

Jejunal Microbiome and Bile Acids Induced by Diets in Angus Beef Cattle

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Abstract

Background: The small intestine, while serving as the main absorption organ, also possesses a unique microbiome environment and holds the critical function of conversion of primary bile acids. Bile acids are, in turn, able to regulate microbiome structure and promote the growth of bacteria that convert primary bile acids to secondary bile acids. However, in beef cattle, few studies have explored the microbiome compositions of the jejunum and its relationships with bile acids. Therefore, the hypothesis is that microbiome compositions in the intestine are influenced by diet style and also related to bile acids.

Results: We demonstrated that the influences of diets on the intestinal microbiome could be observed in young beef cattle after weaning. A significantly higher level of microbial diversity was evidenced in feces of grass-fed animals comparing to grain-fed cattle. Top 20 essential genera were obtained with random forest analysis on the fecal microbiome to identify candidate microbial biomarkers. Moreover, the jejunal microbiome of adult Angus beef cattle exhibited significant differences in microbial composition and metabolic potential under different diets. Global balances and microbiome signatures of bile acids were identified.

Conclusions: The findings from this study provided deep insights into microbiome compositions in fecal and jejunal and identified relationships between jejunal microbiome and bile acids with diets in Angus beef cattle. Our results should help us better understand grass-fed beef in production practice and potential health benefits.

Background

Beef is a flavorful, nutritional, and expensive meat product than many other types of meat. In the beef cattle feeding industry, while the grain-fed method feeds young cattle in feedlots with high-grain diets, the pasture-fed mode allows cattle to graze naturally with grass-based land. As the demand for healthy and palatable beef is increasing worldwide, grass-fed beef products gradually attract more attention in the meat market, and a growing group of consumers is willing to pay a higher unit price for grass-fed beef than grain-fed beef [1]. Other than animal behavior aspects, the diet component is one of the main differences between grain-fed and grass-fed methods to determine cattle performance [2]. One critical influence of diets is that diets primarily learn gut microbiome structures and drive microbial adaption in animals and humans [3]. The compositions and functions of the gut microbiome in ruminant species are critical. Bacteria are the dominant kinds of microbes in the ruminant gut, which provide a significant contribution of metabolomic activities on cattle [4]. So far, many gut microbiome studies have focused on rumen microbiome to assess animal's gut and calves performance, but roles of microbes in small intestine and colon are less explored. However, different segments of the ruminant abdomen possess unique ecological and microbial characteristics [5, 6]. After the digestion happening in the upper GI tract, peristalsis and a portion of undigested fibrous residuals together push digesta downward, to enter the

small intestine and be in contact with villi of the intestinal epithelium where absorption happens. The small intestine is the main gut segment for the absorption of glucose, amino acids, fatty acids, and glycerol with the aid of bile and enzymes [4]. Because of the limited contact with the outside environment, low oxygen level, and little development time here, 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 [5, 7–9]. Although the abundance is relatively low, the microbiome in the small intestine played essential roles to aid digestion, including the conversion of primary bile acids, and the protection against pathogens in both human and animals [10, 11]. Importantly, by microbial actions in the small intestine, primary bile acids are mediated to produce secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA), which are conjugated and finally reabsorbed by the enterohepatic circulation for the recycling in the liver to regulate digestion [12, 13]. Diet, as the primary determinant of the gut microbiome, may also post significant influences on the microbiome structure of small intestine in cattle. Therefore, the small intestine microbiome and their relationships with bile acids are worth the exploration.

Previous studies of cattle microbial communities throughout the GI tract demonstrated compositional differences of the microbiome in the small intestine compared to other segments in both dairy and beef cattle [5, 14]. But not much information could be found to illustrate the influence of diet on the jejunal microbiome on beef cattle. In the case of grass-fed and grain-fed beef cattle, the jejunal microbiome is also critical to reveal diet influence on cattle performance. Besides, few studies showed the relationships between jejunal microbiome and bile acid concentration in cattle. In this study, we hypothesized that microbiome compositions in the intestine are associated with diet components. To test the hypothesis, we used Wye Angus beef cattle from the University of Maryland (UMD). The Angus herd in the Wye Angus farm has been closed to any animal importation for almost 70 years. Hence it produces very homogeneous progeny [1]. The heifers were treated and raised equally until weaning. After weaning, the heifers were randomly assigned to either grass or grain-fed style. Our previous studies using the same animals already pointed out several advantages of the grass-fed method over the grain-fed method [15, 16]. In this study, we would like to probe further from the aspect of the gut microbiome to explore the potential roles of microbiome in the jejunum and their relationships with bile acids to influence cattle metabolism. Since the influences may start from the early age of cattle and jejunum is not accessible in young animals, we first examined the fecal microbiome in the rectum to obtain an initial impression of the influence of the two different diets. After the reach of market weight, jejunal digesta were collected for exploration. In addition to the analyses of the microbial community in the jejunal and fecal microbiome, we also examined whether the components of bile acids associated with the composition of the intestinal microbiome. We profiled the microbial structure of jejunum with the 16S rRNA amplicon sequencing, together with bile acid quantification, to characterize the influences of different diets on the jejunal microbiome.

Results

Fecal microbiome structure influenced by diets in young beef cattle

Microbial composition of the feces in the rectum of calves aged seven months old was examined based on the OTU table generated from the QIIME (Quantitative Insights Into Microbial Ecology) closed reference pipeline [17]. In total, there were 19 microbial phyla identified from grass-fed and grain-fed groups (Figure S1). The most abundant phylum was *Firmicutes*, ranging from 38.36–68.42% of relative abundance percentages, followed by *Bacteroidetes* (37.77%), *Proteobacteria* (3.96%), and *Verrucomicrobia* (1.20%).

Microbiome diversity indices were examined for the fecal microbiome of grass-fed and grain-fed cattle. Alpha-diversity indices, including Chao1, Shannon, Simpson, and Phylogenetic diversity (PD_whole_tree) were calculated (Table 1). A Welch's t-test was used to perform a differential test between the two groups and obtain p-values. 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 ($p < 0.05$). Therefore, it was suggested that diets significantly influenced microbial diversity between the two groups, and the grass-fed group tended to have higher microbial diversity than the grain-fed group ($p < 0.05$). PCA (Principal component analysis) was also performed to examine beta diversity to explore the differences between groups based on the most abundant microbial families, such as *Ruminococcaceae* (10.92%), *Rikenellaceae* (6.20%), *Lachnospiraceae* (4.61%), and *Paraprevotellaceae* (4.34%), etc. The biplot of PCA (Fig. 1) showed a clear separation of the fecal microbiome of young beef cattle under grass-fed and grain-fed diets.

