UTILIZATION OF EXOGENOUS FEED ENZYMES AS A WAY TO ENHANCE CREEP FEEDS – IN VITRO, IN VIVO, AND RUMINAL MICROBIOME EVALUATIONS

by

JEFERSON MENEZES LOURENCO

(Under the Direction of Robert Lawton Stewart, Jr.)

ABSTRACT

A multi-step approach was used to evaluate the effects of including exogenous feed enzymes in creep feeds formulated for beef calves. The first experiment consisted of testing 4 different enzymes (endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase, and α-amylase) and their combinations in an in vitro batch culture fermentation using rumen fluid collected from 6-month-old beef calves. Traits such as production of volatile fatty acids, production of methane, and in vitro dry matter digestibility were quantified, and the best enzyme supplement was chosen to be used in the second experiment: an in vivo feeding trial. This second trial was conducted over a period of 2 years and used 4 different cow-calf herds at 2 research stations. The cow-calf herds were split into similar groups and their performances were monitored for approximately 100 days in each year. Upon conclusion of this feeding trial, the third step took place. In this final step, the contents of the forestomach of 27 calves were collected by esophageal tubing, and immediately after this procedure, and all calves were weaned, grouped together, and offered a common diet. Four weeks later, a second collection

procedure was performed on the same 27 calves, and samples obtained on both days were subjected to microbiome analysis using 16S rRNA gene paired-end sequencing. Results from the first step identified endo-1,4-β-xylanase at 3,000,000 units per tonne of dry matter as the best candidate for the subsequent in vivo trial, and after testing this treatment for 2 years, this conjecture was confirmed: calves fed this treatment had greater average daily gains than calves receiving other treatments. Results from the microbiome analysis revealed some shifts in calves' ruminal microbial population; however, most of the fluctuations were not of great magnitude. Therefore, we concluded that factors such as an increased amount of energy intake due to supplementation, and increased metabolizable energy due to addition of endo-1,4-β-xylanase were probably more important than any shifts observed in the microbial community. Consequently, we presumed that these factors had a greater contribution on the observed differences in calf growth when the feeding trial was conducted.

INDEX WORDS: 16S rRNA, Creep feeding, Feed enzymes, Xylanase.

UTILIZATION OF EXOGENOUS FEED ENZYMES AS A WAY TO ENHANCE CREEP FEEDS – IN VITRO, IN VIVO, AND RUMINAL MICROBIOME EVALUATIONS

by

JEFERSON MENEZES LOURENCO

B.S., State University of Maringa, Brazil, 2003

M.S., The University of Georgia, 2014

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2017

UTILIZATION OF EXOGENOUS FEED ENZYMES AS A WAY TO ENHANCE CREEP FEEDS – IN VITRO, IN VIVO, AND RUMINAL MICROBIOME EVALUATIONS

by

JEFERSON MENEZES LOURENCO

Major Professor: Robert Lawton Stewart, Jr.
Committee:
Dennis Hancock
Jacob R. Segers
Joshua C. McCann

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2017

DEDICATION

I want to dedicate this work to my wife – Daniela, who has certainly inspired and supported me during this journey. I also want honor and dedicate this work to all my loved family members, especially to my father – Edinei, my mother – Neide, and my grandparents – Vicente de Paula Menezes and Francisca Valim Menezes. You all are very special to me. Thank you!

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God for this major achievement for He has given me a supernatural strength and taken great care of me, in every little detail, for my entire life – literally! I also have not enough words to thank the sacrifice that He provided on my behalf through His Son, Jesus Christ – my Lord and Savior. Secondly, I want to express my gratitude to every person who has been involved in each of the many steps that brought me to this point in my life. I want to thank my wife and all my loved family members for the unconditional support. I also would like to thank Dr. Robert Lawton Stewart, Jr. for the several opportunities that he has provided me to grow both professionally and as a person. I also would like to thank Dr. Keith Bertrand, Dr. Michael Azain, Dr. Dennis Hancock, Dr. Jacob Segers, Dr. Joshua McCann, Dr. Brian Campbell, Dr. Mark Engstrom, Dr. Mark Froetschel, Dr. Nicolas DiLorenzo, Dr. Martin Ruiz Moreno, Dr. Ignacy Misztal, Dr. Roberto Palomares, Sherie Hulsey, Gina McKinney, Tessa Schulmeister, Andra H. Nelson, Susan Bradley, Dennis Richardson, Valerie Christopher, Michael J. Mathis, David A Fulcher, Randall Chad Westmoreland, Charles Tyler Trumbo, Andrew David Dunn, João H. J. Bittar, Cassandra Bittar, André Garcia, Pattarapol Sumreddee, Camila Silva, and everybody else who has helped me both with their professional or personal skills! Finally, I want to express my appreciation to DSM Nutritional Products for providing all the commercial enzyme preparations, and most of the financial support for my research. Thank you all very much!

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Most beef cattle farms in the southeast United States are cow-calf operations. In such operations, the yearly calf production is usually sold at local auction barns shortly after calves are weaned, and producers are paid on a "per-pound basis". Consequently, as a general rule, heavier animals are more valuable. In spite of this, the most recent report by the National Animal Health Monitoring System (USDA, 2008) shows that the average weaning weight for calves in the eastern U.S. is 228 kg (503 lb). Moreover, USDA market reports for the state of Georgia over the last 5 years have shown that the majority of beef cattle producers in the state are selling their cattle at lighter weights (i.e. below 272 kg, or 600 lb; USDA, 2017). This indicates that most cattle producers in Georgia are not investing their resources in backgrounding/stockering their calves for long periods after weaning, or they are not using these practices at all. In this scenario, it seems logical to promote technologies that increase calves' body weight at weaning, such as pre-weaning supplementation (commonly known as "creep feeding").

The scientific literature shows that creep feeding can be a great tool for weaning heavier calves; however, from a nutrition standpoint, there are still some challenges to be faced, and the efficiency by which calves convert feed into weight gain is undoubtedly one of them. For instance, research has shown that poor feed conversion is usually observed when animals have unlimited access to creep feeding, making it economically unattractive (Brazle et al., 1992; Moriel and Arthington, 2013). Moreover, according to

the latter authors, the poorer feed conversion seen when unlimited creep feed is offered can be attributed to lower NDF digestibility, which leads to a decrease in forage use. Since beef calves are usually introduced to forages at an early age, their forestomach is still not fully developed when they start grazing (Church, 1979). Consequently, digestibility of forages by these animals is expected to be lower than what is normally observed in adult beef cattle. One viable option to overcome this problem may be the inclusion of feed enzymes in the diets of young cattle. Research has demonstrated that supplementing ruminant diets with exogenous enzymes has significant potential to improve feed utilization and animal performance (Beauchemin et al., 2003; Adesogan et al, 2014; Meale et al., 2014).

Due to recent improvements in their manufacturing process, feed enzymes are becoming more economically viable (Balci et al., 2007; Meale et al., 2014), and this fact has allowed the development of commercial products that can improve the profitability of animal production systems. Although utilization of exogenous feed enzymes is a common practice in the poultry and swine industries, the use of these feed additives is still an emerging technology in ruminant nutrition (Meale et al., 2014). Feed enzymes specifically designed for ruminants usually contain xylanase and cellulase activities resulting from bacterial or fungal fermentations. These compounds can enhance fiber digestion in the rumen and improve feed efficiency (Beauchemin et al., 2003; He et al., 2014). However, many nutritionists do not recommend feed enzymes for ruminants because it is assumed that they would be rapidly degraded by ruminal microbes (Beauchemin et al., 1999). In addition, the optimum level of inclusion of feed enzymes for different forage types (e.g. grasses versus legumes) may need to be individually

studied, for they have substantial differences in their cell wall composition and structure (Beauchemin et al., 1995). Furthermore, factors such as the moisture level of the feed, pH, the time required for enzymes to interact with the substrate, among many others, can all influence the observed animal performance responses (Beauchemin et al., 1995).

