may be caused by differences in the microbial species, which are responsible for the fermentation of starches (Enemark 2008). One of the reasons behind our dairy cattle model not

developing ruminal acidosis might be attributed to the sample size. The sample size is an important factor that determines the statistical power of the study. Therefore, the small sample size (n = 6 initially and n = 5 finally) showed 19.23 % of ruminal acidosis development in dairy cattle in comparison to the typical 26 % of dairy cattle that develop ruminal acidosis. The small sample size used in this study, decreased the statistical power of the study showing no ruminal acidosis in the dairy cattle that could conflict with the presence of ruminal acidosis in our dairy cattle. Another reason that the cattle did not develop ruminal acidosis may be because of the time scale of our experiment not being long enough to induce ruminal acidosis.

A previous study showed that a high-grain diet fed to ruminants resulted in a reduction of the diversity, and shift in the composition of the ruminal bacterial community (Y.-H. Kim et al. 2016). In accordance with that study, our findings also showed that a high starch diet reduced the diversity (richness, evenness, abundance) and shifted the composition of the ruminal bacterial community. The plausible explanation for this reduction in diversity and shifts in bacterial composition was likely an increase in the prevalence of the microbes that are capable of degrading undigested starch.

The reduction of the diversity was also mainly driven by a reduction in richness (number of different species) coupled with an increase in dominance (reduced evenness) of some phyla such as Firmicutes (Renee M. Petri et al. 2013). Studies related to metabolic disorders showed an increase in the abundance of Erysipelotrichaceae in mice on high-fat diet (Kaakoush 2015). In accordance with that study, our 16S rRNA gene data also demonstrated that Erysipelotrichaceae was the major Firmicutes family present with the rumen during the treatment diet.

Also, our results demonstrated that Bifidobacteriaceae was one of the Bacteroidetes families that was present in both the control and treatment groups. However, Bifidobacteriaceae was more abundant in the treatment group than in the control group. It is believed that Bifidobacteriaceae family plays an important role in the maintaining and promoting host health by inducing carbohydrate metabolism through utilizing a diverse range of dietary carbohydrates (Pokusaeva, Fitzgerald, and van Sinderen 2011), which might be the reason behind our treatment cows not developing ruminal acidosis.

A previous study found that a high grain diet had an effect on the abundance of some members of the bacterial community, but at variable rates (AlZahal et al. 2017). In accordance with that study, our study's findings showed that the abundance of Lactobacillaceae family was lower in the treatment group than in the control group. It has been reported that Lactobacillaceae and Streptococcaceae are the most common families present in the rumen of dairy cattle undergoing acidotic challenge (Renee M. Petri et al. 2013). In agreement with that, the current study's data also reported that Lactobacillaceae was less abundant in the rumen of the treatment group and our dairy cattle model did not develop ruminal acidosis.

Also, the current study's results showed that the concentration of ruminal lactic acid tended to increase in the treatment group. The plausible explanation for the tendency of increased lactate in the rumen of the treatment group may be the presence of either additional lactate - producing families or fewer bacterial groups responsible for metabolizing lactic acid.

Moreover, the current study's findings showed that rumen fluid-associated bacterial communities' diversity had not varied by cycle number. The bacterial

community across control groups and across treatment groups distributed similarly during the infusion phase. It is worth noting that we did not expect that post-ruminal corn starch infusion would have an impact on the rumen. The post - ruminal corn starch infusion was carried out in the study to directly challenge the intestinal microbiome and to deliver inflammatory response in the intestine without impacting the ruminal microbiome. Also, the reason behind that there was no variation by cycle number across control and treatment groups may be attributed to the season of the study or additional factors across those groups that could play a minor role in affecting bacterial community composition (M. Kim et al. 2014).

Temporal Dynamic Shifts in fecal-Associated Bacterial Community during Infusion Phase

When corn starch was infused into cow's abomasum, corn starch bypassed the rumen and flowed directly into the intestine. We proposed that post-ruminal corn starch infusion may challenge intestinal microbiome, leading to gut wall permeability and LPS translocation into the blood circulation to eventually trigger a pro - inflammatory response in the intestine. A high - throughput sequencing approach proved to be a powerful tool to investigate the bacterial biodiversity of feces, and to reveal the effects of a high grain diet on that biodiversity.