Differentially abundant taxa and important microbial features in fecal microbiome under different diets

The difference in fecal microbiome composition between grass-fed and grain-fed groups was also examined. Relative abundances of taxa were computed by QIIME and analyzed with the Linear Discriminant Analysis (LDA) Effect Size (LEfSe) algorithm [18]. Fourteen phyla were identified to be differentially abundant (LDA score ≥ 2.0) (Fig. 2A). As for the family level, 47 families showed differences in relative abundances. A cladogram was plotted at the family level with a notation of differential taxa under different diets in grass-fed and grain-fed groups (Fig. 2B). Among these differential families, *Ruminococcaceae*, *BS11*, and *Porphyromonadaceae* were the top three discriminative features in the grass-fed group. At the same time, *Succinivibrionaceae*, *S24-7*, and *Lachnospiraceae* were the top three discriminative families in the grain-fed group. At the OTU level, among the detected 4182 OTU, 402 OTU had a significant difference in relative abundance (absolute LDA score $\log_{10} \geq 2.0$). Of them, 144 OTU showed enrichment in the grain-fed group, and the other 258 OTU showed higher plenty in the grass-fed group. The top 20 most abundant significant OTU were listed in Additional file 1: Table S1. The abundance value based on the genus level was further evaluated by applying a random forest analysis

for the group classification ($m_{\text{try}} = 7$, $n_{\text{tree}} = 500$), and predictive accuracy of 100% regarding grass-fed and grain-fed group was achieved (Fig. 3). The decrease in mean accuracy measured the importance of features. The top 20 most important elements were plotted, and their abundance levels were noted on the left side of the plot. The results suggested that these taxa held the highest discriminatory power between grass-fed and grain-fed groups and may be of interest as microbial biomarkers.

Jejunal Microbiome Structure In Beef Cattle Under Different Diets

After the two groups of young animals reached market weight, cattle were slaughtered, and the jejunal microbiome was examined for comparison. QIIME closed reference pipeline was used to analyze 16S-seq data of cattle jejunal contents with Greengenes Database [17, 19], identifying 24 phyla, 44 classes, 77 orders, 149 families, and 263 genera collectively of the two cattle groups. Of the 24 recognized phyla (Additional file 1: Figure S2), the most abundant phylum detected in cattle jejunal microbiome was Firmicutes that accounted for 63.11–98.21% in 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%). 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 typical in cattle, which accounted for approximately 0.99% abundance of jejunal microbiome families.

Also, jejunal microbial diversity was analyzed, including alpha and beta diversities. Common microbial diversity indices were evaluated (Additional file 1: Table S2). No significant differences in diversity indices were detected between grass-fed and grain-fed groups (p values > 0.05). The average values between alpha diversity indices of two groups were examined, the grass-fed group 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 \pm SD) for the grass-fed group, 66.77 ± 18.04 for the grain-fed group. We also performed a rarefaction analysis based on Chao1 values of grass-fed and grain-fed groups. We plotted the rarefaction curve, which suggested that the sequencing depth in the current study was enough (Additional file 1: Figure S3). Jejunal microbiome in grass-fed cattle also showed a higher average of Chao1 than grain-fed animals during a random sampling of rarefaction process. Our results suggested that the grass-fed group tended to have a higher microbial diversity than the grain-fed group, although the diversity was not statistically significant (p values > 0.05). As for the beta diversity analysis, biplot of PCA was plotted based on identified top abundant families across the two groups, which demonstrated a distinct difference in jejunum microbial composition between grass-fed and grain-fed individuals (Fig. 4).

>The diet is the primary determinant of jejunal microbial composition

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 LEfSe [18]. Even though there was limited access to the external environment and low microbial abundance in the small intestine, diets still exerted several critical influences on microbial composition. Nine discriminative taxa at phylum level were depicted (Fig. 5A). At the family level, 67 taxa showed significant differences in relative abundance between the two groups. For example, identified families *Enterobacteriaceae*, *Turicibacteraceae*, *RFP12*, *Elusimicrobiaceae*, and *Bifidobacteriaceae* showed higher abundance in the grain-fed group, whereas *Bacteroidaceae*, *Rikenellaceae*, *Paraprevotellaceae*, *BS11*, and *Nocardioideaceae* were significantly higher in abundance in the grass-fed group. A cladogram based on the family level was depicted (Fig. 5B), displaying taxa with significant differences in the jejunal microbiome. Forty-six named genera showed substantial differences between the two groups (absolute LDA score $\log_{10} \geq 2.0$). For example, *Lactobacillus* and *Ruminococcus* were significantly higher in the grain-fed cattle jejunal microbiome, whereas *Solibacillus* had substantially higher abundance in the grass-fed group (Fig. 6). At the OTU level, 291 OTUs were significantly different in wealth between the grass-fed and grain-fed groups (absolute LDA score $\log_{10} \geq 2.0$).

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 2 with relative abundance (mean \pm SD) in the grain-fed and grass-fed group. The LDA \log_{10} score was calculated using the LefSe algorithm.

Potential Jejunal 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 [20] was used to predict functional profiling of the jejunal microbiome between grass-fed and grain-fed group based on 16S rRNA marker gene sequences. After normalization of read counts in the OTU table from QIIME pipeline output, a total of 6909 Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology gene families were identified. Of them, five KEGG gene families showed significant differences in abundance between grass-fed and grain-fed groups using the LEfSe method [18] with a cutoff value of Linear Discriminant Analysis (LDA) score $\log_{10} \geq 2.0$. Specifically, only one KEGG, insertion element IS1 protein InsB (K07480), was more abundant in the grain-fed group. In contrast, 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 the grass-fed group. In total, as for

predicted KEGG pathways, there were 328 identified microbial biological pathways. Some abundant functional pathways in the jejunal 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 (Fig. 7).

Associations Between The Gut Microbiome And Bile Acids

Bile acids from gallbladder samples of eight cattle in each group were measured using an LC-MS/MS system. In total, 21 bile acids were identified and quantified, including both primary and secondary bile acids and bile acid conjugates. Among them, nine were significantly different between grass-fed and grain-fed groups (Table 3). The conjugated form of cholic acid and deoxycholic acid was detected at a relatively high $\mu\text{mol/mL}$ concentrations. For example, the level of taurocholic acid, cholic, and glycocholic acids were significantly higher in the grass-fed than the grain-fed group (Table 3; $p < 0.05$).