Because ruminant animals are greatly affected by the diversity and relative abundance of microorganisms in their forestomach, characterization of their microbial community is very important. However, traditional culture-dependent methods are not able to reveal the whole ruminal ecosystem, as many species cannot thrive under the conditions provided in laboratories. New approaches such as next-generation DNA sequencing are facilitating a better understanding of the complete, actual rumen microbiome. The combination of these new techniques with animal performance and fermentation parameters has produced significant advances and opened new areas for study (McCann et al., 2014).

To our knowledge, there is no published research on utilization of feed enzymes as enhancers of beef cattle creep feeds. Therefore, the objective of this study was to evaluate the effects of including different feed enzymes in creep feeds formulated for beef calves. Specifically, this evaluation was performed in a multi-step process in which the 3 main steps were: 1) In vitro evaluation of 4 different enzymes: endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase. These enzymes were tested both alone and combined in an in vitro batch culture experiment, in order to identify the best candidate for a future feeding trial; 2) Feeding trial using the best option identified in the previous step (in vitro) in commercial beef cattle; 3) Analysis of the ruminal microbiome of calves that participated in the previous step (feeding trial).

LITERATURE CITED

Adesogan, A. T., Z. X. Ma, J. J. Romero, and K. G. Arriola. 2014. RUMINANT NUTRITION SYMPOSIUM: Improving cell wall digestion and animal performance with fibrolytic enzymes. J. Anim. Sci. 92:1317–1330. doi: 10.2527/jas.2013-7273.

Balci, F., S. Dikmen, H. Gencoglu, A. Orman, I. I. Turkmen, and H. Biricik. 2007. The effect of fibrolytic exogenous enzyme on fattening performance of steers. Bulg. J. Vet. Med. 10:113−118.

Beauchemin, K. A., L. M. Rode, and V. J. H. Sewalt. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75:641–644. doi: 10.4141/cjas95-096.

Beauchemin, K. A., L. M. Rode, and D. Karren. 1999. Use of feed enzymes in feedlot finishing diets. Can. J. Anim. Sci. 79:243–246. doi: 10.4141/A98-124.

Beauchemin, K. A., D. Colombatto, D. P. Morgavi, and W. Z. Yang. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. J. Anim. Sci. 81:E37–E47. doi: 10.2527/2003.8114_suppl_2E37x.

Brazle, F. K., G. L. Kuhl, C. E. Binns, K. O. Zoellner, L. R. Corah, and R. R. Schalles. 1992. The influence of limited creep feed on pre- and post-weaning performance of spring born calves. Prof. Anim. Sci. 8:55–57. doi: 10.15232/S1080-7446(15)32139-2.

Church, D. C. 1979. Growth and development of the ruminant stomach. Digestive physiology and nutrition of ruminants. Volume 1: Digestive Physiology. 2nd ed. Oxford Press Inc., Portland, OR.

He, Z. X., M. L. He, N. D. Walker, T. A. McAllister, and W. Z. Yang. 2014. Using a fibrolytic enzyme in barley-based diets containing wheat dried distillers grains with solubles: ruminal fermentation, digestibility, and growth performance of feedlot steers. J. Anim. Sci. 92:3978–3987. doi: 10.2527/jas.2014-7707.

McCann, J. C., T. A. Wickersham, and J. J. Loor. 2014. High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. Bioinform. Biol. Insights. 8:109–125. doi: 10.4137/BBI.S15389.

Meale, S. J., K. A. Beauchemin, A. N. Hristov, A. V. Chaves, and T. A. McAllister. 2014. Board-Invited Review: opportunities and challenges in using exogenous enzymes to improve ruminant production. J. Anim. Sci. 92:427–442. doi: 10.2527/jas.2013-6869.

Moriel, P., and J. D. Arthington. 2013. Effects of trace mineral-fortified, limit-fed preweaning supplements on performance of pre- and postweaned beef calves. J. Anim. Sci. 91:1371–1380. doi: 10.2527/jas.2012-5469.

USDA. 2008. Beef 2007-08, Part I: Reference of Beef Cow-calf Management Practices in the United States, 2007-08. USDA-APHIS-VS, CEAH. Fort Collins, CO.

USDA. 2017. Georgia Cattle Auctions Weekly Summary. USDA-GA Dept. of Ag. Market News Service, Thomasville, GA.

CHAPTER 2

THE REVIEW OF THE LITERATURE

Pre-Weaning Supplementation in Beef Cattle

The scientific literature shows that supplementation of calves during the suckling stage of their lives (commonly known as creep feeding) can significantly improve calves' weaning weights (Prichard et al. 1989; Tarr et al., 1994; Sampaio et al., 2002; Viñoles et al., 2013); however, less than one third of the cow-calf operations in the southeast United States use this strategic supplementation (Parish and Rhinehart, 2009). In addition, some cattlemen use this supplementation at the wrong time during calves' suckling period. To address this, Prichard et al. (1989) conducted an experiment comparing 2 different creep feeding periods: 1) for the last 155 days prior to weaning calves (long term); or 2) for the last 65 days before their weaning (short term). Both groups received a commercial high-energy creep feed with 14% crude protein. A third group of calves from the same herd was used as a control group (i.e. no creep feed offered). All calves in their study (a total of 200) were weaned at an average age of 210 days. Their results show that the weaning weight of non-creep fed calves was the lowest, followed by the calves supplemented for the short term, and by the ones supplemented for the long term (231, 257, and 264 kg, respectively; $P = 0.001$). However, ADG during the last portion of the trial (the last 65 days) was greatest for the short term group, followed by the long term and the control groups (1.25, 1.20, and 0.90 kg/day, respectively; $P = 0.001$). Moreover, calves

supplemented for the short term had better feed efficiency compared to the ones supplemented for the long term: 5.3 versus 6.7 kg of creep feed/kg of additional gain. The researchers concluded that although all supplemented calves had their performance improved, little benefit was observed in calves supplemented for the long term. For that reason, these authors proposed that calves should be creep fed from about 5 months of age until weaning in order to maximize feed utilization and yield a similar increase in weight gain, when compared to calves supplemented for longer terms.

In an effort to determine the optimum level of protein in creep feeding rations, Lopes et al. (2014) tested supplements ranging from 8 to 41% in crude protein. Their findings revealed a quadratic effect for the increasing levels of protein, and the highest gain was achieved when the supplement contained 19% of this nutrient. The authors also observed that regardless of the protein level, improved daily gains and nutrient digestibility were observed for all supplemented calves. Thus, they concluded that creep feeding optimized the performance of beef calves and promoted greater weights at weaning.

Creep Feeding Effects on the Post-Weaning Phase

In addition to greater gains during the suckling phase, a work by Brazle et al. (1992) showed that creep fed animals also had improved post-weaning performance when compared to non-creep fed animals, regardless of the type of feed they received during their suckling phase (high protein or high energy creep feed). While non-creep fed animals gained 1.05 kg/day in their first 53 days in a feedlot, the group of animals that had previously received any type of creep feed had greater gains ($P = 0.01$) averaging 1.13 kg/day. In addition, the post-weaning feed conversion of these animals was

numerically improved (5.2 vs. 5.4 kg of feed for each kg gain, on average), compared to the ones that were not creep fed. These results were attributed to an improved condition of the rumen due to supplementation during the suckling phase. According to the authors, supplemented calves had their rumen adapted to consume processed feeds earlier in life, which in turn resulted in improved animal performance during their post-weaning phase. Similarly, Moriel and Arthington (2013) found that calves that received preweaning supplementation during the last 100 days of the suckling phase had greater (P = 0.05) body weight at weaning (229 vs. 219 kg), and greater voluntary dry matter intake during the first week post-weaning (1.12 vs. 0.99 % of BW; P = 0.04).

