ABSTRACT

Title of Thesis: COMMUNITY STRUCTURES OF GUT MICROBIOME UNDER DIFFERENT DIETS IN ANGUS BEEF CATTLE

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The microbiota of animals' digestive tract plays important roles in health, nutrient utilization, and performance. For ruminants, the digestive tract, including rumen and other gastro-intestinal regions, hosts a unique and diverse microbiome that aids feed fermentation, digestion, and absorption. We hypothesize that compositions of diets can influence microbiomes and further affect the production of VFA and the composition of bile acids in beef cattle. We used rectum microbiota as a baseline and examined the microbiome in rumen and jejunum by16S-seq, together with quantifications of VFAs,

lactate, and bile acids under grass-feeding and grain-feeding. We found that microbial communities displayed significant differences in microbiome structure and VFA production. Moreover, increased VFAs in grain-fed group may trigger lactate-associated bacteria growth. Bile acids could promote the growth of bacteria that had abilities of secondary bile acids conversion. These results provided deep insights into differences in beef quality and bovine biology under different diets.

COMMUNITY STRUCTURES OF GUT MICROBIOME UNDER DIFFERENT DIETS IN ANGUS BEEF CATTLE

by

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Dedication

To my parents, thanks for your support!

Acknowledgement

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List of Abbreviations

16S-Seq	16S rRNA gene sequencing
ABC	ATP-binding cassette
ADG	Average daily gain
BSH	Bile salt hydrolase
DCA	Deoxycholic acids
ESI	Electrospray ionization
GCA	Glycocholic acids
GCDCA	Glycochenodeoxycholic acids
GI tract	Gastrointestinal tract
KEGG	Kyoto Encyclopedia of Genes and Genomes
KW	Kruskal-Wallis
LC-MS	Liquid chromatography–mass spectrometry
LCA	Lithocholic acids
LDA	Linear discriminant analysis
LefSe	Linear discriminant analysis Effect Size
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PAGE	Polyacrylamide gel electrophoresis
PCA	Principle component analysis
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME	Quantitative Insights into Microbial Ecology
RSLC	Rapid separation liquid chromatography
TCA	Taurocholic acids
TCDCA	Taurochenodeoxycholic acids
UMCP-IACUC	Institutional Animal Care and Use Committee, University of Maryland, College Park
UPLC	Ultra-Performance Liquid Chromatography
VFA	Volatile fatty acids

1. Literature Review

Introduction

Beef is a more flavorful, nutritional, and expensive meat product than many other types of meat. In beef cattle feeding industry, grain-fed finishing method feeds young cattle in feedlots with high-grain diets during finishing, while pasture-fed method allows cattle to graze naturally with high-grass diets. Other than animal behavior factors, diet components are the key differences between grain-fed and grass-fed regimens [1]. As the demand for healthy and palatable beef is increasing worldwide, grass-fed beef products gradually attract more attention in the meat market, and an increasing group of consumers are willing to pay higher unit price for grass-fed beef than grain-fed beef [2]. Several studies have proven differences between grass-feeding and grain-feeding from the aspects of nutrition, meat palatability, and genetics [3-5]. One important influence of diets is that diets primarily determine gut microbiome structures and drive microbial adaption in animals and humans [6]. Several studies have proved that different dietary habits in human can shape gut microbial composition and community structure [6-8]. Diet-induced changes in gut microbiota could further influence micro-environment in the gastrointestinal (GI) tract, and then influence host physiology and metabolism. Therefore, community structure of gut microbiome under different diets is highly significant and meaningful to study to promote microbiota balance and host health.

As for the ruminant species, the gastrointestinal tract is particularly unique regarding microbial community structures and functions. A variety of microorganisms, including bacteria, protozoa, fungi, and archaea, dynamically adapt to diets and manage to occupy their niches in the gut to help nutrient breakdown and absorption [8]. Bacteria are the dominant microbes in ruminant gut, and provide great contribution of metabolomic activities in the gut [9]. Rumen is a unique organ where microbes function to ferment and digest complex carbohydrates to produce volatile fatty acids (VFA), which serve as the primary energy source for ruminant [9, 10]. Many gut microbiota studies have focused on rumen microbiome to assess gut microbiota and cattle performance, but the role of microbes in small intestine and colon has been paid less attention. However, different segments of cattle gut possess unique microbial and ecological characteristics [11, 12]. The small intestine is the main segment for the absorption of glucose, amino acids, fatty acids, and glycerol with the aid of bile and enzymes. Therefore, bacterial diversity and taxonomic composition are also necessary and meaningful to explore [11, 13]. Compared to the conventional culture techniques, high throughput sequencing methods such as shotgun sequencing and 16S rRNA gene sequencing (16S-seq) can characterize a much higher portion of rumen bacterial genera [14]. Combined with bioinformatic tools and gut metabolite measurements, in-depth and comprehensive profiling of gut microbiome structure can be achieved. In this study, we focused on diet-induced changes on gut microbiome between grass-fed and grain-fed Wye Angus beef cattle and provided scientific evidence to explore if grass-fed beef cattle are worth paying additional attention in the beef industry.

Diets and gut microbiome

Diet as a major determinant of gut microbiome

Dietary habit is known to be the dominant determinant that can shape gut microbiota [15]. The quantity, type, and balance of the major dietary nutrients, especially macronutrients such as carbohydrates, proteins, and fats, have significant influences on gut microbial composition [16, 17]. For example, plant fiber is an essential dietary component that helps to regulate the contents and micro-environment of the digestive tract, and also changes how other chemicals are mobilized in human [18]. Relationships of gut microbiota and fiber content have also been reported in several studies [15, 19-21]. According to a study using gnotobiotic mice with characterized human gut microbiota, low-fiber diets on mice promoted a higher abundance of mucus degrading bacteria and their activities. Fiber-deprived gut microbiota could lead to aggressive colitis associated with enteric pathogen [21]. Not only for human, several research studies have been done in diet-induced changes in gut microbiome of livestock species such as chicken, goat, cattle, and swine, and various feeding trials, administration of different feeding regimens, and different nutrients were explored among these studies [20, 22-24]. Understanding how diets can modulate community structure and metabolic functions of gut microbiota enables researchers to improve both animal and human health.

Rumen microbiome and metabolism

Microbes in rumen

Rumen microbial micro-environment is dynamic and complicated and has been explored extensively. Rumen microbes include bacteria, protozoa, fungi, archaea, and viruses. Bacteria are the predominant kind of microbes in rumen, and they could reach approximately 10^{10} to 10^{11} cells/ml of rumen liquid, and occupy 60% to 90% of total rumen microbial mass [9]. Ruminal bacteria are mostly attributed to phyla Bacteroidetes and Firmicutes, but several other phyla can also be identified, such as Proteobacteria, Spirochaetes, Fibrobacteres, and Verrucomicrobia. Ruminal bacteria are also diverse in higher taxonomic levels. Core microbiome was defined as a common set of microbial taxa that are shared by most individual samples [25]. Prevotellaceae, Lachnospiraceae, Ruminococcaceae, Veillonellaceae, Acidaminococcaceae, and Porphyromonadaceae were some examples of dominant core bacteria families that could commonly be found in cattle rumen microbiome [25-28]. Examples of common bacteria genera included *Prevotella*, *Dialister*, *Succiniclasticum*, *Ruminococcus*, *Butyrivibrio*, *Mitsuokella*, and *Fibrobacter* [11, 28, 29]. Bacteria in the rumen can utilize primarily carbohydrates (cellulose, hemicellulose, pectin, starch, and sugars), cellulodextrins, pentoses, glucose, lactate, succinate, formate, and hydrogen as their energy sources [9]. Bacteria in the rumen can also be categorized according to their functions such as their substrates and products during metabolism. Selected representative species in ruminants are listed in table 1.1 [9]. In addition, differences in diet regimens fed to cattle can induce considerable changes in gut microbiota. For example, *Butyrivibrio* was more abundant in rumen of cattle fed mixture of forage and

concentrates, and *Fibrobacter* was most abundant in bovines fed forage than concentrates [28].

Table 1.1. Selected dominant bacteria in ruminants and their related functions.

Species	Gram stain	Morphology	Substrate	Products
Structural CHO fermenters				
Ruminococcus albus	Positive	Cocci	H F C, F X	1, 2, Et, H2, CO2
R. flavafaciens	Positive	Cocci	H F C, F X	1, 2, Su, H2, CO2
Fibrobacter succinogenes	Negative	Bacillus	H F C, H Hm, F Dx	1, 2, Su
Butyrivibrio fibrisolvens	Negative	Bacillus curve	H F C, F X, F Pr	1, 2, 4, Et, La, H2, CO2
Pectinolytic species				
Succinivibrio dextrinosolvens	Negative	Bacillus curve	F Pn, F Pc	1, 2, Su, La
Lachnospira multiparus	Positive	Bacillus curve	F Cb, F Pc	1, 2, Et, La, H2, CO2
Nonstructural CHO fermenters				
Bacteriodes ruminicola	Negative	Bacillus	F Cb, F X, F Pc, F S, F Hx, F Pr	1, 2, 3, Su
Selenomonas ruminantium	Negative	Bacillus curve	F Cb, F Pn, F S, F Hx, F Gl, F Pr	2, 3, 4, Su, La, H2
Succinomonas amylolitica	Negative	Oval	F S, F G	2, 4, 5, Su, H2
Streptococcus bovis	Positive	Cocci	F Cb, H Pc, F S, F Hx, F Pr	1, 2, Et, La
Megasphaera elsdenii	Positive	Cocci	F Ml, F Su, F Gl, F Pp, F La	2, 3, 4, 5, 6, H2, CO2
Lipolytic species				
Anaerovibrio lipolytica	Negative	Bacillus	F Fr, F Tg, A, F La	2, 3, Su, CO2, H2
Proteolytic species				
Peptostreptococci sp.	Positive	Cocci	Fr, F Pr A	2, 4, Br, NH3, CO2
Clostridia sp.	Positive	Bacillus	F Cb, F X, F Pc, F S, F Sc Fr, F Pr A	1, 2, 4, Br, Et, La, H2, NH3, CO2
Organic acid fermenters				
Megasphaera elsdenii	Positive	Cocci	F S, F Ml, F Gl, F Pp, F La	2, 3, 4, 5, 6, H2, CO2
Veillonella alcalescens	Negative	Cocci	F La, U H2	2, 3, H2, CO2

Note:

A = amino acids	C = cellulose	Cb = cellobiose
CO2 = carbon dioxide	Dx = dextrin	Et = ethanol
Fr = fructose	F = ferments and utilizes substrate	
G = glucose	Gl = glycerol	H2 = hydrogen
H = hydrolyzes substrate but does not use products		Hx = hexose
Hm = hemicellulose	La = lactate	Ml = maltose
Pn = pentose	Pp = peptides	Pr = protein
S = starch	Sc = sucrose	Su = succinate
U = utilize	X = xylan	1 = formate
2 = acetate	3 = propionate	4 = butyrate
5 = valerate	6 = caproate	

(Sources: This table was adapted from [9, 30, 31].)

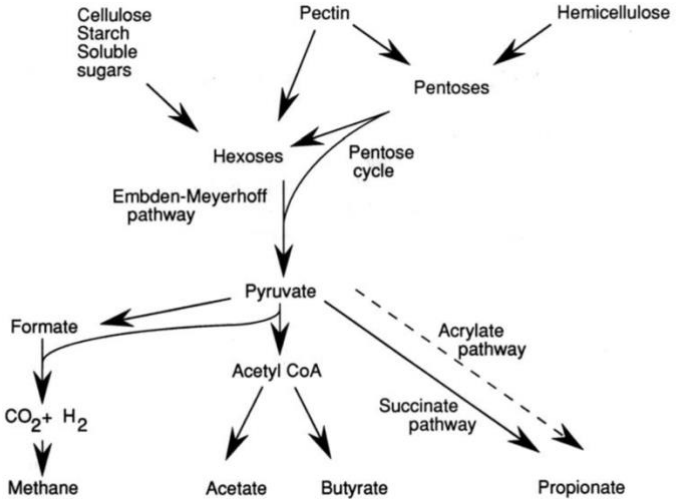
Volatile fatty acids and lactate production in rumen

In the rumen, the volatile fatty acids (VFA), also known as short-chain fatty acids, are produced from carbohydrates and proteins by fermentative microbes, and they contribute approximately 70% to the energy requirements of ruminants [32]. The principle VFA in the rumen are acetate, propionate, and butyrate, which account for 95% of VFA products found in rumen fluid, and are important to rumen metabolism [9, 33]. Pathways of these major VFA are summarized in Figure 1.1. Acetate is an end product of fiber fermentation and is necessary for producing milk fat in dairy cows [10]. Propionate is an end product of starch and sugar fermentation in the rumen, which serves as an efficient energy source for cattle. The production of butyrate does not have reduced cofactors such as NADH or $FADH_2$, so it generates less wasted methane and carbon dioxide (Figure 1.1 (B)) [10]. Lactate is also an important product of VFA production. There are two pathways for propionate synthesis, the succinate pathway and the acrylate pathway, and lactate serves as an intermediate (Figure 1.1). The

succinate pathway is the main pathway in the rumen to produce propionate, and the intermediate product is succinyl-CoA [9, 20]. The succinate pathway also produces more acetate than does the acrylate pathway [9]. However, it was reported that this pathway was less observed in cattle fed with a high-forage diet [20]. As for butyrate, other than energy sources, butyrate is known for its regulation of animal physiology [34]. For instance, butyrate is a preferred substrate for rumen epithelial metabolism and can regulate several metabolic pathways related to rumen development [34-36].

The amount of these major VFA in rumen largely depends on diets [10]. For example, cattle diets that contain high fibrous components can produce higher ratio of acetate to propionate, which generates more methane and in turn leads to wasted energy [9, 10]. However, grain-rich diets for cattle may promote the production of propionate and butyrate because of increased numbers of relevant bacteria population. In extreme cases, if the percentage and level of starch are high in diets, there could be significant increases in VFA production and lactate accumulation, and as a result, ruminal pH decreased, and ruminal acidosis can occur [9, 20, 37]. Bacteria species are also associated with this process. For instance, *Streptococcus bovis* was reported to be a dominant species when high-starch diets were fed. It produced lactic acid as its primary fermentation product, and may cause ruminal acidosis or feedlot bloat [38]. Even if lactate-consuming strains such as *Megasphaera elsdenii* also exist in the rumen, this could not compensate for the rapid accumulation of lactate in this case [39]. Therefore, an appropriate forage: concentrate ratio in diets and stepwise adaption to new regimens are necessary to maintain a balanced ruminal environment.

A.



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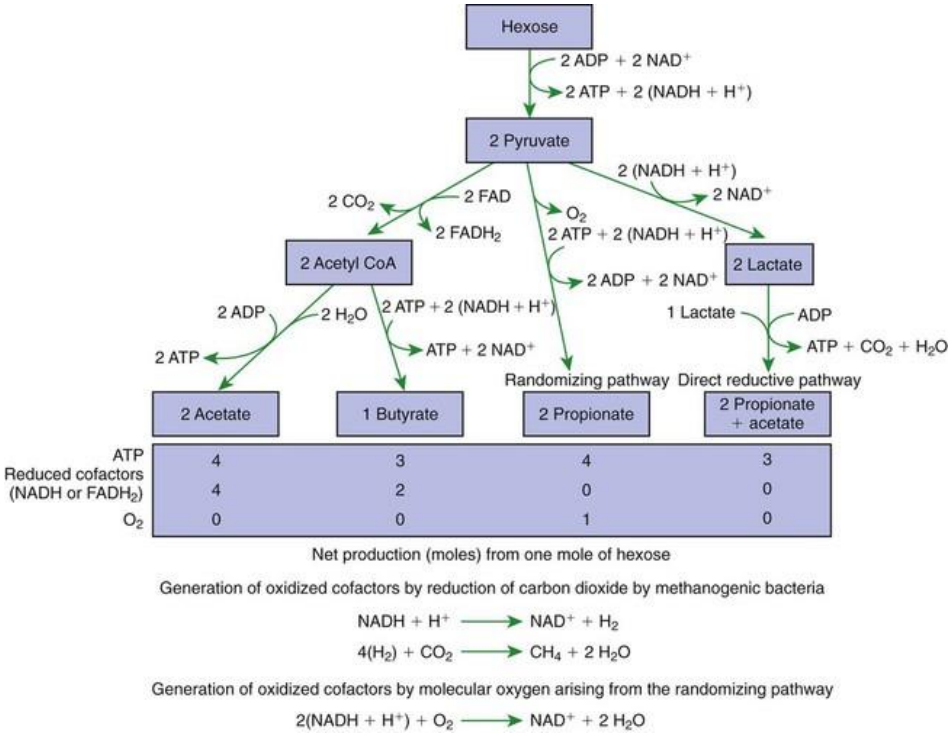


Figure 1.1. Pathways of volatile fatty acid production by the rumen.
(A) Dietary carbohydrates are fermented by anaerobic microorganisms in rumen and converted to VFAs. (B) Detailed biochemical pathways of VFA and methane production. *(Sources:Reprinted from A.http://www.em-la.com/archivos-de-usuario/base_datos/grass_silage_treated_with_em_silage.pdf.*
B. https://veteriankey.com/digestion-the-fermentative-processes/)

Small Intestine microbiome and metabolism

Microbiome in small intestine of cattle

After the digestion happening in upper GI tract, peristalsis and a portion of undigested fibrous residuals together play a major role in pushing digesta downward, to enter the small intestine and be in contact with villi of the intestinal epithelium where absorption happens [9]. Nutrients such as proteins, nucleic acids, vitamins, minerals, and fats, are mostly absorbed in the small intestine. Specifically, microbial proteins and rumen undegraded proteins are broken down to peptides in the upper gut, and finally got absorbed in the small intestine as amino acids. Dietary fats are broken to fatty acids and glycerol in small intestine and absorbed. Glucose from carbohydrates is also absorbed here [9, 10].

Studies of cattle microbial communities throughout the GI tract demonstrated composition differences of microbiota in the small intestine compared to other segments in both dairy and beef cattle [11, 13]. The small intestine of cattle GI tract is the segment where abundance and diversity of microorganisms are significantly reduced compared to rumen and hindgut (cecum and colon) in ruminant species [11, 29, 40, 41]. In a study published in 2017 that tracked the entire digestive tract with 15 gut sites in a beef steer, it has been reported that, after digesta moved to the small intestine, the number of OTUs decreased for nearly half when compared with rumen, but OTU number increased again in the large intestine and downward sections. Shannon index denoting alpha diversity of microbiota was also reduced in the small intestine, especially in duodenum and jejunum [13]. Similar trends were observed in a

metagenomic study of bacterial communities along the digestive tract of dairy cattle and a sheep study [11, 12]. In both of the above studies, taxonomic pattern differences were still present between the small intestine and other gut locations starting from the phyla level [11, 13].