Further, at least six bile acids, including the conjugated form of primary, secondary bile acids, such as lithocholic acid and deoxycholic acid, were significantly higher in the grain-fed group. The other 11 detected bile acids were not significantly different between grass-fed and grain-fed groups (Additional file 1: Table S3), such as the secondary bile acids, deoxycholic acid, ursodeoxycholic acid, indicating their relatively low susceptibility to diet influences in the gut of beef cattle. Together, our data suggest that the grain diet may promote bacterial activities in converting primary to secondary bile acids.

A critical concept of compositional balance has been introduced [21, 22]. The identification of the global microbial balance is to find predictive microbial signatures of a phenotype of interest by *Selbal* [22]. In our study, the predictive microbiome signatures were most likely secondary bile acids, obtained by using *Selbal* with default parameters. In the process, six secondary bile acids related to bacterial bile acid conversion activities were used as the response variables for prediction in *Selbal*. Each time, one of six bile acids was tested using the microbial abundance data at the genus level to perform modeling and variable selection. In total, twelve different taxa were identified among all the taxa, with some of them being selected in more than one balance for different bile acids (Table 4, Additional file 1: Figure S4). The taxa in the numerator and denominator of the global balances predictive of the corresponding bile acids were listed. As expected, among these genera, there were known bile acid producers, *Clostridiaceae*, *Clostridium*, and *Veillonellaceae* [23–25]. For example, the balance (log ratio) of *SMB53* (numerator) and *Clostridium* (denominator) were identified as a microbial signature that could readily help the prediction of glycodeoxycholic acid. The results suggested that these taxa likely played vital global roles to influence bile acids composition in beef cattle under different diets and were worthy of further investigation.

Discussion

Grass-fed and grain-fed methods are two feeding regimens in beef cattle production. As diets are directly related to the microbiome, exploration of diet influences on the gut microbiome is necessary to guarantee a proper functioning gut and determine its further impacts on cattle performance. Because gaining access to forestomach and small intestine is difficult in live animals before slaughtering, the effects of diets on the gut microbiome are always determined using rectal microbial communities as baseline data. Besides, microbial communities and their potential functions are critical to aid nutrient absorption in lower gut segments of cattle. In the jejunum, the large number of villi and microvilli increase the surface area of the small intestine, enhancing the chance of food particles to encounter bile acids and digestive enzymes. Microbes function actively to convert primary bile acids into secondary bile acids to aid digestion, especially the lipid. Nutrients could then be absorbed across epithelium by passive or active transport to blood vessels [26]. In this study, we first focused on the fecal microbiome in 7-month young cattle as a baseline to initially explore the influence of diets on young beef cattle and identify candidate biomarkers. Then we measured jejunal microbiome and examined its association with bile acids when these cattle reached market weight. The results of our study indicated grass-fed and grain-fed regimens altered microbial community's structure, diversity, and biological functional categories of the microbiome in feces and jejunum. Also, bile acids were influenced by diets, which exerted an influence on the jejunal microbiome.

From the feces of 7-month-old young cattle under the two different diets, significant microbiome differences could already be observed from the phylum level to the OTU level. The dominant phyla were in agreement with previous studies of cattle gut microbiome that Firmicutes was the most abundant phylum near the colon, followed by Bacteroidetes and Proteobacteria [8, 27]. We identified significantly different taxa in relative abundance between grass-fed and grain-fed. Specifically, Firmicutes were 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) was approximately 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 these results were also in agreement with similar studies about influences of diets and fed in cattle [28, 29]. At higher taxonomic ranks, several common taxa were identified to be significantly different between grass-fed and grain-fed cattle fecal microbiome (Log_{10} LDA \geq 2.0). For example, *Ruminococcaceae*, the top abundant identified family in the fecal microbiome, was ~ 5 fold higher in effect size in the grass-fed group than the grain-fed group; *Ruminococcaceae* was also the essential family identified by random forest classification analysis and had a higher abundance in the grass-fed group, as it relies on dietary fiber as an energy source [30]. Some named genera were also identified to provide clues of possible influences of diets on fermentation strains in the upper forestomach. For example, a named species *Succinivibrio* was analyzed to be 2-fold greater in effect size of abundance in the grain-fed group than the grass-fed group of cattle fecal microbiome (Log_{10} LDA = 2.3). *Succinivibrio* species ferments glucose to produce high amounts of succinic acid as their main products [31]. It was also found that when fed cattle with diets that contain high levels of rapidly fermented carbohydrates, mainly starch, *Succinivibrio* species would always exist in cattle rumen [31, 32]. Our study indicated that this trend of *Succinivibrio* could already be observed in the fecal

microbiome of 7-months young cattle under different diets, and, as expected, it was higher in fecal microbiome of the grain-fed group. These fecal microbiome explorations served as a baseline data and provided a reference for the jejunal microbiome characterization.

As for the small intestine, the unique environment and function of the small intestine made microbiome here to be distinctive with the upper digestive tract [14]. It was not surprising that microbial profiles we analyzed within jejunum also had various unique characteristics. From several previous studies, microbial diversities were all observed to decrease in this segment of cattle gut. 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 a reduced oxygen level in the small intestine [5, 9, 14, 27]. In our study, we also observed that Firmicutes became the only one dominant phylum in the jejunal microbiome. The microbiome diversity analysis was then calculated. Alpha diversity indices of the jejunal microbiome did not achieve statistically significant differences between the two groups. A relatively small sample size in this study and animal variations within the same group could potentially be the reasons. Another possible reason was that the unfavorable environment of the small intestine itself and fast rate of digesta passage was infeasible for microbial growth compared to rumen and rectum, so significant differences in diversity might not be statically obtained. However, we did observe a clear separation of the two groups in beta-diversity analysis, demonstrating with a biplot of PCA (Fig. 4). In higher taxonomic levels, many named genera and species were identified. It was observed that families such as *Clostridiaceae*, *Peptostreptococcaceae*, and *Ruminococcaceae*, genera *Clostridium*, *Butyrivibrio*, and *Ruminococcus* were common dominant taxa in the jejunal microbiome of beef cattle. This was also in agreement with previous studies regarding the profiles of the ruminant small intestine microbiome [14, 27, 28].