Our data showed that there was no difference in the diversity of fecal-associated bacterial community of both the control and treatment groups during the diet phase. The logic explanation is that the bacterial community in the control and treatment groups tend to adapt to the seven weeks period in which a barley diet fed to cows.

A previous study found a high grain diet can decrease the diversity and shift the composition of the microbiome in the digestive tract of ruminants (E. Khafipour et al. 2016; Wang et al. 2017). In accordance with that study, the current study's data also showed that there was a reduction in fecal-associated microbial diversity, including richness and evenness, during the weeks of the corn starch infusion. Then, the fecal microbial diversity drifted back toward the control community during recovery weeks, returning to a state similar to the control community by recovery week 3. The fact that fecal-associated bacterial communities drifted back to the baseline by the end of the starch infusion cycle number, implies that short-term acute starch challenges do not cause lasting effects in the microbial communities. These results suggest that fecal-associated bacterial communities are resilient to the short-term corn starch insults.

It is reported that fermentation of different polysaccharides has been found to increase production of VFAs like acetate and butyrate, which are mainly associated with starch breakdown (Macfarlane et al. 1998). In agreement with that, our present study's findings showed that the periodic corn starch infusion increased the concentration of fecal butyric acid and decreased fecal pH level. The decreased fecal pH could be an indication of bacterial fermentation in the intestinal tract. The possible explanation to not see an increase in the total of VFAs may be due to that the prolonged exposure to the periodic corn starch was not enough to cause high fermentation. The increase of VFA concentrations and the decrease of pH level in the feces of the treatment group may cause the fecal - associated microbial community to alter (Wang et al. 2017). This may explain the reason for the alteration of fecal - associated microbial diversity in the present study.

The present study showed that Spirochaetaceae was one of the less abundant families detected in the treatment group during the infusion phase. It has been found that Spirochaetaceae is a natural intestinal bacterium associated with cellulolytic bacteria, which may explain the lower abundance of Spirochaetaceae in the treatment group that was periodically infused with corn starch in the present study (Ehsan Khafipour et al. 2009).

Also, the current study showed that the Bifidobacteriaceae family was more abundant in the treatment group, suggesting that starch derived from the diet was used by fecal-associated bacteria, resulting in an increase in the percentage of polysaccharide-degrading bacteria such as Bifidobacteriaceae (Ye et al. 2016). It has been reported that Bifidobacteriaceae has the ability to inhibit enteropathogenic infection (intestinal disease) through the production of acetic acid, which stimulates intestinal health (Fukuda et al. 2011).

Also, it has been found that butyric acid plays a key role in improving tight-junction integrity (tight junctions maintain the barrier function of epithelial cell) (Ríos-Covián et al. 2016). This is consistent with our findings. Our fecal butyrate concentrations increased during the periodic corn starch infusion. At the same time, mRNA expression levels of cytokines (tight junction protein genes) remained unchanged, which suggests that the higher levels of butyric acid could make the tight - junction integrity more stable.

Also, the current study's data showed that Prevotellaceae and Clostridiaceae 1 were more abundant in the treatment group during corn starch infusion, suggesting their role in degrading polysaccharides was derived from diet with the highest starch contents (Thoetkiattikul et al. 2013; M. Kim et al. 2014). It has been found that certain species

of Prevotellaceae may be associated with metabolic syndromes such as obesity and inflammatory bowel disease (SitaoWu et al. 2012; Carding et al. 2015). It has also been reported that some Clostridium spp. can cause intestinal disease in goats and affect the colonic mucosal barrier (M. Kim et al. 2014). Therefore, the corn starch infusion may become a selective force to select certain community members to open the door for metabolic diseases.

Intestinal-Associated Bacterial Community in Response to Diet Shift

Despite the fact that there are numerous published studies that assess intestinal microbial community changes due to diet-induced ruminal acidosis in dairy cattle, no published studies exist, to our knowledge, that have assessed shifts of microbial community in digesta of both small (jejunum and ileum) and large (cecum and colon) intestinal contents in response to periodic corn starch infusion using high-throughput methods in dairy cattle.