As a specific segment belonging to the small intestine in ruminant, jejunum is the main organ to absorb proteins and carbohydrates, bacterial composition also differs a lot with rumen and colon [41]. Regarding the detailed microbial composition differences in the jejunum, one obvious difference is that, compared to rumen where Bacteroidetes is the most dominant phylum, Firmicutes populations are the most abundant phyla in the jejunum, whereas Bacteroidetes only contributes few percentages of the entire phyla in the jejunum of cattle [13, 29, 41]. A study focusing on jejunum microbiota of steers have characterized jejunum bacterial structure by 16S-seq on beef cattle with different feed efficiencies. Besides Firmicutes that possessed up to 90% of jejunum phyla, remaining phyla that accounts for more than 1% in relative abundance included Acinobacteria (6% to 13%), Proteobacteria (0.8% to 5.8%), Bacteroidetes (0.4% to 1.1%), and Tenericutes (0.4% to 4%) [41].

Another distinctive feature of small intestine is the microbial structure similarity between digesta and mucosa of the small intestine in mature cattle. Two of the gut microbiota studies mentioned above in beef and dairy cattle also compared microbial compositions along the most gut segments between digesta and corresponding gut tissues (mucosa). Bacterial characteristic differences between digesta and mucosa were

observed in four upper gastric compartments and large intestine, but not in small intestine [11, 13]. Therefore, it was suggested that cattle small intestine's mucosa and digesta share a similar microbial taxonomic structure.

Overall, small intestine is the middle segments of the entire digestive tract, and its differences with other gut segments are distinctive. It is normal that small intestine always possessed the least amount of microbial numbers, because small intestine has limited contacts with outside environments and lower oxygen levels. Although priority research in cattle gut microbiota always focused on rumen and feces in ruminant to study animal nutrient, animal health, and environmental effects, there are possibilities that middle gut segments in between could affect microbial structures and function of others and also influence animal metabolism [13]. Characterizing small intestine microbial profiles are also valuable. High-throughput sequencing methods would be recommended for studying small intestine microbiome that has lower bacteria number in total. It would be critical to have adequate sequencing depth and sample size to profile small intestine microbiome accurately in scientific studies [14].

Bile acids in small intestine of cattle

Bile acids are produced in liver and stored in gallbladder, and secreted into duodenum to aid absorption of fatty acids in the small intestine of ruminants [42]. Synthesis of primary and secondary bile acids and their metabolism are summarized in Figure 1.2 [43]. Primary bile acids are those synthesized in the liver from cholesterol. Cholic acids and chenodeoxycholic acids are primary bile acids, as well as their conjugates with

taurine or glycine in liver, which are tauro(glyco)cholic acid (T(G)CA) and tauro(glycol)chenodeoxycholic acids (T(G)CDCA) (Figure 1.2) [42, 43]. By microbial action in the small intestine, primary bile acids were mediated to produce secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA). Most of the bile acids can be reabsorbed by the enterohepatic circulation and recycled in the liver [42, 44]. As for bovine, besides bile acids mentioned above, several other kinds of bile acids could be found in cattle bile, such as 3α, 7α, 12α-Dihydroxy-5β-cholanoic acids and 12a-dihydroxy-3-keto-5β-cholanoic acids [42]. Ursodeoxycholic acid, the product of bacterial transformation of chenodeoxycholic acid, could also be detected in a relatively low concentration in cattle gut [42, 45].

Bile acids also interact with gut microbiota in the small intestine. Several publications explored the relationship between bile acids and gut microbes [43, 46, 47]. Bile acids deconjugation and 7α/β-dehydroxylation are catalyzed by bacteria. It has been reported that gram-positive species such as *Lactobacillus, Enteroccocus, Bifidobacterium,* and *Clostridium* performed bile acids deconjugation with an enzyme, bile salt hydrolase (BSH) [47]. Next, 7α/β-dehydroxylation activities converted primary bile acids into secondary bile acids by many kinds of bacteria. Examples were bacterial activities of *Clostridiales* and families of *Lachnospiraceae* and *Ruminococcaceae*, and genera of *Blautia*, *Clostridium*, and *Eubacterium* [43, 46-48].

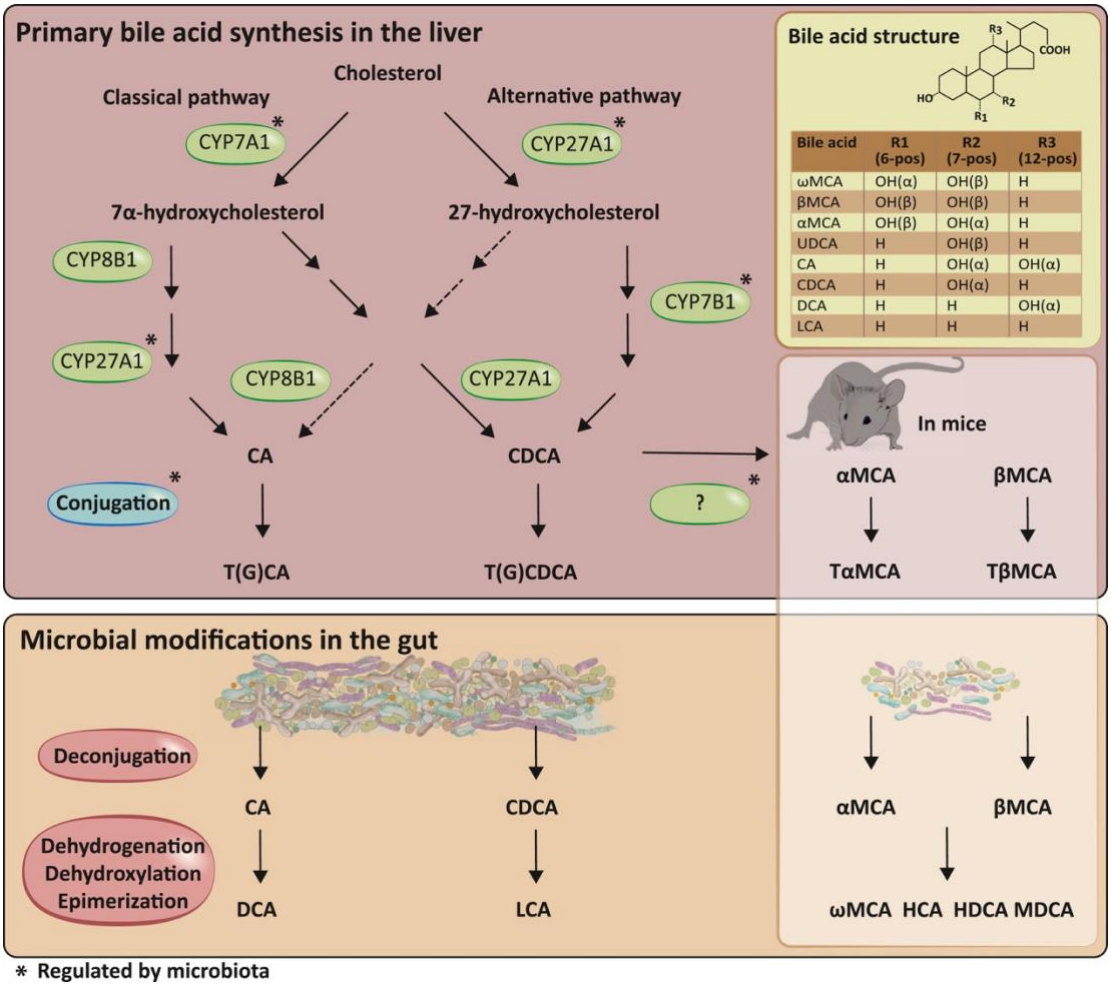


Figure 1.2. Synthesis of primary and secondary bile acids.
Schematic representation of synthetic pathways of primary bile acids in hepatocytes (pink) and secondary bile acids in the intestine (orange). Top right table provides a summary of hydroxylation on steroid nucleus of common bile acids.
(Source: Reprinted from [43].)

Colon and Rectum microbiota

Digestive tract of ruminant is complicated and contains varieties of segments to have different biological functions and microbial structures. The digesta from one compartment flows to be the input for the next neighboring compartment. Thus, different segments of gut are also closely connected. Intestine and colon microbiome are also pertinent to diets because of their functions of post-ruminal degradation of cellulose and starch [49]. Feces is the end products from sequential digestion bioactivities of ingesta from the beginning of digestive tracts [13]. Microbiota of the upper intestines can also be largely and conveniently conjectured through the microbial profiles of feces in a noninvasive manner, which can get around the problem of inaccessibility of intestinal contents in live animal and human.

Because of the complexity and length of the ruminant digestive tract, colon gut microbiota also possess a distinct microbial structure compared with forestomach and small intestine [13, 29, 50, 51]. In early age as pre-weaned calves, this dissimilarity already appeared [51]. Unlike small intestine where Firmicutes accounted for more than 90% abundance of gut microbiota, Bacteroidetes in colon was the dominant phylum (~50%) followed by Firmicutes in pre-weaned calves [51]. Colon microbiota also shared more bacteria genera with rumen than with small intestine [13, 51]. However, as calves grow, Firmicutes was reported to be the dominant phylum in several studies in mature cattle colon [13, 50]. As for specific taxa of Firmicutes in the large intestine, members of *Clostridiaceae, Ruminococcaceae,* and *Lachnospiraceae* were commonly observed dominant families [50, 52]. In addition, 70% of the rumen

OTU that belongs to Firmicutes could also be found in the rectal microbiome [13]. As for the second dominant phylum Bacteroidetes, *Bacteroidaceae* was most abundant in the large intestine. *Rikenellaceae* was a unique family of Bacteroidetes characterized in large intestine, but not in other segments in a study of gut microbiota across GI tract of a Brazilian Nelore steer [50]. At the genus level, *Prevotella*, *Ruminococcus*, *Coprococcus*, *Dorea*, and *Oscillospira* were present with relatively higher abundance in cattle colon [50, 52]. *Prevotella* was commonly present in cattle feces and was reported to be positively associated with high-grain diets [53]. *Oscillospira* in cattle colon was found to be associated with average daily gain (ADG) and may be potentially related to bypass starch from forestomach of cattle. In comparison with forestomach and small intestine, rectum microbiota also had significantly lower abundances of predicted microbial pathways, including carbohydrate metabolism, amino acid metabolism, and energy metabolism, which was expected because of the limited microbial functions in rectum [11].

Rationales and hypothesis of this project

There have been controversies between grass-fed and grain-fed beef products in the beef market, and several studies profiled the differences between the two diets from the aspects of nutrition, transcriptome, and metabolome [54-56]. In addition, some studies also focused on the microbiome of beef cattle digestive tract from the two diets, and most attention were the associations of microbes with the production of volatile fatty acids in rumen [20, 27, 28, 57]. However, lack of knowledge in microbial profiles of other segments of cattle digestive tract needs to be solved. The advantage of 16S rRNA gene sequencing has also allowed researchers to characterize gut microbiome profiles with finer resolutions in recent years. Therefore, in this project, we would like to reveal the diet-induced changes in gut microbiome of beef cattle under different diets, grass-fed and grain-fed diet, to further explore how these induced changes in microbiota could potentially influence meat quality of beef cattle. Overall, we hypothesized that different diets, grass-fed and grain-fed diets, could result in significant changes in metabolites and gut microbiome of beef cattle, which further post impacts on meat quality of beef cattle. We combined microbiome profiles of the three gut segments, rumen, jejunum, and rectum, and used different metabolite measurements, including VFA production in rumen and bile acids composition from bile liquid using Wye Angus beef cattle. The aim was to provide the scientific evidence to the market need of beef and research field of the beef cattle industry. We would also like to exert a more in-depth and detailed level of exploration that combines diets, gut microbiome, and key host metabolic chemicals altogether to promote the understanding between grass-fed

and grain-fed regimens, and ultimately provide potential implications to beef quality for the beef cattle industry.

2. Rectal microbiome differences induced by diets in beef cattle

Introduction

In beef production, grass-feeding beef has appealed much attention in recent years, and it is marked a higher unit price than grain-fed beef. One of the key differences between the two feeding regimens is the diet component difference. Diets have been proved to be a dominant factor to determine the microbiome in the digestive tract. Several studies reported that differences in diets lead to significant changes in gut microbiota, resulting in substantial differences in microbial structures and functions [15, 17, 19, 20]. As for cattle, diet components can also induce several changes in gut microbiota to further influence animal health and performance [20, 40, 58]. Within cattle digestive system, bacteria, fungi, and protozoa are main types of microorganisms that coexist in the gut. Among the three, bacteria are the dominant and most important kind of microbes that could occupy 60% to 90% of total rumen microbial mass [9]. The grass-fed method contains a large portion of dietary fibers that get processed in cattle gut, and also serves as the source of energy for rumen bacteria fermentation [9]. Characterizing and comparing gut bacteria profiles between grass-fed and grain-fed cattle are necessary to clarify diet-induced differences on gut microbiome and their further potential influences on cattle performance. It was also reported that diet regimens could post their influences on gut microbiome in early ages [59]. Therefore, in this chapter, we focused on the effects of grass-fed and grain-fed diets on young beef cattle gut microbiome and hypothesized that diets could induce significant changes in bacterial

communities in the gut of young cattle. Besides, because grass-fed beef cattle take longer to reach market weight and always are slaughtered four months later than grain-fed cattle, rumen and intestine gut contents cannot be collected at the same time for the two groups. This unmatched sample collection time in the future may introduce biases. Thus, we first profiled gut microbiome of young cattle from the two groups at their same age to provide a baseline for our future gut microbiome comparison of rumen and small intestine fluid between grass-fed and grain-fed cattle.

Materials and Methods

UMD Wye Angus herd and experimental design

Steers selected for this study came from a population of cattle in the University of Maryland Wye Angus beef cattle herd. This herd has been kept closed for over 50 years and maintained a very similar genetic background for all offspring, which reduces the genetic variations of groups for our study. Two kinds of feeding methods, grain-fed and free-range grass-fed methods, were used in this herd as two experimental groups in this study. The grain-fed group received finishing diets including corn silage, shelled corn, soybean and trace minerals, which were high in energy and protein. The grass-fed group had free access to grazed alfalfa and consumed bailage during cold seasons. The alfalfa contained no fertilizers, pesticides or other artificial chemicals. Steers from the grass-fed group in this herd were not fed with any animal, agricultural, or industrial byproducts, and were not fed with any types of grain. Individual steer's date of birth, birth weight, dam, and sire information were all recorded. Every 24 to 28 days, body weight was measured, and average daily gain (ADG) was calculated. All these metadata of the steers were kept in record.

Rectal contents sampling and preparation

Twenty-two young male cattle were obtained and raised at UMD Wye Angus herd. 12 cattle were fed with grass *ad libitum*. Other ten cattle were fed with grain diets. Around seven months of age, luminal digesta contents at the rectum of both groups of cattle were sampled at UMD Wye Angus farm (Queenstown, MD) and immediately frozen in dry ice prior to storage at – 80 °C freezer in the lab until microbial DNA extraction.

Steers of this study were handled according to the protocol (protocol # R-11-72) approved by the Beltsville Area Animal Care and Use Committee, and the Institutional Animal Care and Use Committee at the University of Maryland (UMCP-IACUC). Sample collection was performed strictly according to this protocol.

DNA extraction, 16s rRNA gene amplification, and sequencing

Microbial DNA from rectal contents was extracted using QIAamp DNA stool kit (Qiagen, Valencia, CA) with slice modifications that replaced the lysis procedure in protocol with an eight-minute 95 °C incubation in a water bath. DNA concentration was then measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). 16S rRNA gene sequencing was performed. The commonly used hypervariable V3-V4 regions of the 16S rRNA gene were amplified with 20 cycles through PCR from 20 ng of total DNA with PAGE-purified Illumina platform-compatible adaptor oligos. These adaptor oligos included sequencing primers, sample-specific barcodes, and 16S PCR primers (forward primer, 341/357F: NNNNCCTACGGGNGGCWGCAG; reverse primer, 805/785R: GACTACHVGGGTATCTAATCC). The PCR reaction included 2.5 units of AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a 50-µl reaction buffer, which contained 200 nM primers, 200 nM dNTP, 60 mM Tris-SO_4, 18 mM $(NH4)_2SO_4$, 2.0 mM $MgSO_4$, 1% glycerol, and 100 ng/ul bovine serum albumin (New England BioLabs, Ipswich, MA). PCR cycling detail was as follows: initial denaturing at 95 °C for two min followed by 20 cycles of 95 °C 30 sec, 60 °C 30 s, and 72 °C 6 s. Amplicons were finally purified using Agencourt AMPure XP bead kits

(Beckman Coulter Genomics, Danvers, MA). DNA concentration and sizes were quantified using a BioAnalyzer 2000 with high-sensitivity DNA chip and a QuantiFluor fluorometer (Agilent, Palo Alto, CA). Purified amplicons from all samples were pooled together in equal molar ratios. The purified amplicon pool was further spiked with approximately 25% of whole-genome shotgun libraries with a compatible adaptor barcode to guarantee sequence diversity during the first few cycles of sequencing to have better cluster differentiation. The final library pool was quantified with BioAnalyzer and then was sequenced in an Illumina MiSeq sequencer with an Illumina MiSeq Reagent Kit according to the manufacturer's protocol.

Bioinformatics and data analysis

Raw sequences were first analyzed with FastQC (version 0.11.5) to examine the quality of sequencing. The first four maximally degenerate bases ("NNNN") at the most 5' end was designed to maximize the diversity during sequencing run to better identify unique clusters and improve base-calling accuracy, were trimmed with Trimmomatic version 0.38 [60]. Then, the processed pair-end reads were merged using PandaSeq v 2.11 with default parameters to join the reads into representative complete nucleotide sequences (contigs) [61]. Overlapped reads with high mismatches and low-quality scores were filtered and removed during this process. QIIME (Quantitative Insights Into Microbial Ecology) version 1.9.1 pipeline was used to analyze the processed 16S rRNA gene sequencing data [62]. OTU (operational taxonomic unit) was identified using a closed-reference OTU picking protocol with a threshold of 97% similarity. Taxonomic assignment was performed based on GreenGene database v13.8 [63]. Alpha (α) -

diversity and beta (β) diversity were calculated and examined using QIIME and R. PCA (Principal component analysis) was performed, and graphs were plotted with R packages ade4 [64] and factoextra [65]. PICRUSt (v1.1.2) was used with default parameters to predict metagenomics, KEGGs gene families with regarding contributions of OTU, and functional categories [66]. The software takes the OTU table from QIIME as input. After reads normalization with *normalize_by_copy_number.py*, the workflow *predict_metagenomes.py* was applied to predict metagenomes and obtain KEGG Orthologs. Predicted metagenome functions were finally analyzed by *categorize_by_function.py* to collapse thousands of KEGG Orthologs into higher functional categories (pathways). LEfSe algorithm [67] was used in the differential analysis to identify significantly different OTUs, KEGGs, and pathways between the grass-fed group and grain-fed groups. LEfSe is able to identify abundant differences of representative features between two or more sample conditions, considering samples' biological categories as well as statistical significance to perform comparative analysis based on feature abundance. Non-parametric factorial Kruskal-Wallis (KW) sum-rank test, Wilcoxon rank-sum test, and a Linear Discriminant Analysis were used together to identify differential features and effect sizes [67].