Besides, significant differences in the relative abundance of specific taxa in the jejunal microbiome were observed between the two groups and posted health implications for beef cattle production. The genera, *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* that can produce lactic acid [33, 34], and the genera, *Megasphaera*, a commonly known lactic acid utilizing genus [35], all showed higher relative abundance levels in the jejunal microbiome of grain-fed cattle. These genera have long been observed to have a higher abundance in high-grain diet rumen, and in animals with rumen acidosis [36, 37]. Our study further showed that from the rumen to the jejunum, these higher abundances in grain-fed cattle remained significant to have more senior activities related to lactate. 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 [38]. A similar result that *Solibacillus* had reduced abundance under high-grain diets was observed in the jejunal microbiome in our study. From the aspect of cattle health, these observations, all together, suggested that grain-fed cattle tend to cause an acidic gut environment globally, and influence significantly not only the rumen but also jejunal microbiome. The grass-fed method could be a better choice to avoid this phenomenon.

The microbiome of the small intestine was associated with bile acid composition. Bile acids were secreted from the duodenum and flow along the small pipe to aid lipid digestion, and processed into secondary bile acids, and finally could get recycled through the enterohepatic circulation. 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, tauroolithocholic acid, glycolithocholic acid, glycodeoxycholic acid, and taurodeoxycholic acid, all showed significantly elevated levels in the grain-fed group than the grass-fed group. Taxa that were potentially related to secondary bile acids conversion were examined, and *selbal* was used to find predictive microbiome signatures for significantly different secondary bile acids. Six OTUs belong to family *Ruminococcaceae* were found to be more abundant in the grain-fed group, such as the genus *flavefaciens*. It has been reported that gram-positive bacteria in the order of *Clostridiales*, including families of *Ruminococcaceae*, could perform 7 α -dehydroxylation to convert primary bile acids into secondary bile acids [39]. These higher amounts of OTUs belonging to *Ruminococcaceae* in the grain-fed cattle jejunal microbiome might contribute to the higher secondary bile acids recycled from the intestine. Besides, specific genera, such as *Lactobacillus*, *Clostridium*, and *Bifidobacterium*, could potentially mediate bile acids by performing deconjugation with bile salt hydrolases (BSH) to make bile acids available for further biotransformation [39]. As mentioned above, *Lactobacillus* and *Bifidobacterium* had increased abundance in the grain-fed jejunal microbiome in our study.

Moreover, *Lactobacillus* was identified to be the numerator that constructed the global microbial balance predictive of glycolithocholic acid. *Clostridium* was also recognized in the microbial balances regarding glycodeoxycholic acid and the total of taurooursodeoxycholic/taurohyodeoxycholic acid. These increased bacteria in grain-fed cattle jejunum could potentially aid to deconjugate the amide bond between bile acids with glycine or taurine, to make bile acids available as substrates for further biotransformation by other microbes [39]. Also, this elevated level of bile acids and bile acid utilizing taxa in grain-fed cattle might give us clues about its less healthy fatty acids profile in grain-fed beef, which needed further explorations focusing on these associations. Overall, these identified microbial taxa, both in the differential analysis and *selbal* analysis, indicated novel associations between the gut microbiome and higher secondary bile acids in the grain-fed cattle and were also suggestive in other ruminant species.

The jejunal microbiome shared many similar functional categories between grass-fed and grain-fed cattle. Only a few KEGG gene families and potential pathways of the 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. By using the PICRUST algorithm [20], lots of core microbiome were identified to be associated with these KEGGs in contribution analysis of OTU, including 19 phyla such as *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Cyanobacteria*. These could also explain the increased abundance of the functional category, transcription machinery, in the jejunal microbiome of the grass-fed cattle. The lipid metabolism pathway, glycerophospholipid metabolism, was the only pathway that showed a significant increase in the grain-fed group. Glycerophospholipids were the dominant class of complex

lipids, which were composed of glycerol, two fatty acids, phosphate, and amino alcohol [40]. Grain-fed regimen contained higher fats, together with digestion by bile acids, could provide higher levels of glycerol and fatty acids in the small intestine that serves the organ of nutrient absorption, triggering more activities of glycerophospholipids metabolisms of microbes in grain-fed cattle jejunum.

Conclusions

In ruminants, few studies have focused on the lower GI tract; and a comprehensive survey of microbial composition in the small intestine digest of grain- and grass-fed beef cattle is lacking. In this study, microbiome profile and candidate microbial biomarkers were identified in fecal contents of 7-month animals, suggesting an early influence of diet on the microbiome in beef cattle. The jejunal microbiome was observed to have lower microbial diversities to have only Firmicutes accounts for more than 90% relative abundance, which potentially caused by the lower pH and higher rate of digesta passage. Differences in microbial diversities between the grass-fed and grain-fed were not significant, but microbial composition and pathways differed significantly for the jejunal microbiome.

Most importantly, the gut microbiome, in association with bile acids and microbiome signatures were discovered as a novel aspect. There were increased secondary bile acids in the grain-fed group, as well as increased bacteria populations that could modulate bile acids. Besides, microbiome signatures were identified with minimal taxa but could still be associated with secondary bile acids. Furthermore, 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 the high-concentrate dietary components in the grain-fed diet. Our results could provide important clues about the microbiome in the lower GI tract of beef cattle and help the comprehensive understanding of the jejunal microbiome and bile acid metabolism with much higher resolution.

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 bailedage during cold seasons. The hay 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 supplied with any types of grain. Each 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.

Sample collection of fecal and jejunal contents

Twenty-two young male cattle were obtained and raised at UMD Wye Angus herd. Twelve animals were fed with grass *ad libitum*. The other ten cattle were fed with grain diets. Around seven months old, feces at the rectum of both groups of animals were sampled at UMD Wye Angus farm (Queenstown, MD) and immediately frozen in dry ice and stored at -80°C freezer in the lab until microbial DNA extraction. After the steers have reached market weight, they were slaughtered, and jejunal contents were immediately collected for microbial DNA extraction.

DNA extraction, 16 s rRNA gene amplification, and sequencing

Microbial DNA from feces and jejunal 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 as previously described [40]. Briefly, the 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 were 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 manufacturer's protocol.

Bile acids measurements

Bile samples were measured in a commercial lab (Creative Proteomics, Shirley, NY) according to a procedure published previously [41]. After ultrasonication and centrifugation, five μl of cattle bile was mixed with 995 μl of methanol in Eppendorf tubes. Supernatant 8 μl of each sample was combined with 992 μL of 50% methanol. After preprocessing, 20 μl aliquots were used for quantitation by UPLC(-)ESI-MRM/MS according to a procedure published previously[55]. Standard curves for bile acid concentrations were performed, as followed: an 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 S1 standard solution was diluted in a series at the 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. Twenty μl aliquots were injected to record the data files to prepare calibration curves using linear regression (A_s/A_i peak area ratio versus concentration) for calculation of bile acid concentrations measured in the individual samples. SciexMultiQuant was used

for data processing. T-test was used to compare the difference of bile acid from grass-fed to grain-fed group with a selected threshold for p-values < 0.05.