A study by Gadberry et al. (2012) also evaluated performance of creep fed animals during the pre and post-weaning periods. They found that performance of spring-born calves was improved during their suckling phase due to creep feeding, but there was no difference on average daily gain during their post-weaning phase. However, creep fed animals tended to be last days on feed during the feedlot finishing phase, and this finding alone can represent an additional benefit to feedlot operators, suggesting that a premium might be paid to cattlemen producing creep fed animals.

Exogenous Feed Enzymes in Animal Production Systems

Feed enzymes are becoming more economically viable due to recent improvements in their manufacturing process (Balci et al., 2007). This fact has allowed the development of commercial products that can improve the profitability of animal production systems. The use of exogenous feed enzymes is a common practice in the

poultry and swine industries, however, utilization of these feed additives is still an emerging technology in ruminant nutrition (Meale et al., 2014).

Feed Enzymes in Monogastric Nutrition

According to Barrera et al. (2004), the nutritive value of feeds such as wheat is diminished in diets of poultry and swine due to certain physical and chemical characteristics of this ingredient. The non-starch polysaccharides in wheat include cellulose, pectins, β-glucans, and arabinoxylans. This last compound (arabinoxylans) represent about 70% of the total non-starch polysaccharides in wheat. These are linear polymers of variable length consisting of D-xylose joined with β (1–4) linkages, and single residues of arabinose attached along the primary xylan chain. Arabinose renders a polymer soluble, which in solution, interacts with other polymers resulting in the formation of a viscous digesta (Barrera et al., 2004). Several studies showed that an increase in the viscosity of intestinal contents is closely associated with a decrease in nutrient digestibility (Choct et al., 1999; Mathlouthi et al., 2002; Barrera et al., 2004; Wu et al., 2004).

In poultry diets, Choct et al. (1999) fed a diet rich in non-starch polysaccharides (wheat-based diet containing 68% wheat) to 48 individually caged birds. They tested if the inclusion of xylanase in this diet would affect their feed intake, rate of growth, feed conversion, starch digestibility and digesta viscosity in the small intestine, production of VFA in the distal gut, and apparent metabolizable energy value. They included 1 g/kg of a xylanase derived from *Trichoderma longibrachiatum*, which resulted in an activity of 2,500 units per kg of diet. Their results were positive: the inclusion of xylanase resulted in a better feed conversion (2.1 versus 1.9 g feed/g gain), reduced digesta viscosity in the

small intestine, and increased starch digestibility. Furthermore, although the ratios of VFA did not change for the 2 treatments, total production of VFA was higher in the ceca of enzyme-treated birds, indicating that greater fermentation occurred. The resulting effect was a greater apparent metabolizable energy for the diet containing xylanase, compared to control (14.5 versus 13.7 MJ/kg DM), which they attributed mainly to the improvement in nutrient utilization due to the reduction in digesta viscosity.

In another study using growing chickens, Mathlouthi et al. (2002) evaluated the effects of feeding a commercial enzyme preparation containing xylanase and β-glucanase in a rye-based diet. They distributed their chickens in 3 groups: the first was fed a corn-based diet, the second and third groups received a rye-based diet – the difference being the inclusion of enzymes in the diet of the third group (xylanase and β-glucanase). The diets in the 3 groups were formulated to be isocaloric and isonitrogenous. They also were equivalent in calcium, available phosphorus, and essential amino acids. The commercial enzyme preparation was added at the level of 20 mg/kg of diet, which resulted in 560 units of xylanase and 2,800 units of β-glucanase per kg of diet. They observed similar feed intake (478 vs. 524 g) and weight gain (281 vs. 313 g) in birds consuming the corn-based diet and the rye-based diet enhanced with enzymes, however, these traits were significantly impaired in birds fed the rye-based diet without the enzymes (397 g of feed intake and 178 g of weight gain). Regarding feed conversion, birds fed the corn-based diet were the most efficient, followed by rye with enzyme group, and the rye-based diet (0.656, 0.534, and 0.437 g gain:g feed, respectively; $P = 0.0002$). Individual water intake assessments revealed that birds receiving the rye-base diets consumed more water than corn-fed birds, regardless of the addition of the enzyme, which was attributed to the

capacity of non-starch polysaccharides to bind water. At the end of the feeding trial, the authors conducted a digestibility trial, followed by euthanasia for collection of their intestines for histological analyses. Their findings showed similar digestibility of crude protein, and similar apparent metabolizable energy in the corn and rye plus enzyme groups, with the rye diet being significantly lower. The exact same trend (i.e. rye plus enzyme similar to corn-fed birds) was observed in their histological measurements of intestinal villus length, villus width, and villus surface area. The total concentration of bile acids in the small intestine of chickens was greatest in corn-fed birds, compared to the 2 other groups, however, although not statistically significant, the addition of enzyme to the rye-based diet increased the total concentration of bile acids in the intestine of birds. The authors concluded that the inclusion of the enzyme mix counteracted the negative effects of replacing corn with rye in broiler chicken diets.

In a similar study, Wu et al. (2004) examined the influence of phytase and xylanase, both individually or in combination, on performance, digesta viscosity, apparent metabolizable energy, and gut morphology in broilers fed wheat-based-diets. Their basal diet (composed mainly of wheat and soybean meal – 67% and 25.5%, respectively) was enhanced with xylanase at 1,000 units/kg, phytase at 500 units/kg, or a combination of xylanase and phytase. Compared to the basal diet, individual additions of xylanase and phytase significantly improved the weight gains by 16.5 and 17.5%, respectively, whereas the combination of phytase and xylanase caused a numerical improvement of 19.8% in this trait. A similar trend was observed for feed intake: birds fed the diets supplemented with xylanase, phytase, or a combination of both significantly consumed more feed. The feed efficiency of birds fed the diets with enzymes was

improved, markedly for the treatments containing xylanase alone or the combination of xylanase plus phytase. Individual additions of xylanase and phytase resulted in numerical improvements in apparent metabolizable energy, however, when both enzymes were combined, this trait was significantly improved compared to the control group, and this same effect was observed on ileum villus height. They also reported that digesta viscosity was reduced by the addition of the individual enzymes or their combination. The authors concluded that the tested enzymes are able to improve performance of broilers on wheat-based diets.

Barrera et al. (2004) carried out 2 trials to test the effects of inclusion of xylanase in wheat-based diets offered to growing pigs. Digestibility of amino acids and animal performance were monitored. Animals were individually housed in metabolic cages and fed 1 of 4 wheat-based diets with or without xylanase. Three rates of xylanase inclusion were tested: 5,500, 11,000, and 16,000 xylanase units per kg of diet. Their results showed that the apparent ileal digestibility of crude protein quadratically increased with the increasing levels of xylanase, and it was maximized when the inclusion rate was 11,000 units/kg. The same effect was observed on the digestibility of all the essential amino acids. For instance, the apparent ileal digestibility of lysine went from 56% in the basal diet (i.e. no xylanase) to 66% in the diet where xylanase was present at 11,000 units/kg. In the animal performance trial, the group receiving the diet with 5,500 units of xylanase/kg had the greatest daily feed intake, however, when compared to the basal diet, the ADG of pigs receiving the diet with 11,000 xylanase units per kg was maximized: an increase of 17.4% was observed. This level of 11,000 xylanase units/kg also yielded the best feed conversion among all treatments: while pigs in the basal diet utilized 4.49 kg of

feed to gain 1 kg of BW, pigs in this group only needed 3.85 kg of feed. They concluded that supplementation of xylanase at a rate of 11,000 units/kg in wheat diets can increase apparent ileal digestibility of most indispensable amino acids in growing pigs improve feed:gain ratio.