Results

Microbial population and diversity of grass-fed and grain-fed groups

Microbial diversity of the rectum was examined based on OTU table generated from the QIIME (Quantitative Insights Into Microbial Ecology) closed reference pipeline [62]. In total, there were 19 bacteria phyla identified from grass-fed and grain-fed groups (Figure 2.1). The most abundant phylum was Firmicutes, ranging from 38.36% to 68.42% of relative abundance percentages. The other phyla that had relative abundance greater than 1% were Bacteroidetes (37.77%), Proteobacteria (3.96%), and Verrucomicrobia (1.20%). As for the family level, a total of 93 families were identified. *Ruminococcaceae* was the most abundant family, which accounted for approximately 36.91% of the taxa. 12 families had a relative abundance higher than 1%. The top five were *Ruminococcaceae* (36.91%), *Bacteroidaceae* (10.92%), *Rikenellaceae* (6.20%), *Lachnospiraceae* (4.61%), and *Paraprevotellaceae* (4.34%). For OTUs, 4183 total OTUs were identified using an identity threshold of 97%. Some named species with high abundances included *Clostridium butyricum* (0.18 %), *Clostridium difficile* (0.17%), and *Bacteroides uniformis* (0.10%).

Microbiome diversities were examined for grass-fed and grain-fed groups. Alpha-diversity indices including Chao1, Shannon, Simpson, and Phylogenetic diversity (PD_whole_tree) were calculated (Table 2.1). Because of the unbalanced sample size and the situation of the sample, a Welch's t-test was used to perform a differential test between the two groups and obtain p values (Table 2.1). In general, the grass-fed group had higher alpha diversity values of indices than the grain-fed group, and p values of

all four indices showed significant differences. Therefore, it was suggested that diets significantly influenced microbial diversity between the two groups, and grass-fed group tend to have higher microbial diversity than grain-fed group ($p < 0.05$). PCA (Principal component analysis) was also performed to examine beta diversity to explore the diversity between groups. The analysis was performed based on the top 10 most abundant microbial families and demonstrated in a PCA plot. The results showed a clear separation between the two groups in microbial structure within the rectum of beef cattle under grass-fed and grain-fed diets (Figure 2.2).

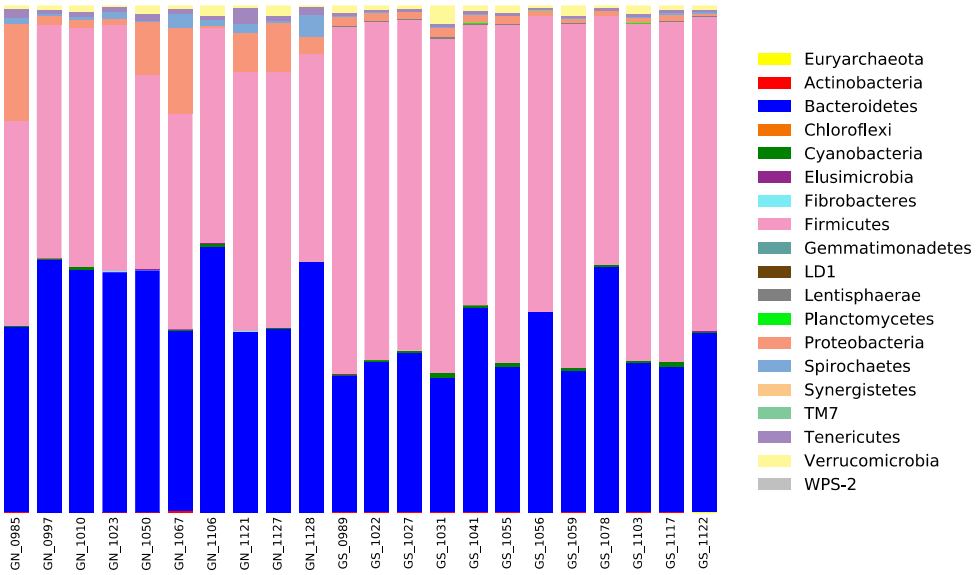


Figure 2.1. Phylum-level microbial composition in cattle rectum.
The first ten samples were grain-fed (GN) individual cattle; the last 12 individuals were cattle from grass-fed (GS) group.

Table 2.1. Alpha diversity indices in cattle fecal microbiome.

Welch's t-test was used to calculate p-values.

Diversity Indices	Grain-fed	Grass-fed	p value
Chao1	1596.03 ± 147.74	1876.16 ± 97.42	0.0001
Shannon	7.09 ± 0.21	7.86 ± 0.20	< 0.0001
Simpson	0.980 ± 0.006	0.990 ± 0.002	0.0004
PD_whole_tree	61.86 ± 3.77	72.25 ± 2.95	< 0.0001

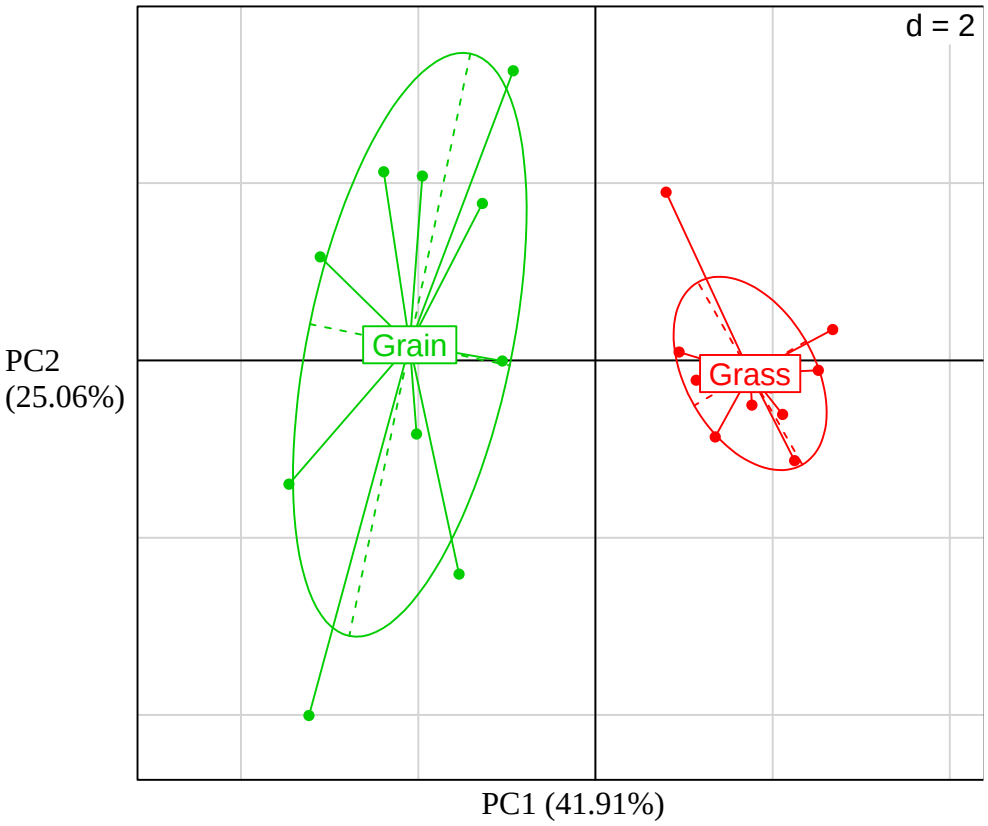


Figure 2.2. Beta diversity assessment of rectum feces microbiota.
Principle Component Analysis (PCA) was performed with ade4 package in R based on top abundant families in the rectal microbiome.

Diets altered the microbial composition of the rectal microbiome

The difference in rectal microbial composition between grass-fed and grain-fed groups was examined. Relative abundances of taxa were computed by QIIME and analyzed with the Linear Discriminant Analysis (LDA) Effect Size (LEfSe) algorithm [67]. At the phylum level, 14 out of 19 taxa were significantly different in relative abundance. Among these 14 taxa, eight phyla showed higher abundance in grass-fed group, while six had greater abundance in grain-fed group (absolute LDA score log10 $\geqslant$ 2.0). As for the family level, 93 named families were identified in total, from which 47 families showed differences in relative abundances (Figure 2.3). For example, Ruminococcaceae, BS11, and Porphyromonadaceae were top three discriminative features in the grass-fed group, while Succinivibrionaceae, S24-7, and Lachnospiraceae were top three discriminative families in the grain-fed group. At the OTU level, 402 OTU had a significant difference in relative abundance (absolute LDA score log10 $\geqslant$ 2.0). Of them, 144 OTU showed enrichment in the grain-fed group, and the other 258 OTU showed a higher abundance in the grass-fed group. The top 20 most abundant significant OTU were listed in Table 2.2.

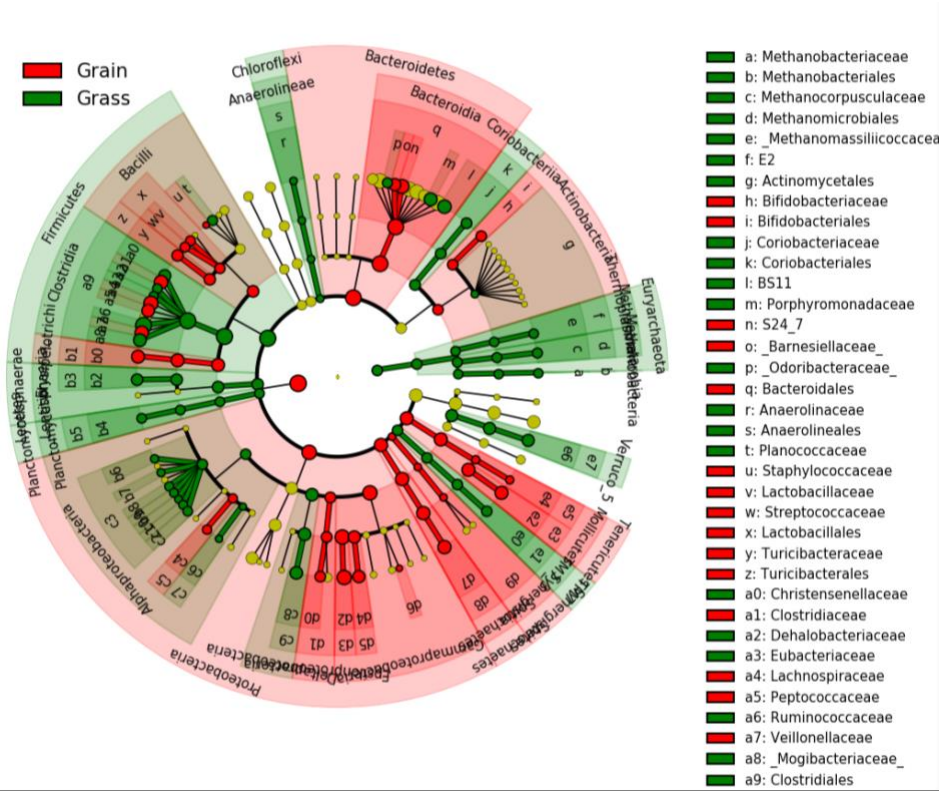


Figure 2.3. A cladogram representing the taxa in rectum microbiota.
Taxa at the family level that had significantly different abundances between grass-fed and grain-fed cattle with an absolute Linear Discriminant Analysis (LDA) score log10 $\geqslant$ 2.0 were displayed.

Table 2.2. Selected 20 significantly different OTUs in relative abundance in the rectal microbiome of grain-fed and grass-fed Angus beef cattle.

The OTU was identified based on Greengenes 16S rRNA database version 13_8. The numbers denoted the relative abundance (Mean ± SD) of corresponding OTUs in the grain-fed and grass-fed group. The LDA log10 score was the calculated logarithm of LDA scores using LefSe algorithm.

GreenGenes ID	Grain-fed	Grass-fed	LDA log10 score	Annotation (Domain|Phylum|Class|Order|Family|Genus|Species)
327184	0.0077 ± 0.0043	0.0319 ± 0.0069	4.08	Bacteria|Firmicutes|Clostridia|Clostridiales
509709	0.0078 ± 0.0039	0.0289 ± 0.0069	4.03	Bacteria|Firmicutes|Clostridia|Clostridiales| Lachnospiraceae
199226	0.0103 ± 0.0066	0.0213 ± 0.0092	3.78	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
298717	0.0178 ± 0.0123	0.0115 ± 0.0055	3.44	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
330414	0.0253 ± 0.0340	0.0000 ± 0.0000	4.08	Bacteria|Firmicutes|Clostridia|Clostridiales
1112614	0.0203 ± 0.0208	0.0038 ± 0.0008	3.92	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
85199	0.0172 ± 0.0098	0.0060 ± 0.0020	3.74	Bacteria|Firmicutes|Clostridia|Clostridiales| Veillonellaceae
146314	0.0233 ± 0.0409	0.0000 ± 0.0000	4.05	Bacteria|Tenericutes|Mollicutes|RF39
341737	0.0125 ± 0.0048	0.0075 ± 0.0026	3.33	Bacteria|Firmicutes|Clostridia|Clostridiales
177109	0.0069 ± 0.0041	0.0110 ± 0.0031	3.28	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae|Oscillospira
593357	0.0062 ± 0.0019	0.0114 ± 0.0060	3.38	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales| Prevotellaceae|Prevotella
323737	0.0111 ± 0.0061	0.0044 ± 0.0014	3.53	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
248126	0.0160 ± 0.0144	0.0000 ± 0.0000	3.90	Bacteria|Firmicutes|Clostridia|Clostridiales| Peptostreptococcaceae
554560	0.0115 ± 0.0061	0.0036 ± 0.0014	3.60	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
287909	0.0004 ± 0.0010	0.0127 ± 0.0076	3.79	Bacteria|Firmicutes|Clostridia|Clostridiales
287798	0.0083 ± 0.0032	0.0056 ± 0.0016	3.12	Bacteria|Firmicutes|Erysipelotrichi|Erysipelotrichales| Erysipelotrichaceae
341730	0.0091 ± 0.0037	0.0049 ± 0.0015	3.32	Bacteria|Firmicutes|Clostridia|Clostridiales| Lachnospiraceae|Coprococcus
332778	0.0036 ± 0.0014	0.0093 ± 0.0022	3.45	Bacteria|Firmicutes|Clostridia|Clostridiales| Lachnospiraceae
2051	0.0125 ± 0.0098	0.0016 ± 0.0016	3.76	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales| Prevotellaceae|Prevotella
353691	0.0073 ± 0.0031	0.0057 ± 0.0007	2.80	Bacteria|Firmicutes|Erysipelotrichi|Erysipelotrichales| Erysipelotrichaceae

Biological pathways and functions predicted from 16S data

Biological functions of microbiota are vital factors that influence the micro-environment within the digestive tract. In this study, we used a method called Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [66] to predict metagenome from sequenced 16S rRNA genes. Then, predicted functional pathways based on 16S-seq data could be used to explore the potential biological functions influenced by grass-feeding and grain-feeding on Angus beef cattle. After normalization of read counts of OTU table, 6910 KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology gene families with KO identifier and annotation were identified by PICRUSt in total from the OTU table. Then, LefSe was performed with a cutoff value of linear discriminant analysis (LDA) score log10 $\geqslant$ 2.0. It was found that under the grain-fed diet, 26 KEGG gene families showed significantly higher abundances, whereas 47 KEGG genes had significantly higher abundances in the grass-fed group. For example, RNA polymerase sigma-70 factor (K03088), antibiotic transport system ATP-binding protein (K09687), and subfamily B of ATP-binding cassette (K06147) were the top three significantly different KEGGs in the grass-fed group. Iron complex outer-membrane receptor protein (K02014), beta-hexosaminidase [EC:3.2.1.52] and hexosaminidase [EC:3.2.1.52] (K12373), and outer membrane protein (K06142) had significantly higher abundance in the grain-fed group.

Diets also had an impact on microbial pathways of gut microbiota. From these identified 6910 KEGG gene families, higher KEGG functional categories were classified using PICRUSt. In total, 328 functional KEGG pathways were identified

from the data, among which 74 pathways showed significant differences between grass-fed and grain-fed groups with Lefse algorithm (LDA score log10 $\geqslant$ 2.0). To select and display the top significant pathways between the two groups, we used a higher cutoff value of $\log_{10}$ LDA $\geqslant$ 2.5, and 21 pathways were depicted in Figure 2.4. Several primary biological pathways involving bacterial activities, cellular signaling, and energy metabolism showed significant differences. Specifically, transporter, ABC transporters, sporulation, and bacterial motility proteins showed higher abundances in the grass-fed group, whereas oxidative phosphorylation, lipopolysaccharide biosynthesis and its proteins, and membrane and intracellular structural molecules were significantly increased in abundances in the grain-fed group.

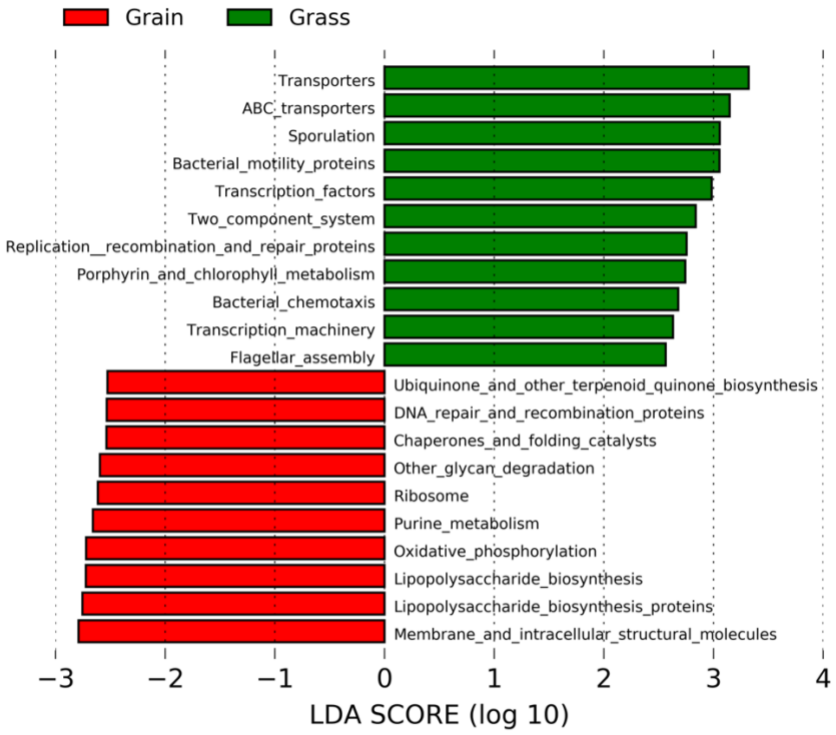


Figure 2.4. KEGG pathways of rectum microbiome significantly impacted by diets between grass-fed and grain-fed Angus beef cattle.