Our data at the conclusion of the experiment revealed that the digesta-associated bacterial community was distinctly clustered by location (small intestine and large intestine), whereas there was no diet influence on tissue-associated bacterial communities.

Moreover, our mRNA gene expression levels of cytokines and immune cell phenotype results showed that there was no evidence of a high starch diet's effect on pro-inflammatory cytokine expression and immune cell population. These results suggest that the homeostasis in the intestinal mucosal immune system may resist the microbial disturbance associated with a high starch diet (Quast et al. 2013). Also, the limitations of timescale or sample size may be additional reasons to not cause a microbial alteration in response to high starch diet.

Additionally, the current study's results showed that there were shared "core" taxa across both control and treatment groups, either in rumen fluid such as Bifidobacteriaceae, Prevotellaceae, Staphylococaceae, and Lactobacillaceae or in fecal-associated microbial communities such as Actinomycetaceae, Rikenellaceae, Clostridiaceae 1, and Spirochaetaceae. This suggests that these microbial species possess shared functionalities which enables the structure of the collective rumen or fecal microbial ecosystem to be more stable and robust (Durso et al. 2010).

There are several factors that influence the challenge of ruminal acidosis and pro-inflammatory responses in this study, that affected the results we observed. One of these factors is the limited number of cows that were treated. Previous studies demonstrated that 26 % of the dairy cattle develop metabolic diseases when they are on high grain diets (Nagaraja and Titgemeyer 2007). This means that 1 cow out of 4 develops adverse health effects. In this study, we started with 3 treatment cows and ended up with 2 treatment cows (19.23 %), which might be a plausible reason that this limited number of cows were not showing the required response to this dietary challenge.

Other factors that may result in the lack of development of any obvious metabolic disease symptoms in the current study may be related to the dose of corn starch infused (initially 1.5 g/ kg BW per day and 4 g/ kg BW per day to the end of the study) to cows. The question that may arise here is: was the starch dose that we used too low to cause pro-inflammation response, or was it a good dose but were our cows able to adapt to that dose over time? One of the dairy cattle studies observed dramatic and severe changes, such as inflammatory response, in animal health receiving 5 g/ kg BW per day starch treatment (Bissell, n.d.2002). In the current study, we did not use the

same amount of starch dose that was used by Bissell, n.d. because our trail lasted several months, and we did not want our dairy cattle to develop severe metabolic diseases which could result in losing them. Hence, the dose of starch administrated was initially 1.5 g/ kg BW per day but was increased during the experiment to 4 g/ kg BW per day when lower doses failed to change fecal consistency or decrease fecal pH. This gradual dose may also justify that the dose of corn starch infusion was not sufficient enough to develop the acidosis challenge. Additionally, starch infusion periods (one - week infusion) may not have been long enough to cause the systemic response.

Also, one of the factors that contribute to ruminal acidosis is periparturient cow factor (the period immediately before and after calving, i.e. 3 weeks before calving and 3 weeks after calving). Periparturient cows are at risk of ruminal acidosis because the rumen and rumen microbes need to adapt from a very fibrous dry cow diet to the high energy lactation diet in a short period of time. In our study, we used non-lactating cows which were not subjected to metabolic or hormonal stressors such as lactating and the abrupt switch to a high grain diet. This might also be another reason which made our dairy cattle more tolerant to the risk of any metabolic acidosis.

Additionally, we were not able to collect intestinal tissue samples before and after infusion week for testing microbial community and gene expression for diagnose if there were any current microbial shifts or gut barrier damage over the time of the study.

Collectively, we hypothesize that the gut microbiome could show distinct shifts during high grain diet-induced ruminal acidosis in dairy cattle. Our results demonstrated that a microbiome shift occurs during high grain diets, as expected. However, no

ruminal acidosis or inflammatory immune response was observed in the gut associated with the microbiome alteration.

The dairy cattle in the current study did not experience any pro-inflammatory response due to the high starch diet, amplifying that the high grain diet did not really impact the cow's health.