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, designed to maximize the diversity during sequencing run to identify unique clusters better and improve base-calling accuracy, were trimmed with Trimmomatic version 0.38 [42]. 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) [43]. 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 [44]. OTU was identified using a closed-reference OTU picking protocol with a threshold of 97% similarity. The taxonomic assignment was performed based on the GreenGenes database v13.8 [45]. 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* [46] and *factoextra* [47]. PICRUSt (v1.1.2) was used with default parameters to predict metagenomics, KEGGs gene families with regarding contributions of OTU, and functional categories [20]. 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 [18] was used in the differential analysis to identify significantly different OTUs, KEGGs, and pathways between the grass-fed group and grain-fed groups. LEfSe can identify abundant differences of characteristic 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 [18]. *Selbal* [22] was used to identify global balances of the two groups and identify microbiome signatures predictive of significantly different secondary bile acids.

Declarations

Ethics approval and consent to participate

The animal handling and experiments in this study were 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) with the protocol #R-11-72.

Consent for publication

Not applicable.

Availability of data and material

The 16S rRNA gene sequencing data are available in the NCBI Sequence Read Archive (SRA) under Bioproject PRJNA608270.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JS and RWL designed the experiment. JL, FL, WC, CJ, YB, and WZ participated in the data collection and performed the experiments. JL wrote the manuscript, and JS and RWL revised the manuscript. All authors read and approved the final manuscript.

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Tables

Due to technical limitations the Tables are available as downloads in the Supplementary Files>

Table 1 Alpha diversity indices in cattle fecal microbiome.

Table 2 Select significantly different OTUs in relative abundance in jejunal microbiome of grain-fed and grass-fed cattle.

Table 3 Nine significantly different bile acids between grass-fed and grain-fed cattle (concentration unit: $\mu\text{mol/ml}$).

Table 4 Predictive global balances of jejunal microbiome for significantly different secondary bile acids.

Additional File Legend

Additional file 1 as pdf

Additional file 1: Figure S1 Phylum-level fecal microbial composition in young beef cattle. **Table S1** Selected 20 significantly different OTUs in relative abundance in the rectal microbiome of grain-fed and grass-fed Angus beef cattle. **Figure S2** Phylum-level microbial composition of jejunal digesta. **Table S2** Alpha diversity indices of jejunum microbiota in grass-fed and grain-fed Angus beef cattle. **Figure S3** Rarefaction curves of jejunal microbiome based on Chao1 values of grass-fed (blue) and grain-fed (red) groups. **Figure S4** Demonstration of global balances for secondary bile acids quantities of grass-fed and grain-fed cattle.

Figures

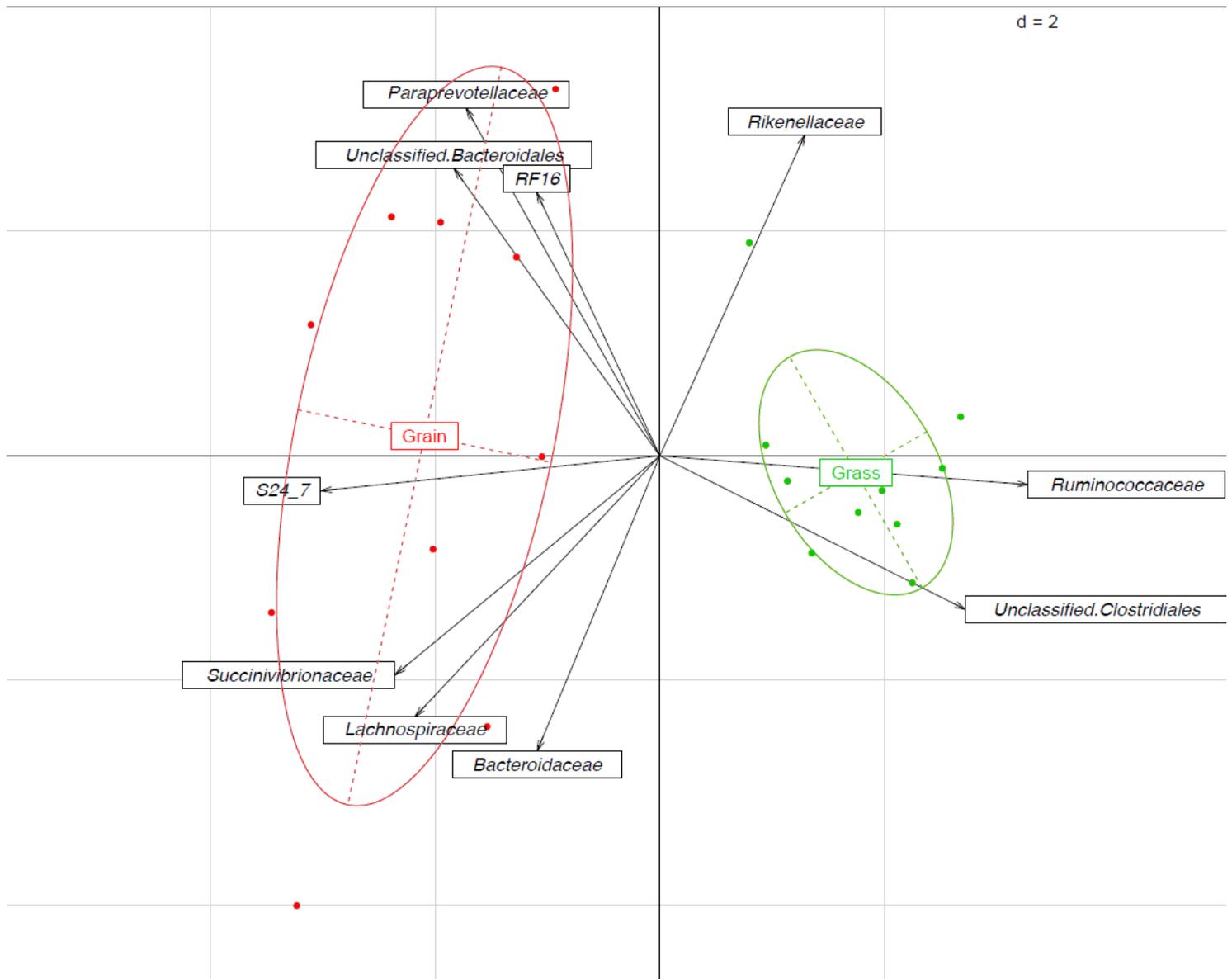


Figure 1

Ordination biplot of principal component analysis (PCA) on the fecal microbiome. Arrows display the directions and relative importance of the top 10 abundant families. The variability in the fecal

microbiome are explained on x-axis with 41.91% and on y-axis with 25.06%.