Discussion

Grass-fed and grain-fed methods are two dominant feeding regimens in beef cattle. As diets are directly related to microbiota, exploration of diet influences on the gut microbiome is necessary to guarantee a properly functioning gut and determine its further impacts on cattle performance. Because gaining access to forestomach and small intestine is difficult in live animals, effects of diets on the gut microbiota are always determined using rectal microbial communities. In this study, we explored the diet effects on rectal microbiome between 7-month grass-fed and grain-fed Angus cattle. We utilized the NGS technique, 16S rRNA gene sequencing, to gather the microbial taxa information in the communities as a whole. The purpose is to compare the community differences induced by diets, and, importantly, to provide a baseline for future small intestine and rumen microbiome studies.

The primary result of our study was that grass-feeding and grain-feeding altered microbial community structures, diversities, and biological functional categories in the rectum. The dominant phyla were in agreement with previous studies of cattle gut microbiota that Firmicutes was the most abundant phylum near the colon, followed by Bacteroidetes and Proteobacteria [29, 50]. However, we identified significantly different phyla in relative abundance between grass-fed and grain-fed using LefSe algorithm (Figure 2.3). Specifically, Firmicutes was almost five-fold higher in abundance in the grass-fed group than the grain-fed group in LDA value (Log_{10} LDA = 4.7), whereas Bacteroidetes (Log_{10} LDA = 4.4) and Proteobacteria (Log_{10} LDA = 4.2) were ~4 fold higher in the grain-fed group. These phyla were commonly found

within the digestive tract of animals, and also were found in colon and feces of steers, and our results were also in agreement with similar studies about diets and feeds [52, 68].

At higher taxonomic levels, several common taxa were identified to be significantly different between grass-fed and grain-fed cattle rectum (Log_{10} LDA $\geqslant$ 2.0). For example, *Ruminococcaceae*, the top abundant identified family in rectum microbiota, was ~5 fold higher in effect size in the grass-fed group than the grain-fed group. A genus, *Provotella* (OTU ID 84913), and a named species *Prevotella copri* (OTU ID 326482) were both high in the abundance of the grass-fed group, suggesting potential differences in feed efficiency and methane production in cattle upper gut compartments [69]. Some named genera were also identified to provide possible influences of diets on fermentation strains of the upper forestomach. For example, a named species *Succinivibrio* was identified to be 2-fold greater in effect size of abundance in the grain-fed group than the grass-fed group of cattle rectal microbiome (Log_{10} LDA = 2.3). *Succinivibrio* species ferments glucose to produce high amounts of succinic acid as their main products [70]. It was also found that when fed cattle with diets that contain high levels of rapidly fermented carbohydrates, especially starch, *Succinivibrio* species always existed in cattle rumen [70, 71]. This evidence could explain our observation that the genus *Succinivibrio* was higher in rectum microbiome of the grain-fed group. The rectum microbiome also served as a baseline data and provided a reference for future ruminal and small intestine microbiota characterization.

KEGG gene families and predicted functional pathways also provided potential influences of diets on gut microbiota metabolism between grass-fed and grain-fed groups. For instance, ABC transporter had a higher abundance in the grass-fed group than the grain-fed group, which was likely because of the higher level of Firmicutes in the grass-fed group. In Firmicutes bacteria, ATP-binding cassette (ABC) transporters were evolved to regulate against antimicrobial peptides, and many ABC transporters almost exclusively existed in Firmicutes species [72].

Conclusions

In this chapter, the characterization of rectal microbiome between grass-fed and grain-fed cattle provided a baseline of the future studies about diet-induced changes in gut microbiota. To solve the problem that rumen and intestine digesta samples cannot be collected at the same age of cattle because grass-fed cattle took longer to reach market weight (470 kg) to be slaughtered, rectal samples were collected at the early time in young cattle at 7-month old in the two groups. Comparisons of rectum microbiome between grass-fed and grain-fed young cattle assured that different diets could induce structural changes in cattle microbiota, reflected in changes in the Bacteroidetes, Firmicutes, and Proteobacteria populations. These proved that the microbiota differences in the rumen and small intestine to be explored in the future between the two groups were not generated by collection time artifact. Results in this gut segment provided a preview of diet-induced effects on gut microbiota, and also provided directions for the following exploration of the rumen and small intestine microbiome differences.

3. Diet-induced changes in rumen microbiome and their potential associations with VFA production in Angus beef cattle

Introduction

Microbial community structure and function in the digestive tract have close relationships with host health and performance. Rumen of ruminants is a representative example. In rumen, microbial fermentation enables the conversion of plant fibers into chemical compounds, which then subsequently get absorbed and digested by the animal [73]. This process makes it possible to utilize the solar energy stored in plant fibers by converting it into food products of the ruminant, such as milk and meat. Characterizing rumen microbial community structures can promote the improvement of energy utilization and feeding efficiency in the cattle industry. Diets are well known to be a major determinant of gut microbiota [15]. For beef cattle, there are two feeding methods in finishing stage of the beef cattle industry, grass-fed and grain-fed methods. As the demand for beef harvested from grass-fed cattle is increasing, understanding the community structure of ruminal microbes between grass-fed and grain-fed beef cattle is necessary to reveal the effect of diets on rumen microbiome. One important feature of ruminal bacteria fermentation is volatile fatty acids (VFA) production from dietary components, which contributes to 70% of cattle's energy supply [9, 32]. Differences in grass-feeding and grain-feeding also influence VFA and lactic acid levels in rumen [9, 25] For example, VFA production involves lactic acids as intermediate products that are often related to ruminal acidosis in high-grain diet [39]. We hypothesized that

different diets can induce significant changes in ruminal microbiome that further post influences on rumen VFA and lactate productions. In this study, we characterized the microbial population of rumen with 16S rRNA gene sequencing, together with VFA and lactic acid quantifications between grass-fed and grain-fed cattle, in order to explore the influences of diets on rumen microbiome and its potential functions in Angus beef cattle.

Materials and Methods

Experimental design and sample collection

Steers selected for this study were originated from UMD Wye Angus farm (Queenstown, MD). Two groups were assigned randomly into grass-fed and grain-fed cattle groups. Feeding method in each group was described in detail in Chapter 2. At around twelve months of age, when steers reached the market weight, they were commercially slaughtered at a slaughterhouse belonging to George G. Ruppersberger and Sons, Inc (Baltimore, MD). Rumen liquid from 9 grass-fed cattle and 15 grain-fed cattle were sampled into sterile 50 ml tubes and then pipette into 2 ml tubes, and immediately frozen in dry ice for transportation, and finally stored in the – 80 °C freezer in lab for later DNA extraction. Steers of this study were handled according to a protocol (protocol # R-11-72) approved by the Beltsville Area Animal Care and Use Committee, and the Institutional Animal Care and Use Committee at the University of Maryland (UMCP-IACUC). Sample collection was performed strictly based on this protocol.

DNA extraction, 16s rRNA gene amplification, and sequencing

Microbial DNA extraction and 16S rRNA gene sequencing were performed as previously described in Chapter 2 Materials and Methods section. Briefly, QIAamp DNA stool kit (Qiagen, Valencia, CA) with slice modifications was used for microbial DNA extraction. DNA concentration was then measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). 16S rRNA gene sequencing was performed. Hypervariable V3-V4 regions of the 16S rRNA gene was

amplified through PCR from 20 ng of total DNA with PAGE-purified Illumina platform-compatible adaptor oligos. DNA concentration and sizes were quantified using a BioAnalyzer 2000 (Agilent, Palo Alto, CA). Amplicons were finally purified using Agencourt AMPure XP bead kits (Beckman Coulter Genomics, Danvers, MA). The library pool was sequenced in an Illumina MiSeq sequencer with an Illumina MiSeq Reagent Kit according to the manufacture's protocol.

Measurements of volatile fatty acids and lactate

Levels of volatile fatty acids (VFA) and lactic acids in rumen liquid were measured in a commercial lab (Dairy One – Forage Laboratory, Ithaca, NY). Rumen liquid samples were stored in the −80 °C freezer until use. 450 ul deionized water was added to 50g thawed samples of each individual. The samples were then mixed well by vortexing for 2 min. The samples were then centrifuged, and the clear supernatant was kept for gas chromatography to measure acetic, propionic, butyric, and iso-butyric acids. Aliquot of extract mixed 1:1 ratio with 0.06M oxalic acid containing 100 ppm trimethylacetic acid was used as an internal standard. Samples were injected into a Perkin Elmer Autosystem XL Gas Chromatograph containing a Supelco packed column with the following specifications: 2m x 2mm Tightspec ID, 4% Carbowax 20M phase on 80/120 Carbopack B-DA. VFA concentrations were determined and expressed as parts per million (ppm). YSI 2700 SELECT (YSI Incorporated Life Sciences, Yellow Springs, OH) Biochemistry Analyzer equipped with an L-Lactate membrane was used for lactic acid measurements. Samples were injected into the sample chamber of YSI Analyzer where L-Lactate diffuses into a membrane containing L-Lactate oxidase. The L-Lactate

was immediately oxidized to hydrogen peroxide and pyruvate. The hydrogen peroxide was detected amperometrically at the platinum electrode surface. The current flow at the electrode is directly proportional to the hydrogen peroxide concentration, and hence to the L-Lactate concentration. Total lactic acid is determined by multiplying L-Lactate by 2.0. T-test was used for differential statistical analysis of VFA and lactic acids between grass-fed and grain-fed groups.

Bioinformatics and data analysis

Bioinformatic pipeline of 16S rRNA gene sequencing used in this study was described in Chapter 2 in detail. Briefly, raw sequence data were first imported to FastQC to examine the quality of sequencing. The first four random nucleotides ("NNNN") were trimmed in R1 reads. R1 and R2 reads for each individual sample were merged into one FASTA files using PandaSeq v2.11 with default parameters [61]. Overlapped reads with high mismatches and low-quality scores were filtered and removed during this process. QIIME v1.9.1 closed reference pipeline was used to analyze the processed 16S rRNA gene sequencing data with an OTU threshold of 97% similarity [62]. Taxonomic assignment was performed based on GreenGenes database v13.8 [63]. Microbial diversities were calculated and examined with QIIME and R. As there were two cattle groups in this study, OTUs that were present in at least 75% of samples were considered as the core microbiome, which could be identified via QIIME compute_core_microbiome.py [62]. PICRUSt v1.1.2 was used for predicting metagenomics and functions with KEGGs and pathways, as well as contributions of OTUs to identified KEGG orthologs [66]. LEfSe algorithm [67] was used in the

differential analysis to identify significantly different OTUs, KEGGs, pathways, and their effect sizes between the grass-fed group and grain-fed groups.

Results

Microbial population and diversity in rumen

QIIME (Quantitative Insights Into Microbial Ecology) closed reference pipeline was used to analyze 16S-seq data with 97% sequence similarities with Greengenes 16S rRNA database. A total of 3756 operational taxonomic units (OTU) were identified across all samples, which were classified into 23 phyla, 51 classes, 85 orders, 154 families, and 299 genera. At the phylum level, the most abundant phylum in rumen microbiome was Bacteroidetes (42.77%), followed by Firmicutes (39.52%), Proteobacteria (3.69%), Fibrobacteres (3.34%), Spirochaetes (2.20%), Verrucomicrobia (1.99%), Tenericutes (1.81%), and Lentisphaerae (1.20%). The kingdom Archaea was also detected. The only one phylum in Archaea was Euryarchaeota, which accounted for 0.12% to 0.72% of phylum abundance in rumen. A stacked bar plot was plotted to represent phylum-level bacteria composition (Figure 3.1). Regarding higher taxonomic levels, among the 154 identified families, there were 15 families that had relative abundances higher than 1%, such as *Prevotellaceae* (18.20%), *Ruminococcaceae* (13.10%), *Lachnospiraceae* (8.53%), *Veillonellaceae* (4.05%), *Fibrobacteraceae* (3.34%), *Paraprevotellaceae* (3.26%), and *Succinivibrionaceae* (2.71%). Core OTUs were identified across all samples of this study, which were considered to be OTUs that were present in at least 75% of samples in this study. Of the total 3756 detected OTU, 1016 OTUs were identified as the core microbiome, which included several named genera and species, such as *Fibrobacter succinogenes*, *Prevotella ruminicola*, *Selenomonas ruminantium*, *Blautia producta*, *Streptococcus luteciae, Ruminococcus flavefaciens*, and *Clostridium aminophilum*.

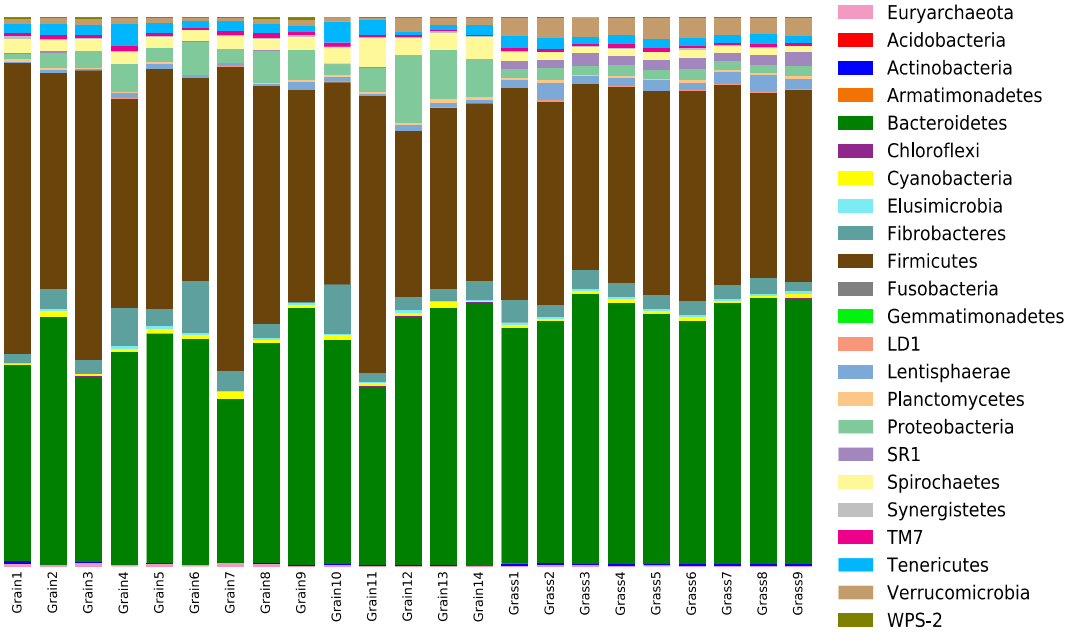


Figure 3.1. Phylum-level composition of ruminal gut bacteria between grass-fed and grain-fed group.
The first 14 bars denoted grain-fed individuals, the last nine bars denoted grass-fed individuals.

Microbial diversities of rumen between grass-fed and grain-fed group were examined. Rarefaction curve was plotted (Figure 3.2) and suggested that the sequencing depth in this study was adequate. Common alpha diversity metrics, including Chao1, Shannon, Simpson, and Phylogenetic diversity (PD_whole_tree), were calculated from QIIME and tested statistically with Welch's t-test and summarized in Table 3.1. All α microbial diversity indices were significantly higher in the grass-fed group than grain-fed group ($p < 0.05$). For example, the Shannon index was 8.70 ± 0.28 (mean ± sd) in the grass-fed group, which was significantly higher in the grain-fed group (mean ± sd = 8.03 ± 0.24). As for the beta diversity examining the differences of microbial

communities between groups, the principal component analysis (PCA) showed a distinct difference based on rumen microbial composition between grass-fed and grain-fed individuals. PCA was plotted based on identified top abundant families of grass-fed and grain-fed groups, and a clear separation of the two groups could be observed (Figure 3.3).

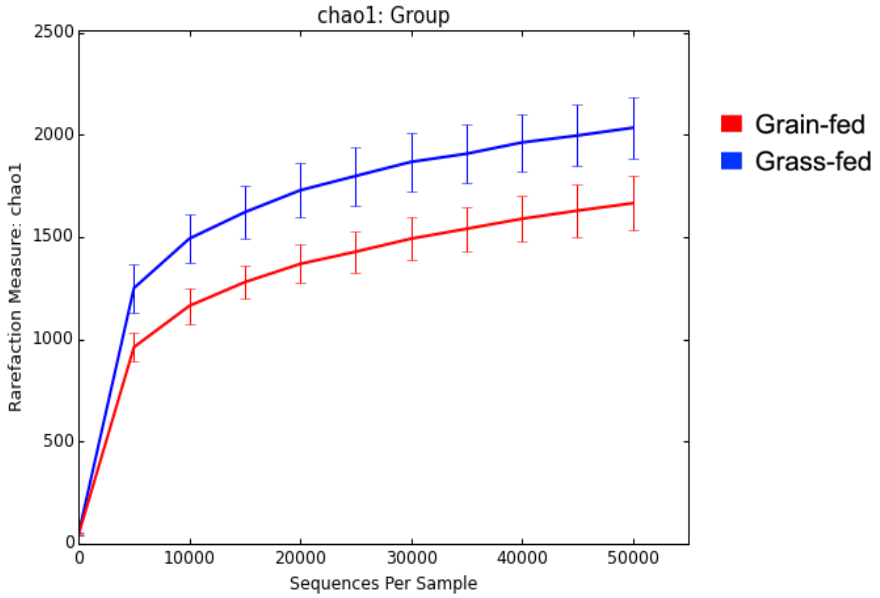


Figure 3.2. Rarefaction curves based on Chao1 values of grass-fed (blue) and grain-fed (red) ruminal microbiome.

Table 3.1. Alpha diversity indices of rumen microbiota in grain-fed and grain-fed Angus beef cattle.

Welch's t-test was used to calculate p-values.