Also, diet had limited to no effect on the microbial community of both small (ileum and jejunum) and large (cecum and colon) intestinal digesta and tissue collected post - study. However, the digesta-associated microbial community significantly was clustered by location (small intestine and large intestine). Moreover, the results of relative gene expression of cytokines and immune cell phenotypes showed that they were not affected by the corn starch infusion.

Chapter 3

CONCLUSION AND FUTURE DIRECTION

In the current study, the alteration in microbial community of dairy cattle's rumen fluid, feces, tissue, and digesta in response to diet shift was assessed via high throughput 16S rRNA gene amplification and barcoding library preparation protocol for Illumina MiSeq sequencing.

We hypothesized that feeding the cows with starch grain contents and infusing the cows' abomasum with corn starch could contribute to the onset of ruminal acidosis and pro-inflammatory response, by altering the pH level, concentrations of VFA and lactate, and the bacterial community biodiversity, in gastrointestinal tract of dairy cattle.

Overall, our study's data showed that short-term corn starch challenge dynamically reduced the diversity and shifted the composition of the ruminal microbial community over time. Also, our findings showed that the short-term corn starch challenge affected the resilience of fecal-associated bacterial community and their ability to recover.

However, the ruminal acidosis challenge model in the current study showed that the short - term acute starch challenge did not result in ruminal acidosis.

As a future approach to overcome the limitations in the current study and further investigate the observed results, a bigger sample size is to be analyzed. A higher number of cows in the treated sample reflects a clearer image of the microbial alteration and pro - inflammatory responses during diet-induced ruminal acidosis.

An additional future approach would be to observe the development of ruminal acidosis challenge, in addition to pro - inflammatory responses in the gastrointestinal tract, through conducting longer - term infusion. A suggested infusion term of 2 weeks

rather than one week or multiple infusion times a day rather than twice a day. It is also possible to start infusing 4 g/ kg BW or 5 g/ kg BW of corn starch immediately rather than graduating infusion, or start the diet and the infusion at the same time to be able to see the effect of the high grain diet that we were unable to detect in our experiment.

Furthermore, processing tissue biopsies from different segments in the gastrointestinal tract before and after the starch infusion is also recommended to observe the intestinal structural changes through gene expression of pro - inflammatory cytokines over the time of the study. Observation of gut barrier proteins, such as tight junction proteins, mucus gene expression, and microbial heptapeptide, to detect any barrier damage due to high grain diet.

Additional diagnosis is requested to test the content of bacterial endotoxins, such as lipopolysaccharide (LPS) in order to further investigate the pro-inflammatory immune response caused by the high starch diet.

Finally, the functional capacity of the microbiome and the immune cell function can be characterized in order to determine the compositional changes that result from significant changes in the function of the microbial community and immune cells.

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Appendix - one

IACUC APPLICATION LETTER

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research
(New and 3-Yr submission)

RECEIVED
JUL 8 2016
IACUC

Title of Protocol: Crosslinkage between gut fermentation, adiposity and immune response activates mesenteric adipose depot inflammation in dairy cows	
AUP Number: 62R-2016-0	**← (4 digits only — if new, leave blank)**
Principal Investigator: Robert M Dyer VMD, PhD	
Common Name (Strain/Breed if Appropriate): Cow/Holstein **Genus Species:** Bos Taurus	
Date of Submission: 5/26/2016	

Official Use Only

IACUC Approval Signature: Glenn Tallon, DVM

Date of Approval: 7/21/16

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to by the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. ***(Teaching Protocols, including cooperative extension demonstrations, Exempt)***
12. I understand that by signing, I agree to these assurances. Robert M Dyer VMD, PhD_____ _____ Signature of Principal Investigator Date 5/26/2016

Rev202/2016

NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

Name	Signature
1. Robert M Dyer	
2. Erin Brannick	
3. Ryan Arsenault	
4. Tanya Gressley	
5. Click here to enter text.	
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7. Click here to enter text.	
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10. Click here to enter text.	

If after hours participation is required by students on project involving **agricultural animals**, please describe how this is handled and the times and days that students may be on site
Students may be on site on the days where abomasal starch infusions will be performed. Any student will be trained and supervised by Drs.Gressley and Dyer and at least one fully trained graduate student.