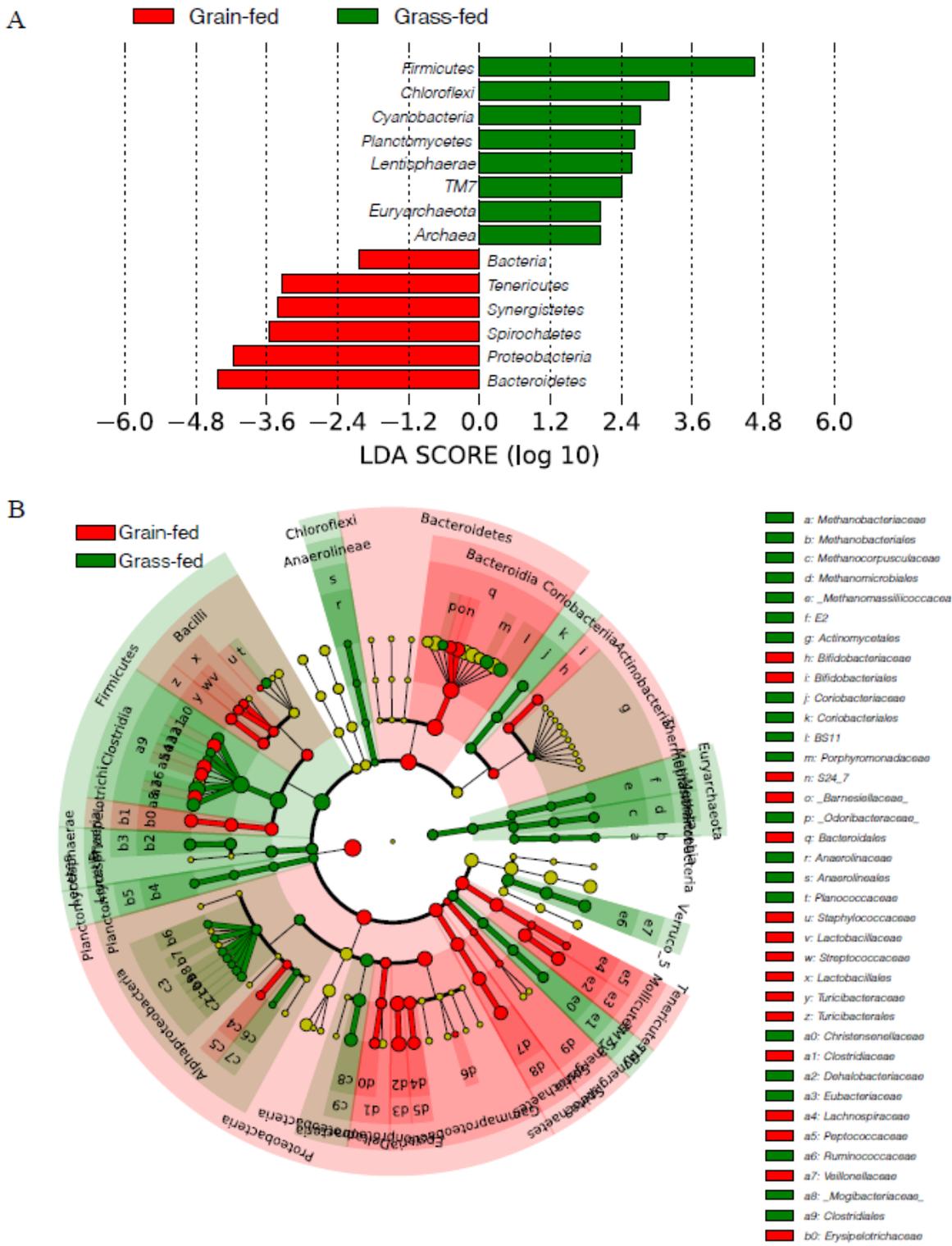


Figure 2

(A) Significantly discriminative taxa to phylum level with absolute Linear Discriminant Analysis (LDA) score ≥ 2.0 in fecal microbiome between grain-fed and grass-fed cattle. (B) The cladogram representing the taxa in the fecal microbiome. Taxa at the family level that had significantly different abundances between grass-fed and grain-fed cattle with an absolute LDA score ≥ 2.0 were displayed.