Feed Enzymes in Ruminant Diets

As previously discussed, the use of feed enzymes is well documented and accepted in the poultry and swine production systems, however, they are not consistently used in commercial cattle diets due to several reasons. According to Beauchemin et al. (1999), many nutritionists do not recommend feed enzymes for ruminants because it is assumed that they would be rapidly degraded by ruminal microbes. In addition, the optimum level of inclusion of feed enzymes for different forage types (e.g. grasses versus legumes) may need to be individually studied, for they have substantial differences in their cell wall composition and structure (Beauchemin et al., 1995). Furthermore, factors such as substrate specificity, moisture level of the feed, time required for enzymes to interact with the substrate, pH, and even the temperature of the feed likely affect the binding of enzymes with the substrate and influence the observed responses (Beauchemin et al., 1995). Feed enzymes specifically designed for ruminants usually contain xylanase and cellulase activities that can enhance fiber digestion in the rumen and improve feed efficiency (He et al., 2014).

Giraldo et al. (2008) performed an in vitro study using 3 fibrolytic enzymes: xylanase from *Trichoderma viride*, cellulase from *Aspergillus niger*, and cellulase from *Trichoderma longibrachiatum*. These enzymes were incubated with treatments varying from 70:30% to 30:70% in their forage:concentrate ratios. Their results showed that,

compared to the control treatment (in which no enzyme was added), all the enzymes used in their study significantly increased ADF digestibility and total production of VFA after 24-h incubation. However, NDF digestibility at 24 h was only improved by the addition of xylanase obtained from *Trichoderma viride*, and the cellulase from *Trichoderma longibrachiatum*. In addition, they found that for the treatment containing high-forage (70% of the total DM), the true dry matter degradability was improved by utilization of the 3 tested enzymes, especially when the highest rate of inclusion was used: 80 enzymatic units/g substrate DM.

Beauchemin et al. (1999) conducted a trial to evaluate, in a commercial setting, the effects of using a fibrolytic enzyme mixture in feedlot cattle consuming a high-concentrate diet. After a period of adaptation, 1,200 crossbred heifers were split into 2 groups (control or enzyme group) and offered a diet composed of 92% concentrate (DM basis) in which dry-rolled barley was the main ingredient. The calculated enzyme activities of the diet offered to the enzyme group was 33.4 units of xylanase and 66.3 units of cellulase per kg of dietary DM. The researchers noticed that the variation in daily DMI was reduced in the group of cattle consuming the enzymes, however, the amount of DM consumed was not different between the 2 groups. In addition, there was a significant difference in ADG in favor of the enzyme group (1.53 versus 1.40 kg/day; P = 0.01). Although their feed conversion rate was not statistically different, there was a numerical improvement of 10% in this trait for cattle fed the enzyme mix (6.95 vs. 7.72 kg feed DM:kg BW gain). Since feedlot diets have a low fiber content, the authors hypothesized that the observed results may have occurred because the optimum pH of the feed enzymes used in this study was below 6.0. It is well documented that the fibrolytic

activity in the rumen is reduced when the pH is below 6.0, and feedlot cattle fed high-concentrate diets experience this acidotic condition on a daily basis. So, the addition of fibrolytic enzymes that perform better in lower pH may have contributed to their results and explain the observed differences in animal performance in this trial.

In another study with feedlot cattle, He et al. (2014) studied the effects of adding fibrolytic enzymes on ruminal fermentation, digestibility, and growth performance of cattle fed a finishing diet containing wheat dried distillers grains with solubles (WDGS). Their study had 2 distinct parts – a digestibility evaluation using cannulated animals, and a growth performance finishing trial. In the first part of their study, 4 heifers with ruminal cannulas were used in a replicated 4 × 4 Latin square design. These heifers were individually housed and adapted to a high-grain diet by gradually increasing the proportion of concentrate (40 to 90%, DM basis) over a 4-week period. They were then randomly assigned to 4 treatments, including one control (10% barley silage and 90% barley grain–based concentrate); a second one replacing 30% of barley grain with WDGS; and 2 other treatments similar to the latter, the difference being the inclusion of fibrolytic enzymes at a low level, or at a high level. The low and the high levels provided 16,150 units of xylanase and 500 units of cellulase, or 32,300 units of xylanase and 1,000 units of cellulase/kg diet DM, respectively. The authors found that the inclusion of the enzymes significantly improved ($P = 0.02$) starch digestibility in the diet, especially when the level of inclusion of the enzymes was greater. Likewise, digestibility of NDF, ADF were numerically improved. No differences were detected on ruminal pH ($P = 0.70$), total concentration of VFA ($P = 0.78$), and molar proportions of the major VFA. On the other hand, in situ ruminal DM digestibility at 48 hours of WDGS linearly increased with the

addition of the enzymes, and the same tendency was observed for NDF digestibility at 48 hours. In the second part of their study the researchers fed the same 4 diets mentioned previously to 160 crossbred steers in a finishing trial that lasted 120 days. The enzymes were sprayed onto WDGS every 3 days and incorporated into a total mixed ration. Investigators did not observe significant differences on DM intake, final BW, ADG, and carcass quality traits. However, as expected, animals were less efficient when WDGS replaced barley grain, but this condition was improved by the addition of the fibrolytic enzymes, especially at the higher rate of inclusion. Similarly, the addition of fibrolytic enzymes reduced the incidence of liver abscesses. Researchers concluded that although the fibrolytic enzymes were not able to improve growth performance of feedlot steers, their addition helped to reduce the adverse effects of including WDGS in their diets.

In a study to establish whether fibrolytic enzyme additives enhance the performance of growing steers, and to determine optimal enzyme concentrations in forage diets, Beauchemin et al. (1995) individually fed 72 weaned calves for a period of 10 weeks. They received one of three forage diets: cubed alfalfa hay, cubed timothy hay, and whole crop barley silage. Supplements were added to each forage to provide a minimum of l2% CP and to supply adequate rumen undegradable protein and minerals. Xylanase and cellulase from fungal sources were applied to the forages at 6 incremental levels, starting from zero (no inclusion) and having the greatest level of inclusion equivalent to 15,800 units of xylanase and 632 units of cellulase. They found that the effects of enzyme addition varied among forages. For alfalfa hay, DMI, ADG, and digestibility of DM were all maximized when the combination of 4,733 units of xylanase and 156 units of cellulase per kg DM was included. This combination also numerically

improved feed efficiency. For timothy hay, DMI, ADG, feed efficiency, digestibility of DM, and digestibility of ADF were maximum for the treatment including the combination of 12,000 units of xylanase and 632 units of cellulase per kg DM. Finally, when the forage used was barley silage, no significant improvements were observed by the addition of the enzymes. In fact, the treatment containing 2,000 units of xylanase and 80 units of cellulase per kg of DM numerically decreased ADG by 11.6% compared to the group receiving barley silage without the addition of any enzyme. Dry matter intake and digestibility of DM were also negatively affected at this inclusion level (reduction of 9.3 and 11.1%, respectively). The authors did not specify the reason for the marked difference in response to enzyme application across forage types, but one possible explanation is the different method of application of enzymes across forages: while barley silage had an aqueous enzyme solution mixed into it just before feeding, alfalfa and timothy hays had the enzyme solution sprayed during the cubing process, which was done several days before feeding them, and consequently allowing more time for enzymes to act on those forages. The investigators concluded that the relationship between enzyme concentration and animal response was non-linear and differed for legumes and grasses, indicating that the optimum combination and application rates of carbohydrases need to be assessed individually for grasses and legumes, given the substantial differences in their cell wall. Moreover, the lack of response obtained using barley silage indicates that factors such as substrate specificity, moisture level of the feed, time required for enzymes to interact with the substrate, and pH of the feed during treatment are likely important and possibly affect the binding of enzymes with the substrate.