Diversity Indices	Grain-fed	Grass-fed	p value
Chao1	1665.40 ± 136.34	2034.52 ± 157.37	< 0.0001
Shannon	8.03 ± 0.24	8.70 ± 0.28	< 0.0001
Simpson	0.989 ± 0.0003	0.994 ± 0.002	0.00056
PD_whole_tree	74.12 ± 3.29	85.14 ± 4.42	< 0.00001

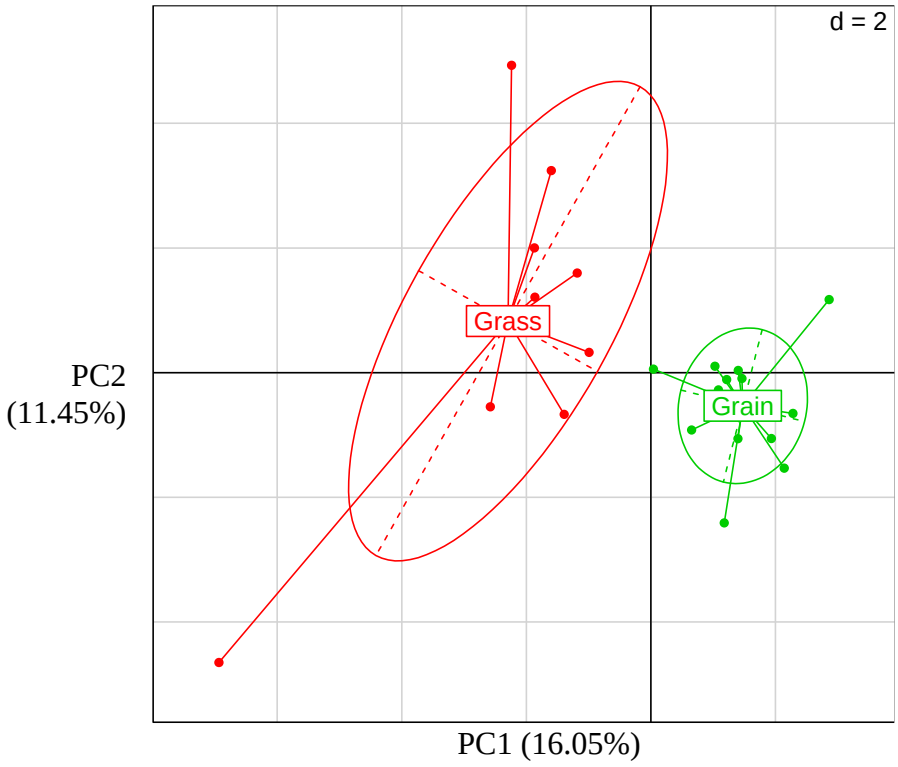


Figure 3.3. Differences in microbial composition of the cattle rumen microbiome between grass-fed and grain-fed cattle.

Principal component analysis (PCA) was performed with ade4 package in R based on the top abundant microbial families.

Diets altered microbial composition of rumen

Microbial composition differences were analyzed to explore the influences of different diets on beef cattle. Relative abundances of various taxa between grass-fed and grain-fed groups were computed by QIIME and examined with the Linear Discriminant Analysis (LDA) Effect Size (LEfSe) algorithm [67]. At the phylum level in the rumen, 12 phyla displayed significant differences (absolute LDA score log10 $\geqslant$ 2.0) (Figure 3.4).

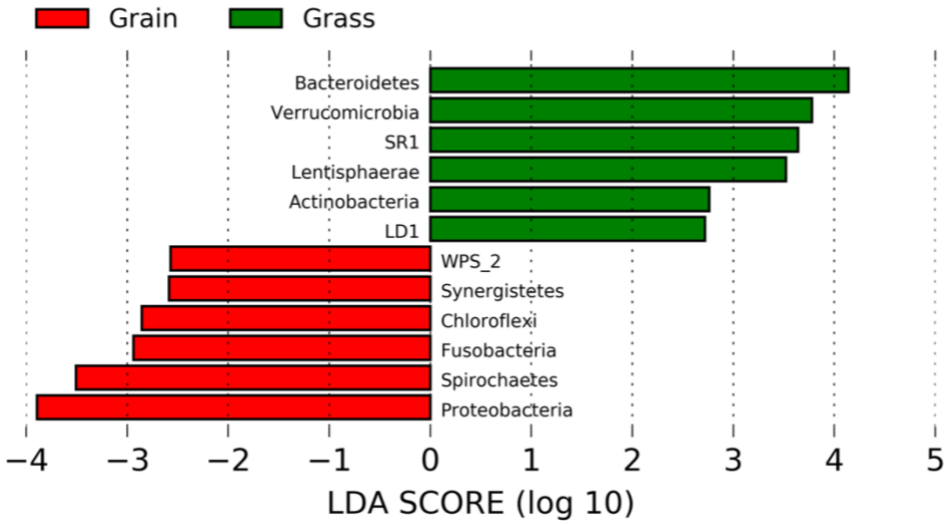


Figure 3.4. 12 significantly discriminative phyla in rumen microbiome between grass-fed and grain-fed beef cattle.

For example, Bacteroidetes and Actinobacteria were significantly higher in relative abundance induced by grass-fed diets, while Proteobacteria and Spirochaetes were higher in the grain-fed group. At higher taxonomic levels, a cladogram based on the family level with emphasis on significantly different taxa was depicted (Figure 3.5). Both groups showed great changes in the composition of the microbial community in

rumen of beef cattle. At the family level, there were 90 significantly distinctive taxa affected by the diets. For named families, 16 families were relatively higher in the grain-fed group, such as *Methanobacteriales*, *Succinivibrionaceae*, and *Lactobacillaceae*. 20 families were relatively higher in the grass-fed group, such as *Methanomassiliicoccaceae, Desulfovibrionaceae,* and *Bacteroidaceae*. At the level of genus, 144 taxa showed significant differences (LDA score log10 $\geqslant$ 2.0). There were 28 named genera enriched in the grain-fed group, while other 116 genera were relatively higher in the grass-fed group. For example, the relative proportion of *Mycobacterium*, *Butyrivibrio, Pseudobutyrivibrio*, and *Ruminococcus* were significantly higher in rumen of grass-fed cattle than grain-fed cattle, whereas grain-fed rumen presented higher abundances of *Megasphaera* (Figure 3.6A), *Prevotella*, *Succinivibrio, Lactobacillus* (Figure 3.6B), and *Clostridium*. At the OTU level, 593 OTUs were significantly different in abundance between grass-fed and grain-fed groups. For example, a commonly observed species in rumen, *Ruminococcus flavefaciens*, was significantly higher in grass-fed rumen microbiome (Figure 3.6C). Selected differentially abundant OTUs between the two groups were listed in table 3.2.

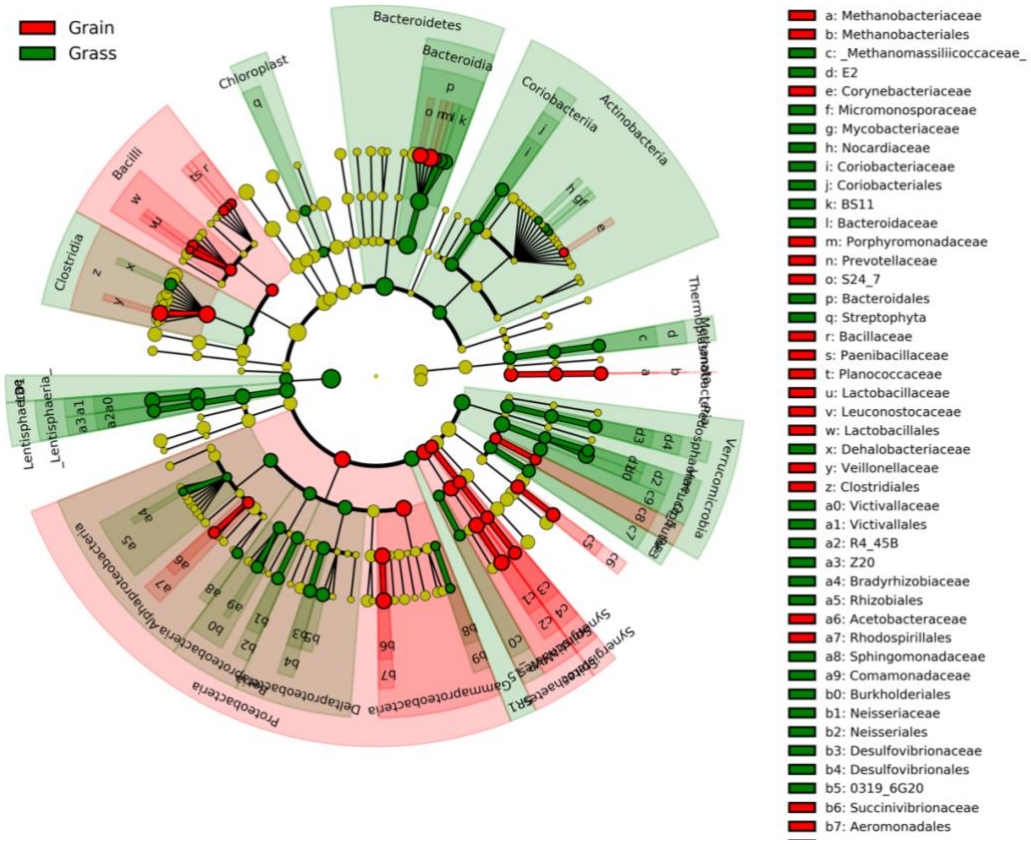
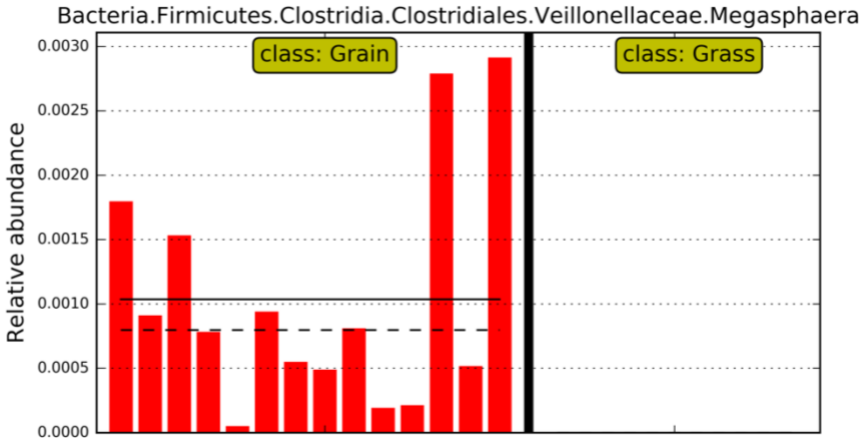
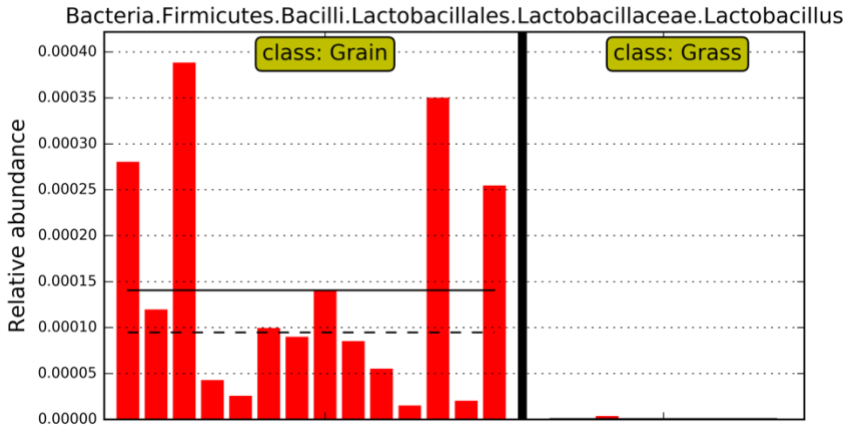


Figure 3.5. A cladogram representing the rumen microbial taxa.
Taxa that had significantly different abundances between grass-fed and grain-fed group were displayed with an absolute LDA score log10 $\geqslant$ 2.0. The taxa were displayed based on family level in rumen.

A



B



C

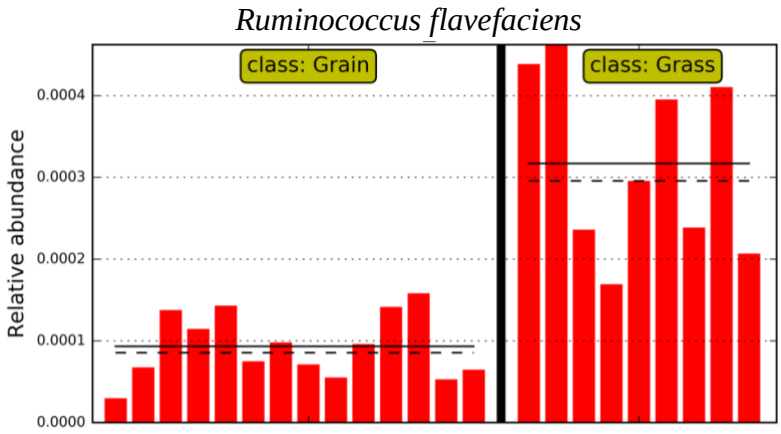


Figure 3.6. Selected significantly different microbial taxa in rumen microbiome between grass-fed and grain-fed cattle.
(A) *Megasphaera.* (B) *Lactobacillus.* (C) *Ruminococcus flavefaciens*. X-axis represented individuals; y-axis represented relative abundances.

Table 3.2. Select significantly different OTUs in relative abundance in the rumen microbiome of grain-fed and grass-fed Angus beef cattle.

OTU ID	Grain-fed	Grass-fed	LDA	Annotation (Domain|Phylum|Class|Order|Family|Genus|Species)
584684	3.0634 ± 1.3145	1.3931 ± 0.3634	3.93	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
589419	2.5215 ± 1.8734	0.5902 ± 0.1881	3.99	Bacteria|Firmicutes|Clostridia|Clostridiales| Veillonellaceae|Succiniclasticum
638485	1.5222 ± 0.8375	0.6533 ± 0.2011	3.65	Bacteria|Fibrobacteres|Fibrobacteria|Fibrobacterales|Fibro bacteraceae|Fibrobacter|succinogenes
815207	0.9289 ± 0.2999	1.3547 ± 0.1579	3.32	Bacteria|Firmicutes|Clostridia|Clostridiales| Lachnospiraceae|Butyrivibrio
574821	0.0079 ± 0.0168	2.3710 ± 0.5280	4.08	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales
342978	0.0829 ± 0.2742	2.0351 ± 1.3858	3.98	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales
539412	0.6324 ± 0.3937	1.1600 ± 0.2114	3.41	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales
578085	0.9950 ± 0.6398	0.4720 ± 0.3092	3.42	Bacteria|Bacteroidetes|Bacteroidia| Bacteroidales|Prevotellaceae|Prevotella
262849	1.2768 ± 1.1830	0.0239 ± 0.0132	3.79	Bacteria|Proteobacteria|Gammaproteobacteria|Aeromonad ales|Succinivibrionaceae|Ruminobacter
584804	0.9731 ± 0.3585	0.4085 ± 0.1107	3.46	Bacteria|Firmicutes|Clostridia|Clostridiales
107308	1.1099 ± 1.4835	0.1566 ± 0.0906	3.69	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|S24-7
545922	1.0392 ± 0.7757	0.2480 ± 0.0774	3.61	Bacteria|Bacteroidetes|Bacteroidia| Bacteroidales|Prevotellaceae|Prevotella
628517	0.4123 ± 0.2118	1.2013 ± 0.6345	3.61	Bacteria|Lentisphaerae|[Lentisphaeria]| Victivallales|Victivallaceae
271582	0.0618 ± 0.0626	1.6945 ± 0.3545	3.91	Bacteria|SR1
341759	0.7018 ± 1.8453	0.4808 ± 0.1492	3.30	Bacteria|Bacteroidetes|Bacteroidia| Bacteroidales
321384	0.1628 ± 0.3832	1.1983 ± 0.2952	3.72	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales
563055	0.3423 ± 0.6851	0.8631 ± 0.2410	3.43	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales
593542	0.8118 ± 0.6916	0.0862 ± 0.0303	3.56	Bacteria|Bacteroidetes|Bacteroidia| Bacteroidales|Prevotellaceae|Prevotella
589852	0.3782 ± 0.1417	0.7499 ± 0.2085	3.26	Bacteria|Firmicutes|Clostridia|Clostridiales| Lachnospiraceae
339090	0.7656 ± 1.0334	0.1373 ± 0.05580	3.52	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales

Ruminal microbial pathways impacted by diets

To obtain insight into the functional categories of rumen microbiota under different diets, we used PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) to predict functional profiling of microbial communities between grass-fed and grain-fed group based on 16S rRNA marker gene sequences. A total of 6910 KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology gene families were predicted. Some top abundant KEGGs included RNA polymerase sigma-70 factor ECF subfamily, ATP-binding cassette subfamily B, and multiple sugar transport system permease proteins. There were 9 KEGGs that showed significant differences. For example, cobalt/nickel transport system ATP-binding protein (K02006) and multiple sugar transport system permease proteins (K02025 and K02026) were enriched in grain-fed rumen microbiome, while RNA polymerase sigma-70 factor ECF subfamily (K03088), and arylsulfatase (K01130) were enriched in the grass-fed group. After classifying KEGG gene families into higher KEGG functional categories (pathways), there were 328 identified microbial biological pathways. Top abundant pathways involved environmental information processing, genetic information processing, and metabolism. The 27 specific microbial pathways that showed significant differences (LDA score log10 $\geqslant$ 2.0) between the two groups were depicted (Figure 3.7).

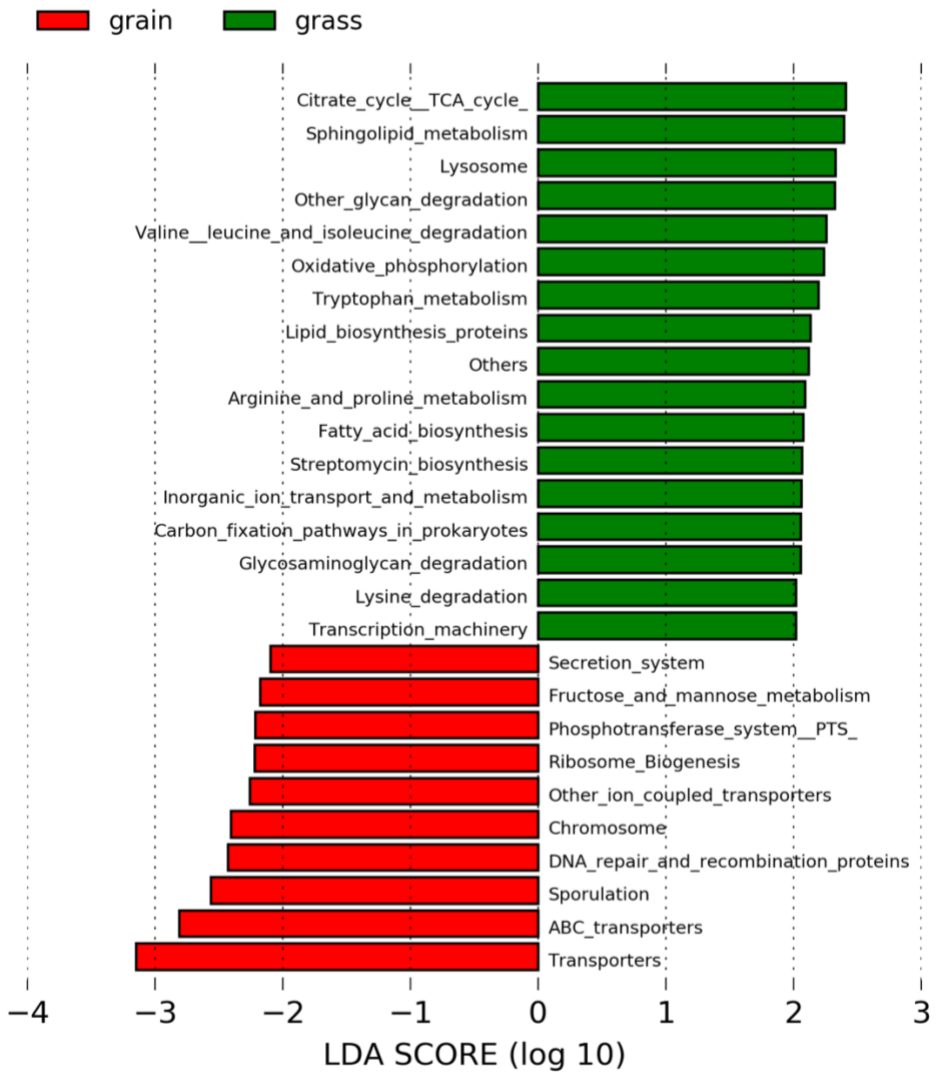


Figure 3.7. Predicted microbial KEGG pathways with significant differences in rumen under different diets.