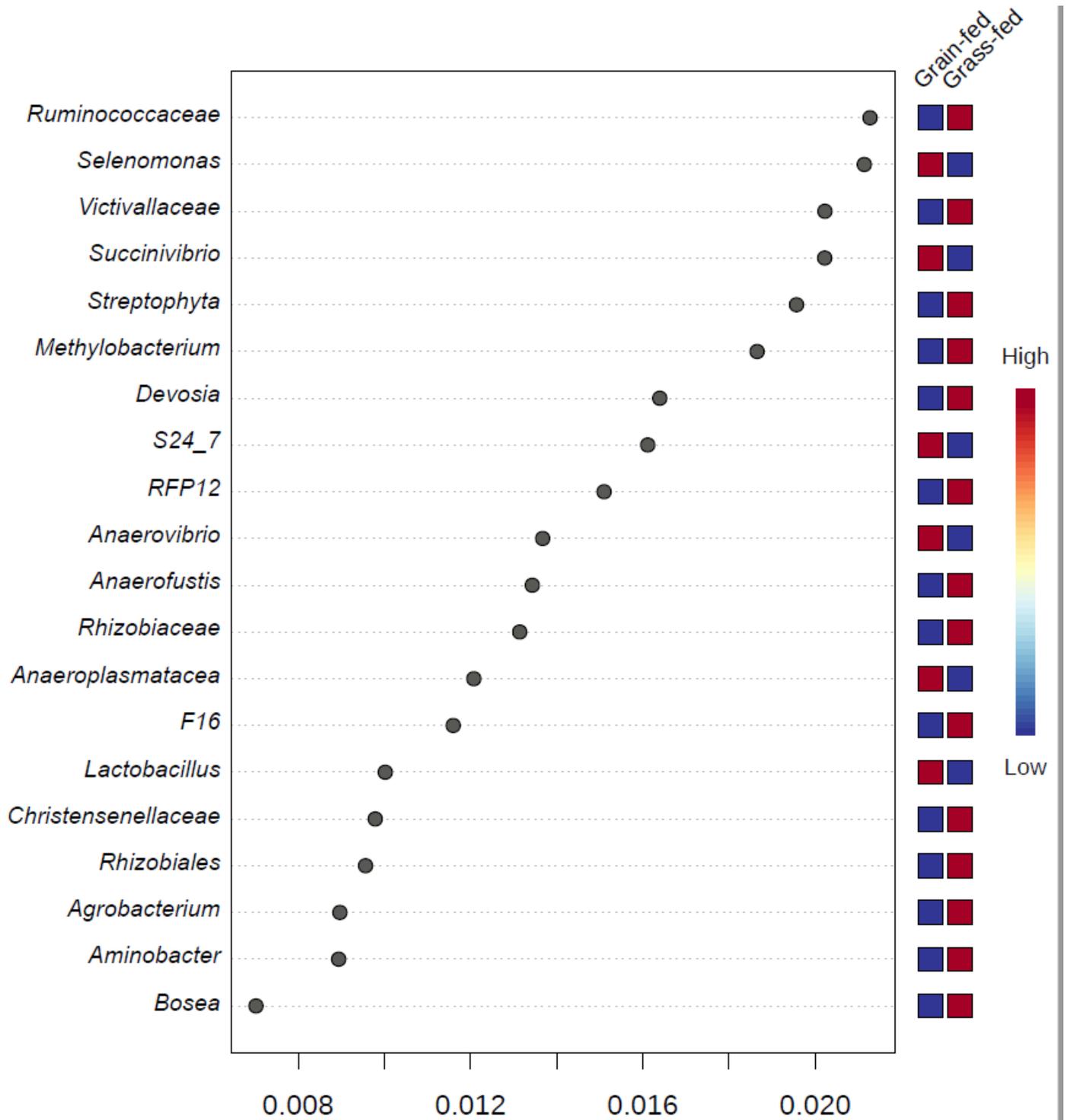


Figure 3

Random forest analysis on fecal microbiome of grass-fed and grain-fed Angus cattle. The y-axis, from top to bottom, displays the taxa ranked by their importance (Mean Decrease Accuracy) for the group classification.

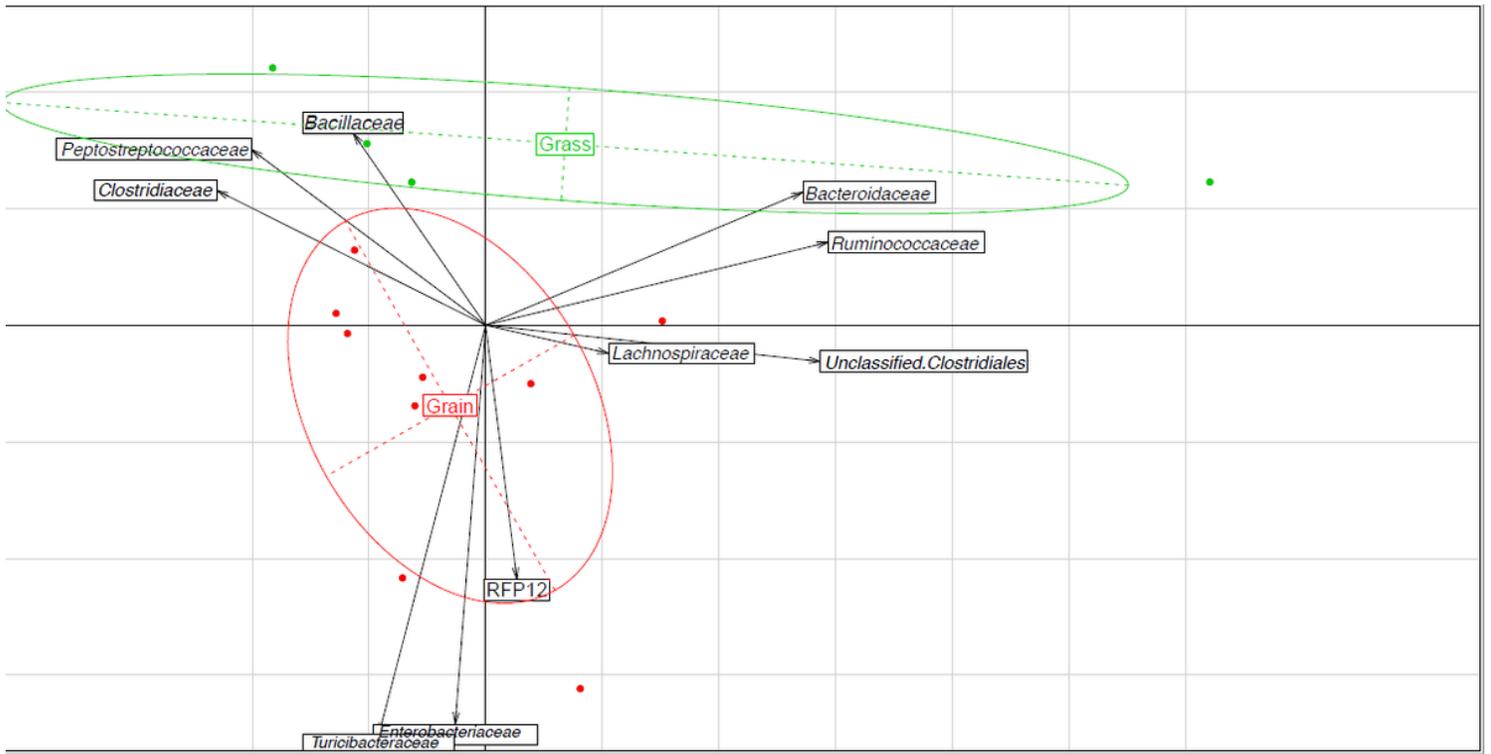
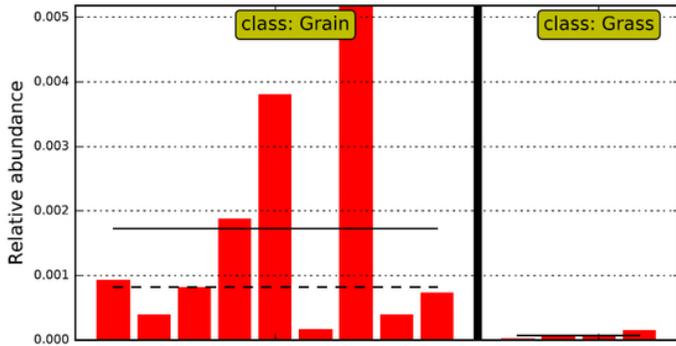