Balci et al. (2007) studied the effects of fibrolytic enzymes with cellulase and xylanase activities on the digestibility of forage and body weight gain of steers. Animals ranged from 9 to 12 months of age and had ad libitum access to a diet composed of wheat straw and concentrate mix. This diet was offered for a period of 80 days to 2 distinct groups: control and enzyme treated. At the end of the experimental period, rumen fluid samples were collected from 6 steers in each group via esophageal tube and used in in vitro procedures. Although steers in both groups had similar body weights at the beginning of the feeding trial, the ones supplemented with feed enzymes were 20 kg heavier at the end of the 80-day trial, which was found to be statistically significant. In addition, the feed conversion rate was significantly improved in the enzyme-treated group. No differences were found in rumen pH (6.19 versus 6.20) but in vitro DM digestibility of wheat straw was significantly higher when rumen fluid from the enzyme-treated group was used for the incubations. Moreover, organic matter digestibility and NDF digestibility were greater than the control group. Numerical differences were also observed when the substrate used in the incubation was a concentrate feed. These improvements in digestibility may explain the greater gains observed for animals receiving the fibrolytic enzymes in their trial.

The Ruminal Microbiome

The microbiome inhabiting the rumen is characterized by its high population density, wide diversity, and complexity of interactions, and it functions as an effective system for the conversion of plant cell wall biomass into microbial protein, short chain fatty acids, and gases (Brulc et al., 2009). The chemical processes required to break down

plant cell wall are rarely carried out by a single species of microorganism. As an illustration, initial colonization of fiber is done by organisms that have enzymes that attack the easily available side chains of complex polysaccharides, followed by another subset of organisms that degrade the main chains of cellulose and xylan (Brulc et al., 2009). Because ruminant animals are greatly affected by the diversity and relative abundance of microorganisms in their forestomach, characterization of their microbial community is very important, however, traditional culture-dependent methods are not able to reveal the whole ruminal ecosystem, as many species cannot thrive under the conditions provided outside the rumen. For instance, *Ruminococci* have been studied in many culture-based experiments, however, recent studies using modern molecular techniques have observed this genus in low abundance in the rumen microbiome. In addition, members of the *Ruminococcaceae* family identified through modern techniques may be different from the ones identified through culture-based techniques, and consequently have different biological functions (McCann et al., 2014a). New approaches such as next-generation DNA sequencing are facilitating a better understanding of the complete, actual rumen microbiome. The combination of these new techniques with animal performance and fermentation parameters has produced significant advances and opened new areas for study (McCann et al., 2014b).

Mammalian gut microbial communities are dominated by members of the phyla *Firmicutes* and *Bacteroidetes*. The ability of bacteria from these phyla to metabolize otherwise indigestible complex polysaccharides is very important for the host animal, as they produce a myriad of enzymes that can target a broad range of substrates. Rosewarne et al., (2014) carried out pyrosequencing of 16S rRNA genes extracted from ruminal

samples, and found a predominance of sequences from the phyla *Bacteroidetes* (47.2%) and *Firmicutes* (42.3%). The *Bacteroidetes* fraction was dominated by sequences from the order Bacteroidales (71% of *Bacteroidetes*), while the *Firmicutes* fraction was dominated by sequences from the order Clostridiales (91%).

Research has been conducted on how the gut microbiota affects body weight gain and body composition of the host animal. Working with mice, Turnbaugh et al. (2006) found that the microbiome of obese animals is rich in DNA sequences that encode enzymes involved in the breaking down of dietary polysaccharides which are otherwise indigestible. In comparison with lean mice, obese mice had greater concentration of acetate and butyrate in their distal gut. Moreover, even though the diet offered was the same, and that feed consumption in both groups was similar, bomb calorimetry revealed that obese mice had significantly less energy remaining in their feces (kcal/g of feces). The authors went further and transplanted gut microbiota from the cecum of both obese and lean mice to germ-free mice. The initial body weight and body fat of the recipients of these microbiotas were identical, however, after a 2-week period, mice colonized with the "obese microbiota" had a significantly greater percentage increase in body fat than mice colonized with a "lean microbiota" (dual-energy X-ray absorptiometry results: 47% versus 27%; or 1.3g versus 0.86g fat). These results indicate that the gut microbiota of obese animals has an increased capacity to harvest energy from the diet, which will consequently affect their body composition (e.g. fat). In regard to the taxonomic classification of the microbiomes, results from all data sets in their study averaged 94% of gene sequences as bacteria, 3.6% as eukaryotic organisms, 1.5% as archaea, and 0.61% were classified as viruses. Within the predominant division of bacteria,

Bacteroidetes and *Firmicutes* consisted of more than 90% of all phylogenetic types in both groups of mice. However, in obese animals, the relative abundance of *Firmicutes* was increased at the expense of *Bacteroidetes*, which was exactly the opposite of what they found in lean mice.

The phylogenetic composition of the rumen microbiome can be influenced by external factors such as the diet, however, animal-to-animal fluctuations are also frequently observed. Brulc et al. (2009) fed the same diet to three 5-year old crossbred steers, and even though the 3 animals were receiving the same diet, the community structure and metabolic potentials in the rumen of 1 animal were markedly different. The phylum *Firmicutes* predominated the sequences of the 2 animals with similar microbiomes (53 – 55%), followed by *Bacteroidetes* (23 – 27%), and *Proteobacteria* (8 – 10%), whereas the steer with the distinct microbiome composition had more *Proteobacteria* (66%), followed by *Firmicutes* (23%) and *Bacteroidetes* (5%).

The Relationship Between Rumen Microbiome and Animal Performance

Ruminococcus flavefaciens is recognized as an essential microorganism for the microbial breakdown of cellulose in the rumen. Præsteng et al. (2013) performed a research to evaluate the effect of feeding *R. flavefaciens* (strain 8/94-32) on rumen microbiome structure of the Scandinavian reindeer. The resulting effects were evaluated by monitoring the fibrolytic capacity of their rumen and characterization of their rumen microbiome by 454-pyrosequencing of bacterial 16S rRNA gene amplicons. In this study, introduction of supplemental *R. flavefaciens* did not increase digestibility of cellulose or the offered supplement itself. In fact, they observed the opposite effect: supplement dry matter disappearance (DMD) decreased 3.7%, and cellulose DMD dropped 11.6% eight

days after the last ruminal dosing of *R. flavefaciens*. The reindeer rumen microbiota was dominated by the phyla *Bacteroidetes* (54.5–77.6%) and *Firmicutes* (18.5–35.7%), which the authors said was consistent with previous metagenomic analysis for the Svalbard reindeer. Family level analysis showed that *Prevotellaceae* (24.2–67.5%) and *Ruminococcaceae* (3.7–19.4%) dominated the samples in that study. Moreover, there was a strong influence of ruminally dosing *R. flavefaciens* on the composition of different microbial lineages, and the most evident changes in relative abundance were an increase in *Prevotellaceae* and a decrease of an uncharacterized *Bacteroidetes* phylotype. Curiously, the relative abundance of *Ruminococcaeceae*, the affiliate family of the dosed *R. flavefaciens*, remained at a similar level throughout the experiment. However, *R. flavefaciens* was below detection levels post-dosing, suggesting that the introduced bacterium did not persist in the rumen 72–96 hours after dosing it. No clear correlation between bacterial diversity or evenness and cellulose DMD was observed, but communities with higher diversity and evenness were more efficient with respect to supplement DMD. In summary, the authors stated that increased fiber digestion by introduced fibrolytic bacteria is dependent on the ability of the bacteria to be established in the rumen, which was not the case in their study. However, they observed that dosing of a fibrolytic bacterium drives phylogenetic variance within reindeer rumen microbiome structure, which ultimately resulted in reduced rumen biomass saccharolytic capacity.