Diets modulated VFA and lactic acid levels

Grass-feeding and grain-feeding have long been studied and reported to be associated with VFA (volatile fatty acids) and lactate levels in ruminants [10, 32]. In order to further explore their relationships with gut microbiota, quantifications of VFA and lactate were performed. Welch's modified two population t-test was used to examine the differences between grass-fed and grain-fed cattle (Table 3.3). Grain-feeding cattle were measured to have significantly higher levels of acetate, propionate, butyrate, iso-butyrate, but a lower acetate: propionate ratio ($p < 0.05$). As for lactate, the grain-fed group was observed to have a relatively higher lactate concentration, but not statistically significant ($p > 0.05$) (Table 3.3).

Table 3.3. Analysis of VFA and lactate levels between grass-fed and grain-fed rumen.

Concentration unit was shown as parts per million (ppm).

Acids	Grain-fed	Grass-fed	p value
Acetate	3990.125 ± 420.756	3197.556 ± 883.497	0.0338
Propionate	1515.625 ± 214.071	834.889 ± 296.516	< 0.0001
Acetate: propionate ratio	2.652 ± 0.262	3.929 ± 0.354	< 0.0001
Butyrate	773.125 ± 90.044	539.444 ± 200.4	0.0088
Iso-butyrate	119.5 ± 10.61	69.111 ± 8.638	< 0.0001
Lactic acid	8.286 ± 6.264	2.667 ± 3.162	0.0605

Discussion

Rumen serves as an important fermentation chamber for ruminants, and microbial breakdown of dietary components plays critical roles in ruminant digestion. Diet-induced differences through microbial community should be explored deeply. VFAs and lactate are bacterial fermentation products and intermediate products, and their association with bacteria also deserves examination. For microbial diversity of rumen, our results suggested that grass-fed diets induced a significant increase in α diversity indices compared with grain-feeding. This was likely to be caused by the higher diversity of microbes of grass in an exposed pasture environment to the grass-fed group.

As for specific taxonomic levels, at the phylum level, shifts in the relative abundances were already apparent between the two groups. Diets induced several significant differences between grass-fed and grain-fed cattle, with absolute LDA score log10 $\geqslant$ 2.0. Several common taxa were identified to be significantly different. For grass-feeding, as structural carbohydrates were rich in the diets, structural carbohydrate fermenters were examined in this study. For example, the genus *Ruminococcus* was overall higher in grass-fed group than grain fed group. Certain bacteria in this genus were known to be able to hydrolyze and utilize cellulose, hydrolyze hemicellulose, and ferment dextrins [9]. An example was a named species, *Ruminococcus flavefaciens*, which was also significantly higher in grass-fed group in this study. This result suggested the influences of high structural carbohydrates on bacteria in grass-fed group. On the other hand, grain-feeding was rich in starch that provided quickly

accessible substrates for fermentation of ruminal bacteria. It has long been reported that higher ratio of fermentable carbohydrates in cattle diet promoted VFA production in rumen, especially for propionate and butyrate [39, 74], but acetate to propionate ratio was higher in high-forage diet [10, 74]. Our results were in agreement with this previous founding. From our data of VFA analysis, in rumen fluid of grain-fed cattle, the concentrate of major VFAs, including acetate, propionate, butyrate, and iso-butyrate, were all higher in grain-fed group. We also observed that increases of propionate concentration were relatively larger than those of acetate. As a result, grain-fed group had a lower acetate: propionate ratio (Table 3.3). Moreover, as for VFA production in grass-feeding rumen, it was reported that acetate was produced mostly by microbial fermentation of fibrous components [39]. In our study, the identified *Ruminococcus* enriched in grass-fed rumen also produced acetate during its function of fiber digestion. Therefore, this microbial abundance enrichment provided further evidence that high levels of cellulose and hemicellulose in grass-feeding could lead to a higher number of fiber-digestion bacteria population that produced a higher proportion of acetate as end products than other types of VFAs and resulted in the higher acetate: propionate ratio.

From our data, bacterial taxa *Lactobacillus* and *Succinivibrio* were enriched by grain-feeding, which were commonly identified lactate-producing taxa in the rumen, and were reported to be associated with ruminal acidosis [75]. Lactate quantification showed that rumen fluid of grain-fed cattle had relatively higher lactate level, though not significant (p-value > 0.05). This higher level of lactate might provide nutrients for

the growth of lactate-utilizing bacteria. Indeed, we observed that the genus, *Megasphaera*, showed significantly higher abundance in the grain-fed group. One potential species, *Megasphaera elsdenii*, a commonly found lactate-utilizing bacteria in rumen, could potentially contribute to this enrichment of genus. Elevated lactate level caused by the increase of lactate-producing bacteria also triggered the growth of lactate-utilizing bacteria. In addition, a named species *Selenomonas ruminantium*, under the family of *Veillonellaceae* and the order of Clostridiales, was also identified with enrichment in grain-fed group. *Selenomonas ruminantium* was a luminal bacteria that could utilize L-lactate in rumen [76]. Our data provided further evidence about how diets could influence VFA production and then interact with lactate-associating bacteria in high-grain diet. A high amount of VFA production first reduced ruminal pH in the grain-fed group, which then promoted the growth of both lactate-producing and lactate-utilizing populations.

Gut microbiome in cattle digestive tract displayed several physiological functions significantly influenced by host diets. For microbial functions, in the grain-fed group, three KEGG genes were enriched in rumen microbiome, including two multiple sugar transport system permease proteins (K02025, K02026), and one multiple sugar transport system substrate-binding protein (K02027) (absolute LDA score log10 $\geqslant$ 2.0). These enriched gene families potentially suggested that fermentable sugar in grain-feeding not only altered microbial types and numbers as discussed above, but also induce differences in microbial gene families to digest the dietary component, sugar, from the host. From a higher level of biological pathways, in grain-fed group,

microbial functions such as transporters, fructose and mannose metabolism, and secretion system were significantly enriched, which potentially were contributed by the enriched sugar transport system proteins found in KEGG gene results. On the other hand, the TCA cycle and oxidative phosphorylation were enriched in the grass-fed microbiome, suggesting that bacteria in rumen of grass-fed cattle might have different energy utilization metabolism compared with grain-fed cattle.

Conclusions

Rumen fermentation is achieved through activities of different kinds of microorganisms that utilize host dietary components in the environment. In this chapter, we explored the structure of ruminal microbiome as well as the primary fermentation product, VFA, under a grass-fed diet and a grain-fed diet, and also predicted microbial pathways. Culture-independent next-generation sequencing method provided detailed microbial community information influenced by diets. Overall, the grass-fed cattle had higher ruminal microbial diversities than the grain-fed group. According to our findings, microbial divergences between the two groups could be observed starting from the differences in taxonomic levels of Archaea and Bacteria. Grass-fed ruminal microbiota was primarily influenced by the high level of structural carbohydrates, so the bacterial population of forage fermenter was increased, which triggered a higher acetate: propionate ratio than the grain-fed group. As for the microbiome in the grain-fed rumen, the level of VFA was significantly higher, and bacteria population associated with lactate metabolism was enriched than the grass-fed group. Diet also exerts significant influences on ruminal bacterial KEGG gene families and pathways. Overall, this chapter examined interactions of diets, bacteria, VFA, and lactate production, and also provided a prediction of microbial pathways influenced by different diets in Angus beef cattle.

4. Diet-induced changes in jejunum microbiome and their potential associations with bile acids components in Angus beef cattle

Introduction

While the majority of efforts in cattle gut microbiota has been centered on rumen because of its metabolic functions, the lower digestive tract has not gotten as much attention as rumen for a long time. Significantly dropped number of microbes and inaccessibility of cattle small intestine are possible reasons [29]. However, small intestine of ruminants also serves an important organ for nutrient absorption. Proteins, fats, some undigested fiber, and carbohydrates in the form of simple sugars (monosaccharides) and starch, can enter the intestine of cattle. Enzymes produced from liver and pancreas function to digest amino acids, monosaccharides, and starch [9]. As for fats, they have been hydrolyzed and fermented in the upper gut into the form of fatty acids in the intestine. Then, calcium and magnesium salts are produced from hydrogenated fatty acids [9, 41]. Additionally, bile with several bile salts is secreted form gall bladder through bile duct into the duodenum to aid lipid digestion. As a result, in cattle, while VFAs are mainly absorbed in rumen, varieties of the other nutrients, including vitamins, minerals, proteins, and lipids, are mostly absorbed in small intestine, through the epithelium of jejunum, into the bloodstream. The remaining portion of leftover nutrients would be absorbed in the ileum [9, 11, 58].

Because of the various nutrient absorption activities, microbial studies are also worth fully interrogation to understand the relationship between gut metabolism and gut microbiota. Some studies characterized the bacterial structure throughout the GI tract of dairy cattle, beef cattle, and sheep, which involved small intestine microbiome and its comparison with other segments of the digestive tract. It has been found that there was a common trend that the number of identified operational taxonomic units (OTU) dropped about half in the small intestine compared with fermentation and gastric compartments (rumen, reticulum, omasum, abomasum) and colon [11-13]. Although with a reduced number of microbes, it has been reported that functional and metabolic characteristics of small intestine were still associated with gut microbiota populations [11, 13, 29, 41]. Currently, rumen microbiota related to high-forage diet and high-grain diets were largely studied using a variety of methods for decades [20, 68, 77, 78]. However, the community structure of small intestine microbiome affected by grass-feeding and grain-feeding regimens has not been deeply characterized. Only a few studies explored the role of bile acids and microbial community in cattle species. Here, we hypothesized that diets can induce significant changes in the microbial community in jejunum microbiome, and that the components of bile acids are associated with the composition of microbiota of intestine. To address the lack of knowledge in this compartment of gut, we profiled the microbial structure of jejunum using 16S-seq, together with bile acids quantification, to characterize the influences of different diets on the small intestine microbiome in Angus beef cattle.

Materials and Methods

Experimental design and sample collection

Steers selected for this study came from a population of cattle in University of Maryland Wye Angus beef cattle herd. Two groups were assigned randomly into grass-fed and grain-fed cattle groups. Wye Angus herd and feeding method in each group were described in detail in Materials and Methods section of chapter 2. At around twelve months of age, when steers reached the market weight (approximately 470 kg), they were commercially slaughtered at a slaughterhouse belonging to George G. Ruppersberger and Sons, Inc (Baltimore, MD). The luminal contents of jejunum were sampled into sterile 50 ml tubes and then pipette into 2 ml tubes, and immediately frozen in dry ice for transportation, and finally stored in the – 80 ℃ freezer in lab for later DNA extraction. Bile liquids of eight cattle from each group were collected from gallbladder by aspiration into a 50ml tubes and immediately frozen in dry ice after collection and during transportation and was stored in -80°C freezers before bile acid composition quantification. Steers of this study was handled according to a protocol (protocol # R-11-72) approved by the Beltsville Area Animal Care and Use Committee, and the Institutional Animal Care and Use Committee at the University of Maryland (UMCP-IACUC). Sample collection was performed strictly based on this protocol.

DNA extraction, 16s rRNA gene amplification, and sequencing

Microbial DNA from jejunum fluid was extracted using QIAamp DNA stool kit (Qiagen, Valencia, CA), and 16S rRNA gene sequencing was performed as previously described in Chapter 2. Briefly, the hypervariable V3-V4 regions of the 16S rRNA gene

was amplified with 20 cycles through PCR from 20 ng of total DNA with PAGE-purified Illumina platform-compatible adaptor oligos. Particularly, for jejunum, because density of total bacteria in this gut segments was lower than rumen and rectum, a second round of PCR was implicated to ensure adequate amplification of targeted 16S rRNA regions. 2 ul PCR product from the first round of PCR was used as template to perform another 10 cycles of PCR amplification. DNA concentration and sizes were quantified using a BioAnalyzer 2000 (Agilent, Palo Alto, CA). Amplicons were finally purified using Agencourt AMPure XP bead kits (Beckman Coulter Genomics, Danvers, MA). The library pool was sequenced in an Illumina MiSeq sequencer with an Illumina MiSeq Reagent Kit according to the manufacture's protocol.

Bioinformatics and data analysis

Bioinformatic pipeline of 16S rRNA gene sequencing used in this study was described in Chapter 2 in detail. Briefly, raw sequence data was imported to FastQC for quality check. The first four random nucleotides were trimmed in R1 reads. R1 and R2 reads for each individual sample were merged into one FASTA files using PandaSeq v2.11 [61]. QIIME closed reference pipeline was used to analyze the processed 16S rRNA gene sequencing data with an OTU threshold of 97% similarity [62]. Taxonomic assignment was performed based on GreenGenes database v13.8 [63]. Alpha (α) - diversity and beta (β) diversity were calculated and examined with QIIME and R. Core microbiome was also identified via QIIME *compute_core_microbiome.py* [62]. PICRUSt v1.1.2 was used for predicting metagenomics and functions with KEGGs and pathways, as well as contributions of OTUs to identified KEGG orthologs [66]. LEfSe

algorithm [67] was used in differential analysis to identify significantly different OTUs, KEGGs and pathways and their effect sizes between grass-fed group and grain-fed groups. T-test and Welch's t-test were used for differential analysis of bile acids and diversity indices between the two groups, respectively.

Bile acids measurements

Bile samples were measured in a commercial lab (Creative Proteomics, Shirley, NY). Five μL of cattle bile was mixed in Eppendorf tubes with 995 μL of methanol. After vortex mixing for 15 s and sonication for 2 min. The tubes were centrifuged at 15,000 rpm and at 10 ^{o}C for 15 min. 8 μL of each supernatant was mixed with 992 μL of 50% methanol. After mixing, 50 μL was mixed with 50 μL of the SIS mix and 100 μL of 50% methanol, thoroughly. 20-μL aliquots were injected for UPLC-(-)ESI-MRM/MS quantitation, according to a procedure published previously [79]. As for the LC-MS/MS system, an Ultimate 3000 RSLC system (Dionex Inc., Amsterdam, The Netherlands) coupled to a 4000 QTRAP mass spectrometer (AB Sciex, Concord, ON, Canada) via a Turbo Ionspray electrospray ionization (ESI) source was operated in the negative ion mode. A BEH C18 (2.1 mm × 150 mm, 1.7 μm) UPLC column (Waters Inc., Milford, MA) was used for the gradient elution, with 0.01% formic acid in water (solvent A) and 0.01% formic acid in acetonitrile (solvent B) as the mobile phase. The gradient was optimized at 25% to 40% B in 12 min and then 40% to 75% B in 14 min. The column was washed with 100% B for 2 min and equilibrated with 25% B for 4 min between injections. The flow rate was 0.35 mL/min, and the column was maintained at 45 ℃. Calibration curves for calculation of bile acid concentrations were used.

Specifically, a STD mix containing standard substances of all the quantified bile acids was dissolved in 50% methanol and this standard solution was used as S1. The S1standard solution was diluted in a series at a same dilution ratio of 1 to 4 with 50% methanol to have standard solutions of S2 to S10. 50 μL of each of S1 to S10 was mixed with 50 μL of 14 D4-bile acid SIS mix (as internal standards) and 100 μL of 50% methanol. 20-μL aliquots were injected to record the data files so as to prepare calibration curves using linear regression (As/Ai peak area ratio versus concentration) for calculation of bile acid concentrations measured in the individual samples. For data processing, SciexMultiQuant was used.

Results

Diversity and composition of jejunum microbiota in beef cattle under different diets

QIIME (Quantitative Insights Into Microbial Ecology) closed reference pipeline was used to analyze 16S-seq data of cattle jejunum contents with Greengenes 16S rRNA Gene Database [62, 63], identifying 24 phyla, 44 classes, 77 orders, 149 families, and 263 genera in total of the two cattle groups. Of the 24 identified phyla, the most abundant phylum detected in cattle jejunum was Firmicutes that accounted for 63.11% to 98.21% in terms of relative abundances. Besides Firmicutes, some phyla with high abundances included Proteobacteria (6.14%), Bacteroidetes (2.52%), Verrucomicrobia (1.92%), Actinobacteria (1.66%), and Elusimicrobia (0.89%) (Figure 4.1). Regarding higher taxonomic levels, among the 149 assigned families, eight families possessed a relative abundance higher than 1%, including *Clostridiaceae* (33.82%), *Peptostreptococcaceae* (27.87%), *Ruminococcaceae* (6.03%), Enterobacteriaceae (5.69%), *Lachnospiraceae* (5.62%), *Turicibacteraceae* (4.60%), *RFP12* (1.76%), *Bacillaceae* (1.68%). The next abundant family, *Bacteroidaceae*, was also a common family in cattle, which accounted for approximately 0.99% abundance of jejunum microbiota families. Of the total 4734 detected OTUs, 300 OTUs were computed as the core microbiome. In this study, because there were two groups, OTUs that were present in at least 75% of samples could be considered as core microbiome. There were 21 named species in the core jejunum microbiome of cattle considering both grass-fed and grain-fed cattle. The top ten abundant species included *Clostridium difficile*, *Streptococcus luteciae*, *Clostridium neonatale*, *Clostridium butyricum*, *Clostridium*

perfringens, *Ruminococcus flavefaciens*, *Clostridium butyricum*, *Lysinibacillus boronitolerans*, *Clostridium perfringens*, and *Ruminococcus flavefaciens*.