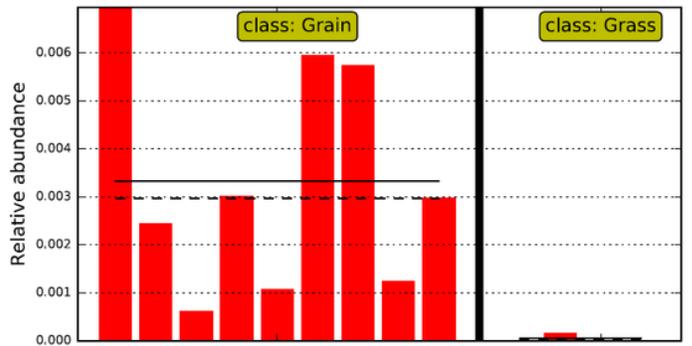
Figure 4

Ordination biplot of PCA on the jejunal microbiome. Arrows display the directions and relative importance of the top 10 abundant families. The variability in the jejunal microbiome are explained on x-axis with 39.91% and on y-axis with 20.05%.

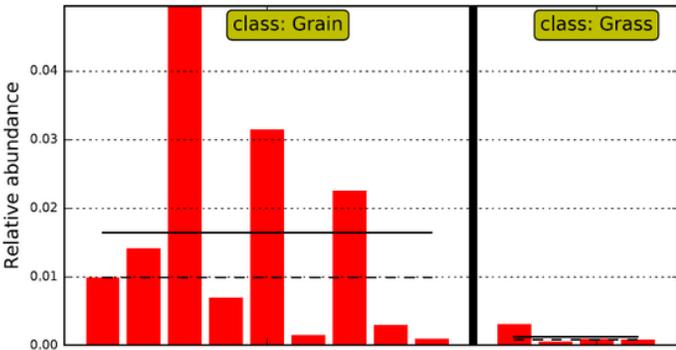
A: *Streptococcus*



B: *Lactobacillus*



C: *Ruminococcus*



D: *Solibacillus*

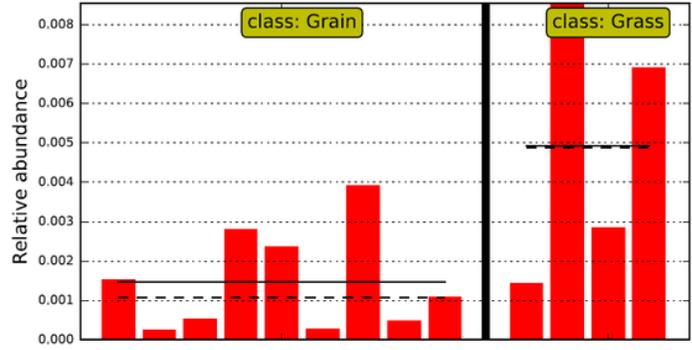


Figure 6

Selected microbial genera are displaying significant differences in relative abundance in jejunal microbiome between grass-fed and grain-fed cattle. (A) *Streptococcus*. (B) *Lactobacillus*. (C) *Ruminococcus*. (D) *Solibacillus*. X-axis represents individuals; y-axis represents relative abundance.

Differential Pathways of Jejunum Microbiome

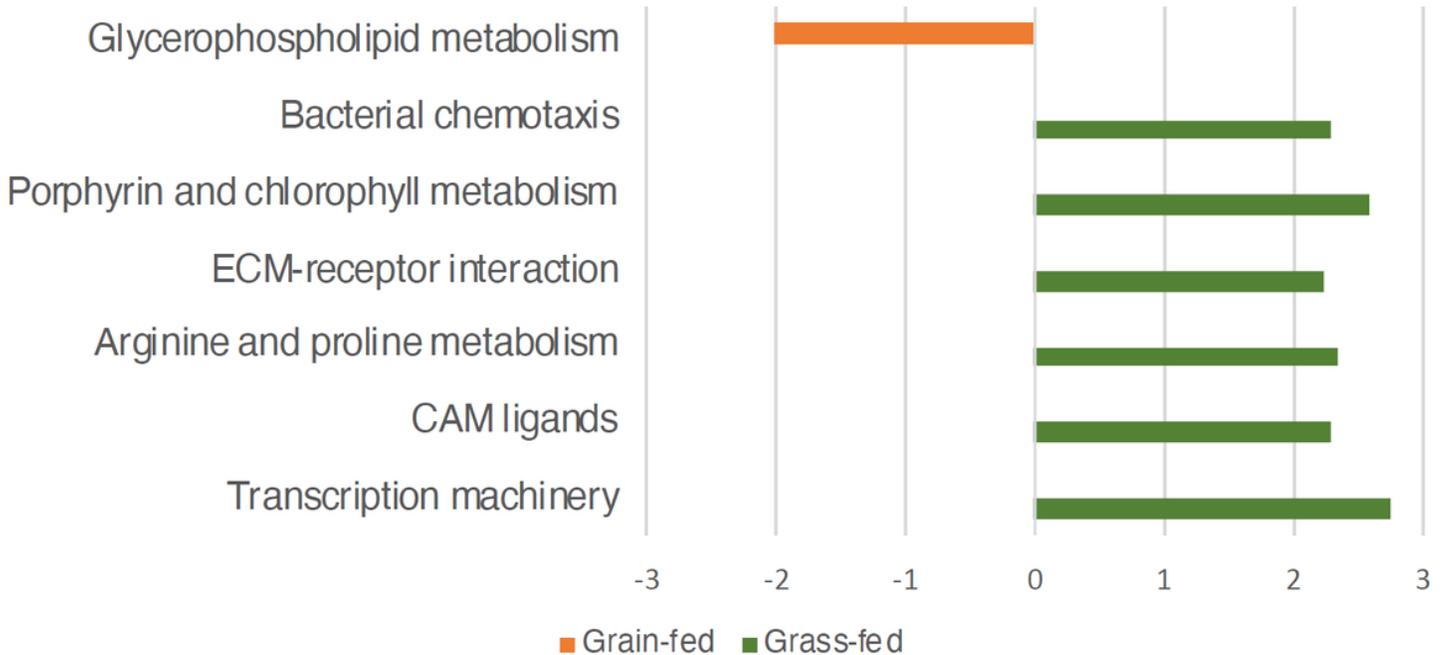


Figure 7

Significantly different KEGG pathways of jejunal microbiome impacted by diets between grass-fed and grain-fed Angus beef cattle (LDA score \geq 2.0).

Supplementary Files

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- [Additionalfile1.pdf](#)
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