The relationship between rumen microbiome and bovine feed efficiency has been investigated by Myer et al. (2015). They fed a high-concentrate diet to a total of 345 steers for a period of 63 days and individually measured ADG and feed intake from each animal. Then, based on their performance during the feeding period, they selected 32

steers with distinct phenotypes for BW gain and feed intake, and ruminal samples from these animals was used in DNA sequencing (bacterial 16S rRNA gene amplicons). *Bacteroidetes* and *Firmicutes* dominated the phyla dataset in terms of percent of the total reads. Although some variability was observed across the different phenotypes, the diversity indexes for each group were not significantly different, however, some differences were observed among phyla and genera. The most efficient group of steers (high ADG with low feed intake) had a smaller percentage of *Bacteroidetes* in their microbiome composition, compared to the less efficient steers (53% versus 57%), which was mainly due to the genus *Prevotella* (45.4% of sequences for the high efficient steers versus 47.6% for the low efficient ones). In contrast, the phylum *Firmicutes* was positively correlated with feed efficiency (33.4% versus 28.9% of the sequences for the high and low efficiency groups, respectively). Within this phylum, the genera *Dialister* had a significantly greater percentage in the most efficient animals (4.1% versus 1.0%), and the same trend was observed for the families *Veillonellaceae* and *Lachnospiraceae*, which were increased 3 and 4-fold in the high efficient group, respectively. These results indicated that many significant changes in the ruminal microbial population may occur as a function of feed efficiency.

McCann et al. (2014a) studied the relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls grazing bermudagrass under 2 levels of forage allowance. The authors reported a tendency for greater operational taxonomic units (OTUs) for bulls in the low stocking intensity group, which may have occurred due to the greater dry matter intake observed in this group, but beyond that, the ruminal bacterial composition was not affected by forage allowance. In contrast, some differences

were observed regarding residual feed intake-efficiency. Overall, the authors classified 67.5% of the sequences as *Bacteroidetes*, and 22.9% as *Firmicutes*. *Prevotellaceae* was the most prevalent family observed in all treatments, representing more than 19% of all sequences, and it was observed in greater relative abundance in bulls that were less efficient. In contrast, these bulls had lesser relative abundance of an undefined family from the order *Bacteroidales*. The third most prevalent family found in their rumen fluid samples was *Ruminococcaceae*, but it was not affected by the residual feed intake phenotype.

The effect of subacute ruminal acidosis (SARA) on the rumen microbiome was investigated by Khafipour et al. (2009). They investigated the shifts in the microbial community structure in rumen-cannulated lactating dairy cows using two models: one based on grain and another based on alfalfa pellets. In both cases, the severity of SARA was determined based on the duration of rumen pH below 5.6, concentration of free rumen lipopolysaccharide, and serum haptoglobin. Based on these parameters, 3 levels of acidosis were observed in their cows: mild grain-induced SARA, severe grain-induced SARA, and alfalfa pellet-induced SARA. In all of the samples analyzed in this study, 9 predominant phyla were detected, however, more than 95% of rumen bacteria were assigned to only 3 phyla: *Firmicutes, Bacteroidetes,* and *Proteobacteria*. Regardless of the level of SARA observed in cows, the phyla composition shifted in the same manner: a numerical increase was observed in *Firmicutes* and a decrease in the phylum *Bacteroidetes*, with minimal changes in the other phyla. Species richness and diversity were numerically lower during SARA than the control period. Furthermore, SARA dropped the number of ciliate protozoa in the rumen compared to the control period.

In another study, McCann et al. (2016) investigated the effects of SARA on the ruminal microbiome and epithelium. They considered having SARA the cows whose ruminal pH remained below 5.6 for more than 3 hours. The ruminal samples obtained from all cows in the study were separated into liquid and solid fractions. The most abundant phylum in the solid fraction of all samples was *Firmicutes* with a relative abundance ranging from 69 to 87.9%, followed by *Bacteroidetes* with a relative abundance averaging 10%. The relative abundance of *Bacteroidetes* and *Firmicutes* remained fairly constant between days 1 and 6 of the experiment in healthy cows, but interestingly, their abundance significantly changed during the same period for cows with SARA: *Firmicutes* decreased from 79.9% to 69%, and *Bacteroidetes* increased from 9.9% to 23% in relative abundance. The changes observed within the phylum *Bacteroidetes* were driven by the genus *Prevotella*, which averaged 77% of the sequences in this phylum. No effects were observed for any particular family within the phylum *Firmicutes*. Similar to the solid fraction, *Bacteroidetes* and *Firmicutes* were the predominant phyla in the liquid fraction of the rumen samples; however, in contrast to the solid fraction, the most abundant phylum in the liquid fraction was *Bacteroidetes*, which ranged from 59.2 to 64.8% of the sequences. Still in this phylum, family S24-7 slightly increased from day 1 to 6 in healthy cows, but it increased more than three-fold in SARA cows, indicating the effect of acidosis. In the phylum *Firmicutes*, family *Lachnospiraceae* abundance increased in the SARA group, while a decrease was observed in healthy animals between days 1 and 6 of the experiment. Overall, the authors observed a reduction in richness in the liquid fraction for SARA cows. In the solid fraction, results indicated that the change in bacterial relative abundance from day 1 to

day 6 was proportional to the severity of the acidotic bout. In addition, beta-diversity results suggested that there was more variation among their liquid samples than in the solid fractions.

The Relationship Between Animal Performance and Individual Bacterial Groups

In an effort to identify specific bacterial groups associated with cattle feed efficiency, Hernandez-Sanabria et al. (2012) conducted a feeding trial in which they offered both a low and a high-energy diet to yearling steers. Each diet was offered to 180 animals for a period of 90 days, and rumen fluid samples were collected during the last week on each diet. Based on their feed efficiency, a subset of 19 steers was selected and their rumen bacterial population was analyzed. Results showed that *Prevotella sp.* (AF21861; phylum *Bacteroidetes*) were absent in the rumen of inefficient steers, regardless of the energy density of their diets. Moreover, when considering only the high-energy diet, which is more consistent with the diets actually offered in commercial feedlots, *Prevotella maculosa* (strain W1609; EF534315), *Prevotella ruminicola* (strain TC2-3; AF218617), and *Succinivibrio dextrinosolvens* (strain 0554; NR_026476) were all found in significantly increased amounts in the rumen samples of high-efficient steers. Conversely, the abundance of *Prevotella sp.* (BP1-56; AB501155), *Clostridium indolis* (AF028351), *Eubacterium sp.*, *Succinimonas amylolytica* (strain DSM 2873; NR_026475), and *Selenomonas ruminantium* (strain S211; AB198441.1) were all significantly decreased.