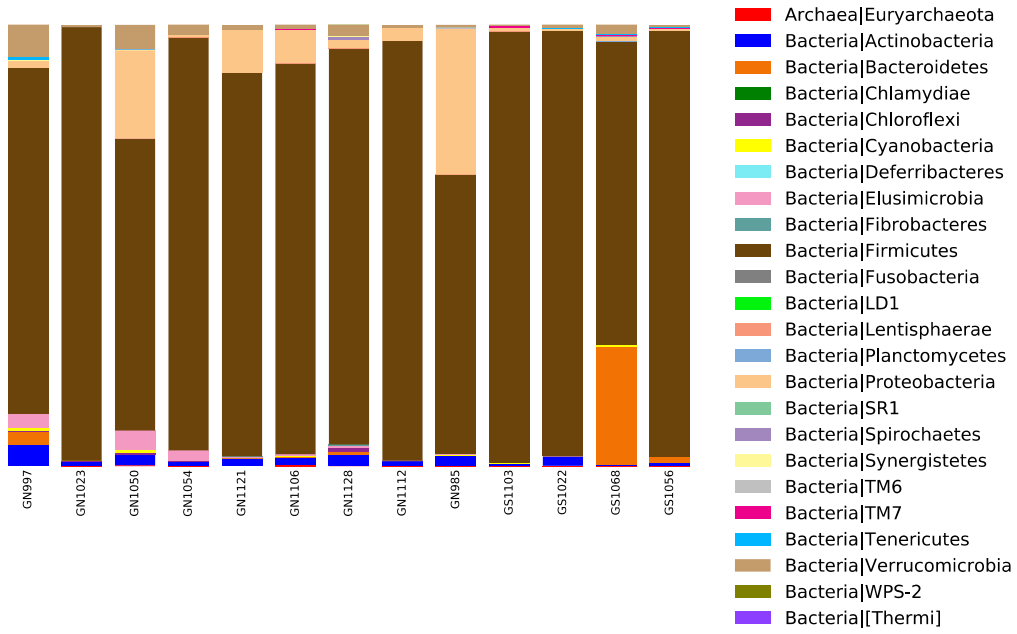


Figure 4.1. Phylum-level composition of jejunum gut bacteria.
The first nine samples were grain-fed (GN) individual cattle, the last four individuals were cattle from grass-fed (GS) group.

Also, microbial diversities were analyzed, including alpha and beta diversities. Rarefaction curve analysis was performed based on Chao1 values of grass-fed and grain-fed groups, suggesting enough sequencing depth in the current study (Figure 4.2). Common microbial diversity indices, Chao1, Shannon, Simpson, and Phylogenetic diversity (PD_whole_tree) were evaluated (Table 4.1). No significant p values of diversity indices were computed between grass-fed and grain-fed groups (p values >

0.05 based on Welch's t-test). However, from rarefaction curves, jejunum microbiome in grass-fed cattle always had a higher mean of chao1 than grain-fed cattle during a random sampling of rarefaction process. By comparing average values between alpha diversity indices of two groups, the grass-fed group also always had higher average values of the alpha index than the grain-fed group. For example, the PD_whole_tree value was 49.02 ± 9.46 (mean ± sd) for the grass-fed group, 66.77 ± 18.04 for the grain-fed group. Our results suggested that the grass-fed group tend to have a higher microbial diversity than the grain-fed group, but the diversity was not statistically significant (p values > 0.05). As for the beta diversity analysis, the principal component analysis (PCA) showed a distinct difference in jejunum microbial composition between grass-fed and grain-fed individuals. PCA was plotted based on identified top abundant families of across the two groups (Figure 4.3).

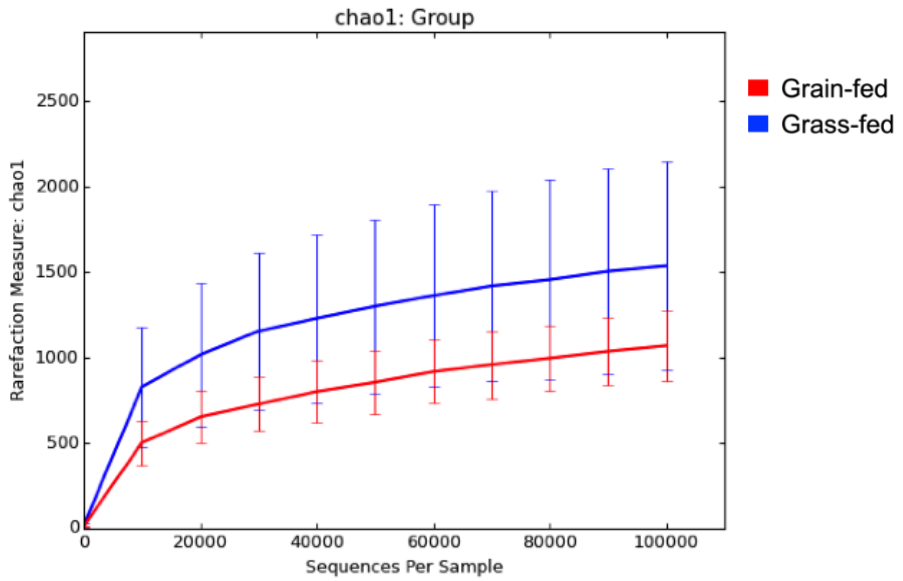


Figure 4.2. Rarefaction curves based on Chao1 values of grass-fed (blue) and grain-fed (red) groups.

Table 4.1. Alpha diversity indices of jejunum microbiota in grain-fed and grain-fed Angus beef cattle.

Welch's t-tests was used to calculate p-values.

Diversity Indices	Grain-fed	Grass-fed	p value
Chao1	1225.62 ± 468.55	1536.97 ± 706.42	0.4575
Shannon	4.07 ± 0.95	4.69 ± 2.40	0.6468
Simpson	0.83 ± 0.08	0.990 ± 0.11	0.9238
PD_whole_tree	49.02 ± 9.46	66.77 ± 18.04	0.1416

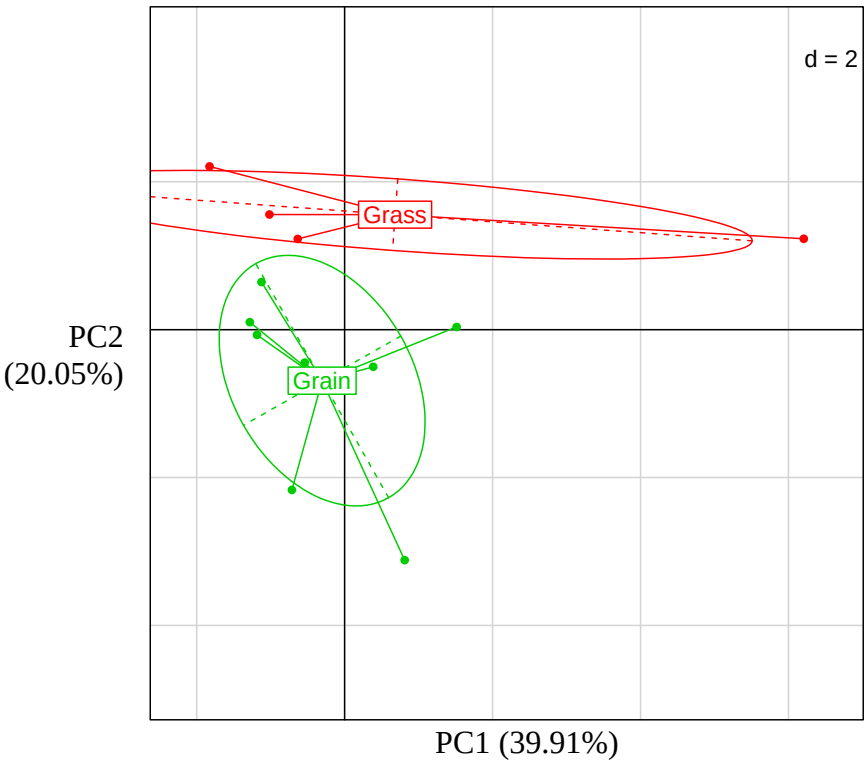


Figure 4.3. Differences in microbial composition of the cattle jejunum microbiome between grass-fed and grain-fed cattle.

Principal component analysis (PCA) was performed with ade4 package in R based on the top 10 abundant microbial families.

Different diets altered microbial composition of jejunum in beef cattle

The difference in microbial composition between grass-fed and grain-fed groups was examined. Relative abundances of taxa were computed by QIIME and examined with the Linear Discriminant Analysis (LDA) Effect Size (LEfSe) algorithm [67]. At the family level, 67 taxa showed significant differences in relative abundance between the two groups. Among them, the abundance of 33 families was significantly higher in the grain-fed group, while the left 34 families were significantly higher in abundance in the grass-fed group. For example, identified families *Enterobacteriaceae, Turicibacteraceae, RFP12, Elusimicrobiaceae,* and *Bifidobacteriaceae* showed higher abundance in the grain-fed group, whereas *Bacteroidaceae, Rikenellaceae, Paraprevotellaceae, BS11,* and *Nocardioidaceae* were significantly higher in abundance in the grass-fed group. A cladogram based on the family level was depicted as in Figure 4.4, displaying taxa with significant differences in jejunum microbiome. 46 named genera showed significant differences between the two groups. For example, *Lactobacillus* and *Ruminococcus* were significantly higher in the grain-fed cattle jejunum, whereas *Solibacillus* had significant greater abundance in the grass-fed group (Figure 4.5). At the OTU level, 291 OTUs were significantly different in abundance between the grass-fed and grain-fed groups (absolute LDA score log10 $\geqslant$ 2.0). In comparison, 215 OTUs had higher relative abundance in the grass-fed group, and 76 OTUs showed higher relative abundance in the grain-fed group. Selected significantly different OTUs impacted by diets between the two groups were listed in table 4.2.

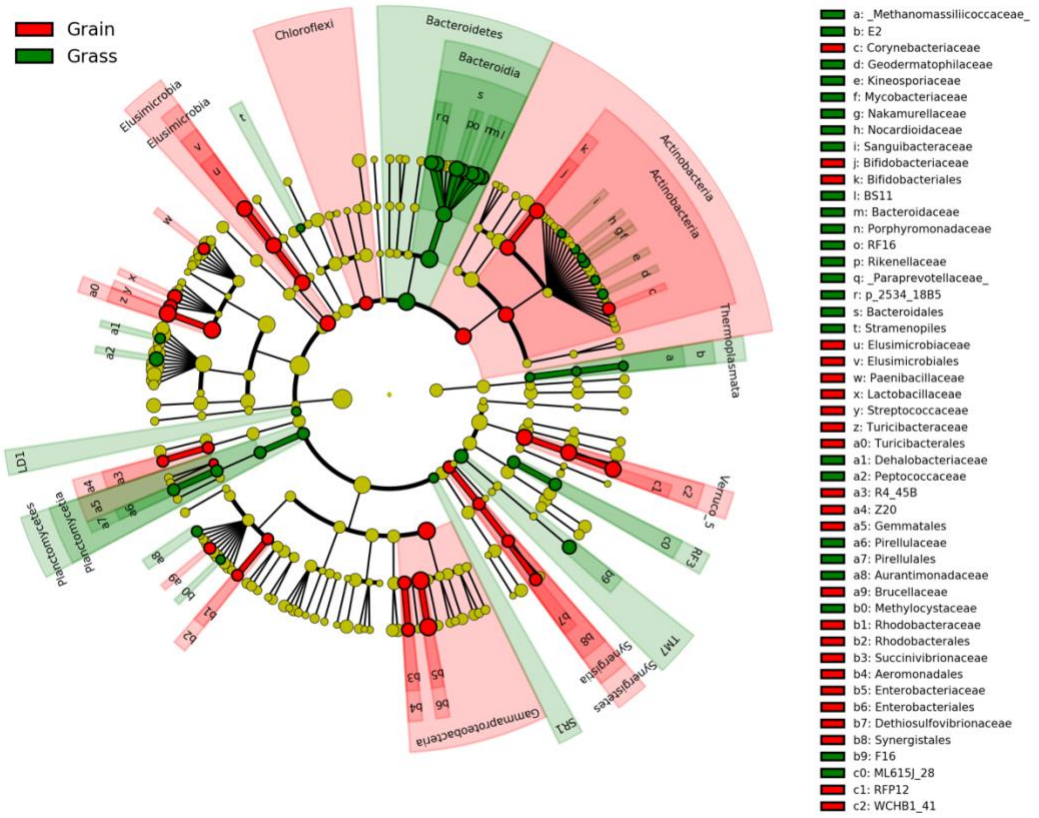
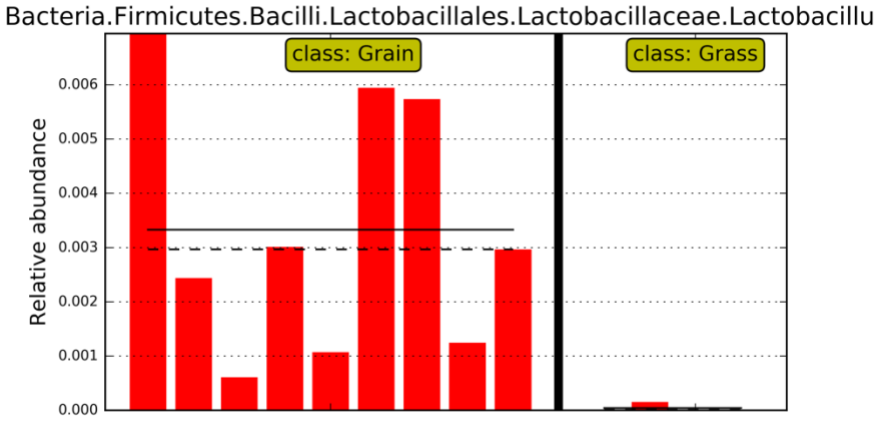


Figure 4.4. A cladogram representing the jejunum microbial taxa based on family level.

Taxa that had significantly different in abundances between grass-fed and grain-fed cattle with an absolute Linear Discriminant Analysis (LDA) score log10 $\geqslant$ 2.0 were displayed.

A



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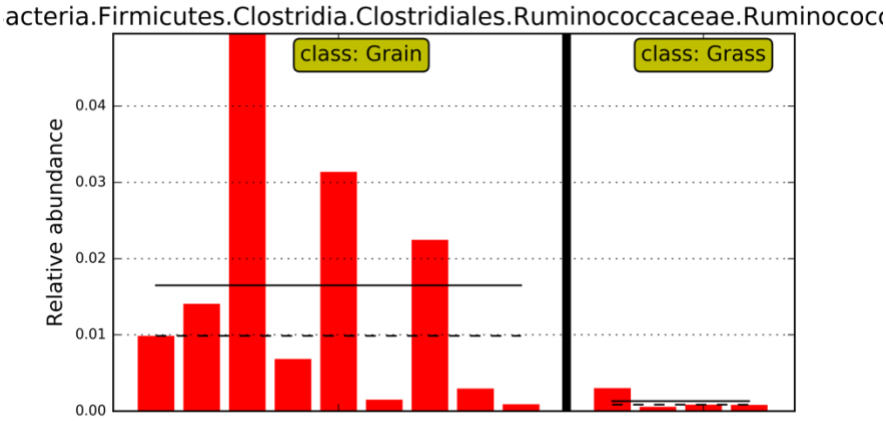

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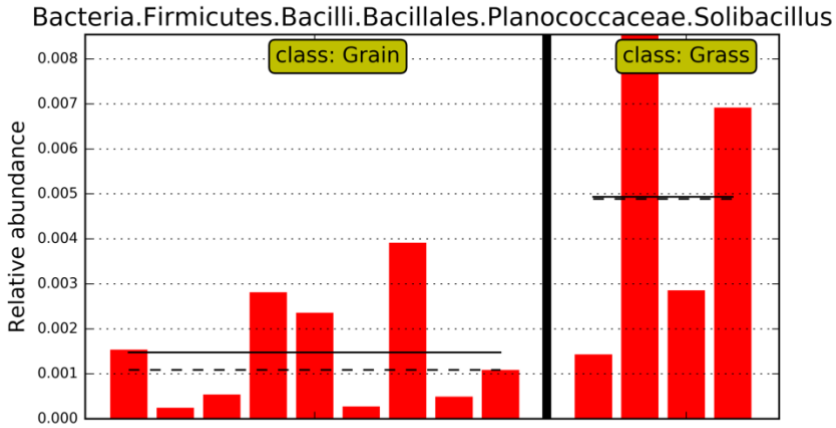

Figure 4.5. Selected microbial genera displaying significant differences in relative abundance in jejunum microbiome between grass-fed and grain-fed cattle.

(A) *Lactobacillus.* (B) *Ruminococcus.* (C) *Solibacillus*. X-axis represented individuals; y-axis represented relative abundance.

Table 4.2. Select significantly different OTUs in relative abundance in the jejunum microbiome of grain-fed and grass-fed Angus beef cattle.

Greengenes ID	Grain-fed	Grass-fed	LDA score	Annotation
347529	6.1592 ± 3.1357	0.7381 ± 0.4641	4.44	Bacteria|Firmicutes|Bacilli|Turicibacterales| Turicibacteraceae|Turicibacter
782953	5.8429 ± 7.3547	0.0838 ± 0.1517	4.46	Bacteria|Proteobacteria|Gammaproteobacteria| Enterobacteriales|Enterobacteriaceae
3829957	1.7923 ± 3.7084	0.0218 ± 0.0411	3.95	Bacteria|Proteobacteria|Gammaproteobacteria| Enterobacteriales|Enterobacteriaceae
533634	1.5224 ± 4.3245	0.0003 ± 0.0004	3.95	Bacteria|Firmicutes|Clostridia|Clostridiales| Lachnospiraceae|Moryella
302219	1.1924 ± 1.5725	0.0577 ± 0.0373	3.71	Bacteria|Verrucomicrobia|Verruco-5|WCHB1-41|RFP12
813277	1.0691 ± 1.2361	0.0001 ± 0.0002	3.69	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
23652	0.9982 ± 1.3779	0.0013 ± 0.0024	3.69	Bacteria|Elusimicrobia|Elusimicrobia|Elusimicrobiales| Elusimicrobiaceae
298198	0.8282 ± 0.5570	0.1283 ± 0.1665	3.52	Bacteria|Actinobacteria|Actinobacteria|Bifidobacteriales| Bifidobacteriaceae
579658	0.7178 ± 1.9300	0.0000 ± 0.0000	3.62	Bacteria|Firmicutes|Clostridia|Clostridiales| Lachnospiraceae|Moryella
3869719	0.6441 ± 0.5425	0.0750 ± 0.0743	3.44	Bacteria|Firmicutes|Clostridia|Clostridiales
570341	0.0001 ± 0.0002	0.8286 ± 1.6200	3.61	Bacteria|Firmicutes|Clostridia|Clostridiales
576472	0.0003 ± 0.0006	0.8228 ± 1.5401	3.59	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales| Bacteroidaceae|5-7N15
347529	6.1592 ± 3.1357	0.7381 ± 0.4641	4.44	Bacteria|Firmicutes|Bacilli|Turicibacterales| Turicibacteraceae|Turicibacter
333300	0.1027 ± 0.1451	0.7123 ± 0.6684	3.48	Bacteria|Firmicutes|Clostridia|Clostridiales| Lachnospiraceae
586562	0.0002 ± 0.0006	0.6750 ± 1.3231	3.50	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
576712	0.0001 ± 0.0002	0.6492 ± 1.2683	3.47	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
592139	0.0000 ± 0.0000	0.5889 ± 1.1536	3.47	Bacteria|Firmicutes|Clostridia|Clostridiales| Ruminococcaceae
288681	0.0005 ± 0.0012	0.5337 ± 1.0051	3.44	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales| Bacteroidaceae
296290	0.0221 ± 0.0351	0.5000 ± 0.4341	3.36	Bacteria|Firmicutes|Clostridia|Clostridiales| Lachnospiraceae
309285	0.0000 ± 0.0000	0.3781 ± 0.7272	3.23	Bacteria|Firmicutes|Clostridia|Clostridiales

Potential jejunum microbial pathways inferred from the 16S data

Differences in microbial communities are always associated with different biological functions of microorganisms. Therefore, in this study, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) method [66] was used to predict functional profiling of microbial communities between grass-fed and grain-fed group based on 16S rRNA marker gene sequences. After normalization of read counts in OTU table from QIIME pipeline output, a total of 6909 (Kyoto Encyclopedia of Genes and Genomes) Orthology gene families were identified. Of them, five KEGG gene families showed significant differences in abundance between grass-fed and grain-fed groups using LefSe method [67] with a cutoff value of Linear Discriminant Analysis (LDA) score log10 $\geqslant$ 2.0. Specifically, only one KEGG, insertion element IS1 protein InsB (K07480), were more abundant in grain-fed group, whereas methyl-accepting chemotaxis protein (K03406), RNA polymerase sigma-70 factor, ECF subfamily (K03088), DNA topoisomerase III [EC:5.99.1.2] (K03169), and ABC-2 type transport system permease protein (K01992) had significantly higher abundance in grass-fed group.