Carberry et al. (2012) studied the association between the rumen microbiota and feed efficiency in low and high-forage diets. Their findings revealed that *Prevotella* abundance was higher in inefficient animals. In addition, a substantial effect of diet was

observed in the abundance of this genus: the presence of *Prevotella* was almost 4 times greater in samples from animals consuming a low-forage diet, compared to the ones on a high-forage diet. Conversely, a lower abundance of *Fibrobacter succinogenes* was observed when animals were offered the low-forage diet, however, no shifts in the relative abundance of this microorganism were observed due to animal feed efficiency phenotype. The researchers concluded that there is an association between both – feed efficiency phenotype and diet – with rumen microbial diversity in cattle. Consequently, differences in the ruminal microflora may contribute to host feed efficiency, although this effect may also be affected by the diet offered.

Bekele et al. (2010) fed 2 distinct diets to fistulated sheep: one composed of 91% forage, and another containing only 33% forage. Like Carberry et al. (2012), their results showed that animals receiving a diet with lower percentage of forage had greater abundance of *Prevotella* in their rumen fluid. Their findings were also similar for *Fibrobacter succinogenes* since they found that diets with lower percentage of forage had reduced amounts of this microorganism. In contrast, Kljak et al. (2017) found that heifers consuming greater amount of starch had a linear decrease in relative abundance of *Prevotella* (as the level of starch was increased from 3.5 to 31.7% of the diet DM). However, abundance of *Fibrobacter succinogenes* remained unchanged as starch levels increased.

Xue et al. (2017) investigated the rumen microbiome of sheep fed total mixed ration (TMR) versus sheep grazing natural grasslands. They found reduced relative abundances of *Methanosphaera,* BF311, CF231, and *Anaerovibrio* when their diet was composed of a TMR. In contrast, relative abundances of YRC22 and *Succiniclasticum*

were found to be significantly increased. Conversely, in a study with lactating goats, Hua et al. (2017) detected significantly lower abundance of YRC22 in the rumen of goats fed a diet containing high quantity of grains (only 35% forage), compared to goats fed smaller quantity of grains (65% forage). Moreover, a study by Jewell et al. (2015) in which researchers analyzed the rumen microbiome of dairy cows during their first 2 lactation cycles reported that cows with poorer feed efficiency had a greater abundance of YRC22 in their ruminal contents when compared to cows with better feed efficiency.

LITERATURE CITED

Balci, F., S. Dikmen, H. Gencoglu, A. Orman, I. I. Turkmen, and H. Biricik. 2007. The effect of fibrolytic exogenous enzyme on fattening performance of steers. Bulg. J. Vet. Med. 10:113−118.

Barrera, M., M. Cervantes, W. C. Sauer, A. B. Araiza, N. Torrentera, and M. Cervantes. 2004. Ileal amino acid digestibility and performance of growing pigs fed wheat-based diets supplemented with xylanase. J. Anim. Sci. 82:1997–2003. doi: 10.2527/2004.8271997x.

Beauchemin, K. A., L. M. Rode, and V. J. H. Sewalt. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75:641–644. doi: 10.4141/cjas95-096.

Beauchemin, K. A., L. M. Rode, and D. Karren. 1999. Use of feed enzymes in feedlot finishing diets. Can. J. Anim. Sci. 79:243–246. doi: 10.4141/A98-124.

Bekele, A. Z., S. Koike, and Y. Kobayashi. 2010. Genetic diversity and diet specificity of ruminal *Prevotella* revealed by 16S rRNA gene-based analysis. FEMS Microbiol. Lett. 305:49–57. doi: 10.1111/j.1574-6968.2010.01911.x.

Brazle, F. K., G. L. Kuhl, C. E. Binns, K. O. Zoellner, L. R. Corah, and R. R. Schalles. 1992. The influence of limited creep feed on pre- and post-weaning performance of spring born calves. Prof. Anim. Sci. 8:55–57. doi: 10.15232/S1080-7446(15)32139-2.

Brulc, J. M., D. A. Antonopoulos, M. E. B. Miller, M. K. Wilson, A. C. Yannarell, E. A. Dinsdale, R. E. Edwards, E. D. Frank, J. B. Emerson, P. Wacklin, P. M. Coutinho, B. Henrissat, K. E. Nelson, and B. A. White. 2009. Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. Proc. Natl. Acad. Sci. U.S.A. 106:1948–1953. doi: 10.1073/pnas.0806191105.

Carberry, C. A., D. A. Kenny, S. Han, M. S. McCabe, and S. M. Waters. 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. Appl. Environ. Microbiol. 78:4949–4958. doi: 10.1128/AEM.07759-11.

Choct, M., R. J. Hughes, and M. R. Bedford. 1999. Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. Br. Poult. Sci. 40:419–422. doi: 10.1080/00071669987548.

Gadberry, M. S., P. A. Beck, S. A. Gunter, B. L. Barham, W. A. Whitworth, and J. K. Apple. 2012. Effect of corn-and soybean hull-based creep feed and

backgrounding diets on lifelong performance and carcass traits of calves from pasture and rangeland conditions. Prof. Anim. Sci. 28:507–518. doi: 10.15232/S1080-7446(15)30399-5.

Giraldo, L. A., M. L. Tejido, M. J. Ranilla, and M. D. Carro. 2008. Effects of exogenous fibrolytic enzymes on in vitro ruminal fermentation of substrates with different forage: concentrate ratios. Anim. Feed Sci. Technol. 141:306–325. doi: 10.1016/j.anifeedsci.2007.06.013.

He, Z. X., M. L. He, N. D. Walker, T. A. McAllister, and W. Z. Yang. 2014. Using a fibrolytic enzyme in barley-based diets containing wheat dried distillers grains with solubles: ruminal fermentation, digestibility, and growth performance of feedlot steers. J. Anim. Sci. 92:3978–3987. doi: 10.2527/jas.2014-7707.

Hernandez-Sanabria, E., L. A. Goonewardene, Z. Wang, O. N. Durunna, S. S. Moore, and L. L. Guan. 2012. Impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle. Appl. Environ. Microbiol. 78:1203–1214. doi: 10.1128/AEM.05114-11.

Hua, C., J. Tian, P. Tian, R. Cong, Y. Luo, Y. Geng, S. Tao, Y. Ni, and R. Zhao. 2017. Feeding a high concentration diet induces unhealthy alterations in the composition and metabolism of ruminal microbiota and host response in a goat model. Front. Microbiol. 8:138. doi: 10.3389/fmicb.2017.00138.

Jewell, K. A., C. A. McCormick, C. L. Odt, P. J. Weimer, and G. Suen. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. Appl. Environ. Microbiol. 81:4697–4710. doi: 10.1128/AEM.00720-15.

Khafipour, E., S. Li, J. C. Plaizier, and D. O. Krause. 2009. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. Appl. Environ. Microbiol. 75:7115–7124. doi: 10.1128/AEM.00739-09.

Kljak, K., F. Pino, K. J. Harvatine, and A. J. Heinrichs. 2017. Analysis of selected rumen microbial populations in dairy heifers limit fed diets varying in trace mineral form and starch content. Livest. Sci. 198:93–96. doi: 10.1016/j.livsci.2017.02.012.

Lopes, S. A., M. F. Paulino, E. Detmann, S. C. V. Filho, E. E. L. Valente, L. V. Barros, J. E. G. Cardenas, D. M. Almeida, L. S. Martins, and A. G. Silva. 2014. Supplementation of suckling beef calves with different levels of crude protein on tropical pasture. Trop. Anim. Health Prod. 46:379–384. doi: 10.1007/s11250-013-0500-6.