In total, as for predicted KEGG pathways, there were 328 identified microbial biological pathways. Some abundant functional pathways in jejunum microbiome included membrane transport such as ABC transporters, genetic information processing such as DNA repair and recombination proteins, and nucleotide metabolisms. LefSe analysis identified seven pathways that had significantly different abundance between grass-fed and grain-fed groups. In detail, six pathways showed significantly higher

abundance in grass-fed group, which included Arginine and proline metabolism (Amino Acid Metabolism), Porphyrin and chlorophyll metabolism (Metabolism of Cofactors and Vitamins), Transcription machinery (Transcription), Bacterial chemotaxis (Cell Motility), CAM ligands (Signaling Molecules and Interaction), and ECM-receptor interaction (Signaling Molecules and Interaction). Only one pathway, Glycerophospholipid metabolism (Lipid Metabolism), showed significantly greater abundance in the grain-fed group, and its abundances were depicted in Figure 4.6. These results suggesting that diets could influence biological pathways of jejunum microbial communities between grass-fed and grain-fed cattle.

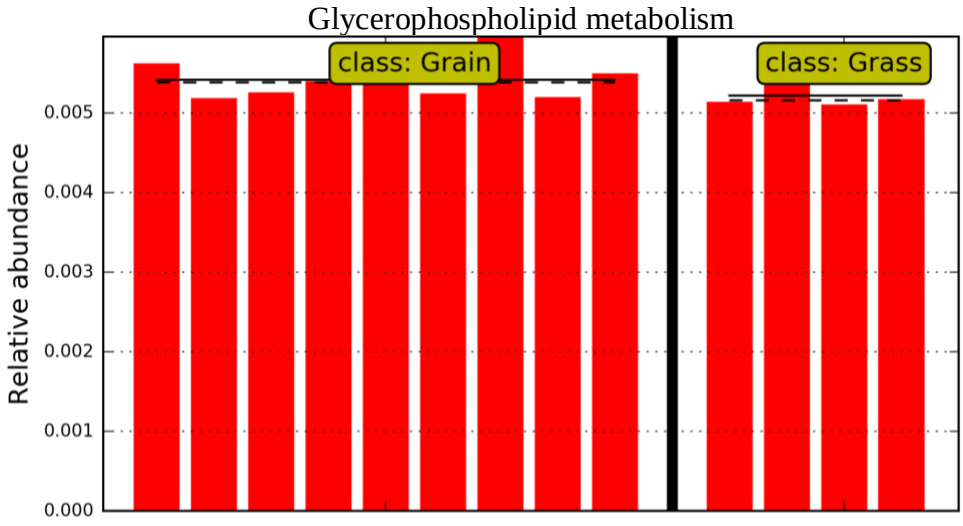

Figure 4.6. Abundance of glycerophospholipid metabolism of jejunum microbiome.
Lipid Metabolism pathway, glycerophospholipid metabolism, displayed significantly higher abundance in jejunum microbiome of grain-fed group than grass-fed group. X-axis represented individual animals.

Diets induced significant changes in bile acid composition of cattle

Microflora of small intestine could also link with bile acid composition. Bile acids were secreted from the duodenum and flow along the small intestine to aid lipid digestion, and processed into secondary bile acids, and finally could get recycled through the enterohepatic circulation. Therefore, in this study, in order to explore the potential association between bile acids and gut microbiota, bile samples from the gallbladder of eight cattle in each group were measured with LC-MS/MS system. In total, 21 kinds of bile acids were identified and quantified, including both primary and secondary bile acids, and corresponding forms of bile acid conjugates. Conjugated form of cholic acid and deoxycholic acid were detected with particularly high concentrations in μmol/mL, such as taurocholic acid (206.139 ± 57.52 for grain-fed group and 358.033 ± 46.647 for grass-fed group), taurodeoxycholic acid (79.243 ± 24.584 for grain-fed group and 54.168 ± 11.158 for grass-fed group). Among the 21 detected cattle bile acids, 9 kinds of bile acids were significantly different between grass-fed and grain-fed cattle. The results of detected bile acids and p values calculated form t-test were listed in Table 4.3. Conjugated form of main kinds of secondary bile acids, lithocholic acid and deoxycholic acid, were significantly higher in grain-fed group, which included taurolithocholic acid, glycolithocholic acid, glycodeoxycholic acid, and taurodeoxycholic acid. These suggested a greater bacteria biological activity to produce secondary bile acids from primary bile acids in the grain-fed group.

Table 4.3. Composition of bile acids identified from bile in gallbladder of grass-fed and grain-fed cattle.

Concentration Unit shown as μmol/mL. Nine out of total 21 bile acids had significant differences between the two groups (p value < 0.05).

Bile acids	Grain-fed	Grass-fed	p value
Taurolithocholic acid	1.075 ± 0.284	0.515 ± 0.08	< 0.0001
Glycolithocholic acid	1.001 ± 0.41	0.334 ± 0.17	0.0002
Glycodeoxycholic acid	57.035 ± 18.609	30.417 ± 8.094	0.0007
Taurodeoxycholic acid	79.243 ± 24.584	54.168 ± 11.158	0.0083
Taurocholic acid	206.139 ± 57.52	358.033 ± 46.647	0.0111
Glycoursodeoxycholic acid	0.073 ± 0.042	0.035 ± 0.013	0.0111
Total of tauroursodexycholic/ taurohyodeoxycholic acid	0.152 ± 0.093	0.08 ± 0.016	0.0203
Cholic acid	0.884 ± 0.403	2.876 ± 2.45	0.0238
Glycocholic acid	171.912 ± 79.959	294.44 ± 63.734	0.0283
7-Ketodeoxycholic acid	0.08 ± 0.057	0.382 ± 0.425	0.0699
Deoxycholic acid	0.828 ± 0.056	0.774 ± 0.076	0.1612
Ursodeoxycholic acid	0.211 ± 0.028	0.201 ± 0.012	0.2352
Glycohyodeoxycholic acid	0.021 ± 0.009	0.018 ± 0.005	0.2574
Glycochenodeoxycholic acidt	19.651 ± 12.122	16.631 ± 3.081	0.3142
Taurochenodexycholic acid	15.947 ± 7.29	14.868 ± 2.207	0.4117
α-Muricholic acid	0.062 ± 0.029	0.078 ± 0.033	0.461
Tauro-ШI-muricholic acid	0.164 ± 0.145	0.143 ± 0.061	0.5654
Tauro-α-muricholic acid	0.018 ± 0.009	0.017 ± 0.008	0.6432
Apocholic acid	0.043 ± 0.028	0.038 ± 0.043	0.7804
Dehydrolithocholic acid	0.032 ± 0.008	0.036 ± 0.007	0.783
ШI-muricholic acid	0.07 ± 0.009	0.073 ± 0.018	0.7948

Discussion

Microbial communities and their potential functions are critical to aid nutrient absorption in the small intestine of cattle. In jejunum, the large number of villi and microvilli increases the surface area of small intestine, which increases the chance of food particles to encounter bile acids and digestive enzymes. Nutrients could be absorbed across epithelium by passive or active transport to blood vessels [80]. This unique environment and function of small intestine also make microbiome of the small intestine to be distinctive in ruminant digestive tract. It was not surprising that microbial profiles we analyzed in jejunum had various distinctive characteristics. Microbial diversities were reduced in this segment of cattle gut. Firmicutes became the only one apparent dominant phylum that could account for more than 90% of abundance. Possible reasons could be that diets already went through several sequential digestions in the upper gut segments, there was limited contact with the outside environment, and the reduced oxygen level in the small intestine. These phenomena were also reported in several previous studies [11, 13, 41, 50]. In this study, differences in microbial abundance and diversity induced by diets were not as distinctive as in rumen or rectum. Alpha diversity indices of jejunum microbiome did not achieve statistically significant differences between the two groups. Relatively small sample size in this study and animal variations even within the same group could potentially be the reasons. Also, the environment of the small intestine and fast digesta passage time were not feasible for microbial growth compared to rumen and rectum, so significant differences in diversity might not be observed.

However, within the jejunum microbiome, in finer levels, many named genera and species were identified, and differences were observed in relative abundance. At the taxonomic levels below phylum, families such as Clostridiaceae, Peptostreptococcaceae, and Ruminococcaceae, genera *Clostridium*, *Butyrivibrio*, and *Ruminococcus* were common dominant taxa in jejunum microbiome. This was also in agreement with previous studies regarding the profiles of ruminant small intestine microbiome [13, 50, 52]. In this study, significant differences in the relative abundance of specific taxa in jejunum were observed between the two groups. For example, the genus, *Lactobacillus*, was significantly higher in the grain-fed group. This species has long been observed to have a higher abundance in high-grain diet rumen, and in cattle with rumen acidosis. It is a dominant lactic acid producing bacteria in the rumen [81, 82]. Our study showed that, even in the small intestine, this trend of higher *Lactobacillus* abundance in grain-fed cattle still remained. Another genus, *Solibacillus*, was observed to have reduced abundance in the grain-fed group. It was previously reported that in dairy cows, *Solibacillus* was decreased significantly in abundance when diets were shifted from control diets into sub-acute ruminal acidosis induction diets, and it was potentially related to valerate metabolism in the gut [83]. Indeed, a similar result that *Solibacillus* had reduced abundance under high-grain diets was observed in jejunum microbiome.

Bile acids could also affect small intestine microbial community structure. In this study, bile acids were measured in the gallbladder of cattle in two groups, and the data showed significant differences in both primary and secondary bile acids. Among the nine

significantly different bile acids, common conjugated forms of secondary bile acids, taurolithocholic acid, glycolithocholic acid, glycodeoxycholic acid, and taurodeoxycholic acid, all showed significantly elevated levels in the grain-fed group than the grass-fed group. This provided clues of associations of bile acids with gut microbiome in cattle, because of bacterial enzymatic capabilities of converting primary bile acids into secondary bile acids through 7α/β-dehydroxylation. Bacteria strains that were potentially related to secondary bile acids conversion were examined. Only restricted types of bacteria, belong to the order Clostridiales, were able to convert primary bile acids to secondary bile acids [46, 48]. Gram-positive bacteria in the order of *Clostridiales*, including families of *Lachnospiraceae, Ruminococcaceae*, and genus *Blautia,* were reported to have 7α-dehydroxylating activities [47]. In this study, relevant bacteria also showed significant differences between the two groups. At the family level, *Lachnospiraceae* had a higher average abundance in the grain-fed group than the grass-fed group, but this difference was not significant, nevertheless. At the OTU level, 18 OTUs showed significantly higher abundance in the grain-fed group, including two named genera, *Moryella* and *Shuttleworthia*. Among these OTUs, six OTUs belong to family *Ruminococcaceae* was found to be more abundant in grain-fed group, such as the genus *flavefaciens*. Certain genera, such as *Lactobacillus, Clostridium,* and *Bifidobacterium*, were reported to potentially mediate bile acids by performing hydrolases of bile salt deconjugation [47]. In this study, *Lactobacillus* and *Bifidobacterium* were all observed to have increased abundance in the grain-fed group. These elevated bacteria in grain-fed cattle jejunum could potentially help deconjugate the amide bond between bile acids with glycine or taurine, to make bile acids available

as substrates for further conversions by other microbial populations. These increased microbial population suggested possible implications of the higher secondary bile acid storage in grain-fed cattle.

The jejunum microbiota shared many similar functional categories between grass-fed and grain-fed cattle. Only a few KEGG gene families and potential pathways of jejunal microbiome showed significant differences between the two groups. Proteins involving microbial genetic activities were higher in the grass-fed group, such as DNA topoisomerase III [EC:5.99.1.2] and RNA polymerase sigma-70 factor, ECF subfamily. Lots of core microbiota were associated with these KEGGs in contribution analysis of OTU, including 19 phyla such as Firmicutes, Bacteroidetes, Actinobacteria, Chloroflexi, and Cyanobacteria. This could also explain the increased abundance of functional category, transcription machinery, in grass-fed cattle jejunum. The lipid metabolism pathway, glycerophospholipid metabolism, was the only pathway that showed a significant increase in the grain-fed group. Glycerophospholipids were the major class of complex lipids, which were composed of glycerol, two fatty acids, phosphate, and an amino alcohol [84]. Grain-fed regimen contained higher lipids, together with digestion by bile acids, could provide higher levels of glycerol and fatty acids in the small intestine as the organ of nutrient absorption. This might also trigger this higher activity of glycerophospholipids metabolism of microbes in grain-fed cattle jejunum.

Conclusions

In ruminants, the lower GI tract has been less focused, and comprehensive exploration of the small intestine is lacking. In this chapter, other than obtaining microbial profiles of the jejunum, the central organ of nutrient absorption, we also integrated bile acids quantification to deeply explore the jejunum microenvironment influenced by different diets in beef cattle. With the culture-independent method and bioinformatic tools, finer resolution of microbial profiles can be achieved. The jejunum was observed to have lower microbial diversities potentially caused by the lower pH and higher rate of digesta passage compared to other gut segments. Differences of microbial diversities between the grass-fed and grain-fed were not significant, but microbial composition and pathways differed significantly. Previously not many studies have examined the relationship between bile acids and small intestine microbiome specifically in beef cattle. In this chapter, we integrated gut microbiota with bile acids composition and found an increased level of secondary bile acids in the grain-fed group, as well as increased bacteria populations that could mediate primary bile acids into secondary bile acids. A microbial pathway, glycerophospholipid metabolism, which was related to lipid metabolism, was also enriched in the grain-fed group. These phenomena reflected the effects of high-concentrate dietary component in the grain-fed diet. Our results could complement the lack of data about microbiome in the lower GI tract of beef cattle and help the comprehensive understanding of jejunum microbiome and metabolism with a greater resolution.

5. General conclusions

Microbial diversities and compositions in cattle GI tract shared similarities, but also possessed significant differences [11, 29]. The ultimate objective of this study is to reveal the diet-induced changes in the gut microbiome of beef cattle under grass-fed and grain-fed regimens, and to explore further how these induced changes in microbiota are associated with VFA production in the rumen, as well as bile acids composition in beef cattle. We have focused on two diet regimens on Angus beef cattle and explored diet-induced changes on the bacterial community in three segments of gut, rumen, jejunum, and rectum. Then, we also applied predicted metagenomics using PICRUSt to investigate functional differences in the gastrointestinal microbial environment and identified differences in effect size with LefSe algorithm.

In general, we found that differences in diet components between grass-fed and grain-fed diets significantly changed microbial diversities, community structures at various taxonomic levels, potential biological functional categories starting from young beef cattle in rectum, and also in rumen and small intestine. Specifically, we observed that gut microbiome was dominated with Bacteroidetes and Firmicutes in the rumen and rectum, while Firmicutes was the only dominant phylum in the jejunum. In addition, bacteria population changes induced by differences in dietary components also trigger a series of changes to the gut ecosystem and the host. Increased butyrate and propionate production by starch-fermenting bacteria in the grain-fed group also promoted the growth of lactate-utilizing/-producing population. In detail, we traced to specific genera and species, and found that lactate-producing populations, *Lactobacillus* and

Succinivibrio, and lactate-utilizing population, *Megasphaera* and *Selenomonas ruminantium*, were both enriched in grain-fed cattle rumen. As for the small intestine, differences in diets also induce significant alterations of microbial bacteria in jejunum. Other than direct influences of diet because of its processing in gut, bile acids were also significantly changed to influence gut microbiome in this study. Bacterial populations that were able to process primary bile acids were observed with higher abundances. Higher abundance of *Lachnospiraceae*, *Lactobacillus*, *Clostridium*, and *Bifidobacterium* population could utilize primary bile acids and perform their conversion into secondary bile acids, which could be the possible reasons of a higher quantity of secondary bile acids in grain-fed group. Our study reinforced the concepts of how grass-feeding and grain-feeding altered specific populations and gut micro-environment of the host. The NGS based 16S-seq also provided additional detailed and more complete information for cattle microbial profiles under different diets in different gut segments, especially the information in the small intestine. These comprehensive results in our study will enhance the overall interrogation of cattle gut microbiome and interaction with host metabolism at different aspects, and also provide a foundation for possible future studies to explore effective targeted manipulation of cattle microbiome in the beef and dairy GI tract to improve beef quality and animal performance.

As for future directions, association analysis can be performed to explore the potential relationships of these three segments of the gut, rumen, small intestine, and rectum, in Angus beef cattle under different diets. Identified OTUs and different levels of taxa can

be analyzed among the three segments in order to explore further the possible links of different microbes in the cattle digestive tract. In addition, bile acids are synthesized by the liver and stored in the gall bladder to secrete into the duodenum. Our group already found that liver gene expressions and liver functions are largely influenced by grass-fed and grain-fed diets. Thus, another potential future direction of this study is to link bile acids profiles with liver studies. For example, the gene *CYP7A1* that codes a rate-limiting enzyme in the classical pathway of bile acids production was observed to have higher expression in the grass-fed cattle group. This provided potential explanations of higher primary bile acids production in the grass-fed group. Integrating all these measurements and analyses will provide in-depth explorations about how different diets influence the gut microbiome and the host metabolism, to ultimately post influences on the beef quality.

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