Mathlouthi, N., J. P. Lallès, P. Lepercq, C. Juste, and M. Larbier. 2002. Xylanase and β-glucanase supplementation improve conjugated bile acid fraction in intestinal contents and increase villus size of small intestine wall in broiler chickens fed a rye-based diet. J. Anim. Sci. 80:2773–2779. doi: 10.2527/2002.80112773x.

McCann, J. C., L. M. Wiley, T. D. Forbes, F. M. Rouquette Jr., and L. O. Tedeschi. 2014a. Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures. PLoS One. 9:e91864. doi: 10.1371/journal.pone.0091864.

McCann, J. C., T. A. Wickersham, and J. J. Loor. 2014b. High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. Bioinform. Biol. Insights. 8:109–125. doi: 10.4137/BBI.S15389.

McCann, J. C., S. Luan, F. C. Cardoso, H. Derakhshani, E. Khafipour, and J. J. Loor. 2016. Induction of subacute ruminal acidosis affects the ruminal microbiome and epithelium. Front. Microbiol. 7:701. doi: 10.3389/fmicb.2016.00701.

Meale, S. J., K. A. Beauchemin, A. N. Hristov, A. V. Chaves, and T. A. McAllister. 2014. Board-Invited Review: opportunities and challenges in using exogenous enzymes to improve ruminant production. J. Anim. Sci. 92:427–442. doi: 10.2527/jas.2013-6869.

Moriel, P., and J. D. Arthington. 2013. Effects of trace mineral-fortified, limit-fed preweaning supplements on performance of pre- and postweaned beef calves. J. Anim. Sci. 91:1371–1380. doi: 10.2527/jas.2012-5469.

Myer, P. R., T. P. L. Smith, J. E. Wells, L. A. Kuehn, and H. C. Freetly. 2015. Rumen microbiome from steers differing in feed efficiency. PLoS One. 10:e0129174. doi: 10.1371/journal.pone.0129174.

Parish, J. A., and J. D. Rhinehart. 2009. Creep feeding beef calves. Mississippi State University Extension Service. Publication 2524.

Præsteng, K. E., P. B. Pope, I. K. O. Cann, R. I. Mackie, S. D. Mathiesen, L. P. Folkow, V. G. H. Eijsink, and M. A. Sundset. 2013. Probiotic dosing of *Ruminococcus flavefaciens* affects rumen microbiome structure and function in reindeer. Microb. Ecol. 66:840–849. doi: 10.1007/s00248-013-0279-z.

Prichard, D. L., D. D. Hargrove, T. A. Olson, and T. T. Marshall. 1989. Effects of creep feeding, zeranol implants and breed type on beef production: I. Calf and cow performance. J. Anim. Sci. 67:609–616. doi: 10.2527/jas1989.673609x.

Rosewarne, C. P., P. B. Pope, J. L. Cheung, and M. Morrison. 2014. Analysis of the bovine rumen microbiome reveals a diversity of Sus-like polysaccharide utilization loci from the bacterial phylum *Bacteroidetes*. J. Ind. Microbiol. Biotechnol. 41:601–606. doi: 10.1007/s10295-013-1395-y.

Sampaio, A. A. M., R. M. de Brito, G. M. da Cruz, M. M. de Alencar, P. F. Barbosa, and R. T. Barbosa. 2002. Use of NaCl in Supplement as an Alternative to Viabilize the Calf Creep-Feeding System. R. Bras. Zootec. 31:164–172. doi: 10.1590/S1516-35982002000100019.

Tarr, S. L., D. B. Faulkner, D. D. Buskirk, F. A. Ireland, D. F. Parrett, and L. L. Berger. 1994. The value of creep feeding during the last 84, 56, or 28 days prior to weaning on growth performance of nursing calves grazing endophyte-infected tall fescue. J. Anim. Sci. 72:1084–1094. doi: 10.2527/1994.7251084x.

Turnbaugh, P. J., R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027–1031. doi: 10.1038/nature05414.

Viñoles, C., M. Jaurena, I. De Barbieri, M. Do Carmo, and F. Montossi. 2013. Effect of creep feeding and stocking rate on the productivity of beef cattle grazing grasslands. New Zeal. J. Agr. Res. 56:279–287. doi: 10.1080/00288233.2013.840320.

Wu, Y. B., V. Ravindran, D. G. Thomas, M. J. Birtles, and W. H. Hendriks. 2004. Influence of phytase and xylanase, individually or in combination, on performance, apparent metabolisable energy, digestive tract measurements and

gut morphology in broilers fed wheat-based diets containing adequate level of phosphorus. Br. Poult. Sci. 45:76–84. doi: 10.1080/00071660410001668897.

Xue, D., H. Chen, X. Zhao, S. Xu, L. Hu, T. Xu, L. Jiang, and W. Zhan. 2017. Rumen prokaryotic communities of ruminants under different feeding paradigms on the Qinghai-Tibetan Plateau. Syst. Appl. Microbiol. 40:227–236. doi: 10.1016/j.syapm.2017.03.006.

CHAPTER 3

INCLUSION OF EXOGENOUS FEED ENZYMES AS A WAY TO ENHANCE CREEP FEEDS – AN IN VITRO APPROACH[1]

---

[1]Lourenço, J. M., B. T. Campbell, N. DiLorenzo, and R. L. Stewart Jr. To be submitted to *Journal of Animal Science*.

ABSTRACT

An in vitro experiment was conducted to investigate if some exogenous feed enzymes, most of them commonly used in rations of monogastric animals, would be effective when included in a creep feed (**CF**) formulated for beef calves. The selected enzymes were included in various experimental treatments both individually and in combination. The treatments consisted of: 1) 100% bermudagrass (**BER**); 2) a mixture of 75% bermudagrass and 25% CF (**BERCF**); 3) BERCF enhanced with xylanase (**BERCF+XYL**); 4) BERCF enhanced with β-1,3-glucanase (**BERCF+BGLUC**); 5) BERCF enhanced with α-amylase (**BERCF+AMYL**); 6) BERCF enhanced with a combination of xylanase, β-1,3-glucanase and β-1,4-glucanase (**BERCF+COMB1**); or 7) BERCF enhanced with a combination of xylanase, β-1,3-glucanase, β-1,4-glucanase and α-amylase (**BERCF+COMB2**). Additionally, 2 rates of inclusion of these enzymes were tested: the dose normally used in rations of monogastric animals (i.e. the label dose of 300 g/t DM for XYL, 400 g/t DM for BGLUC, 650 g/t DM for AMYL, 200 g/t DM for COMB1, and 850 g/t DM for COMB2) and doses 10 times greater than those (**10x**). Five replications per treatment were used. Incubations were carried out for 24 h using rumen fluid collected by esophageal tubing from a group of 6-month-old beef calves. Analysis of variance was conducted as a completely randomized design using fermentation bottle as the experimental unit, with treatments and replications as factors. Digestibility of ADF was lowest (P = 0.02) for BER, however, this trait was improved in all the other treatments, and it was greatest in BERCF+BGLUC 10x. Similarly, IVDMD was lowest (P = 0.02) for BER and highest for BERCF+XYL 10x (27.39 vs. 41.28%). Total production of VFA was also minimal for BER, and it was greatest for BERCF+COMB2

10x (82.01 vs. 97.39 m*M*; $P < 0.01$). Molar proportions of acetate and the acetate:propionate ratio were both greatest ($P < 0.01$) for BER. No differences were found ($P = 0.25$) in molar proportions of propionate, however, molar proportion of butyrate was smallest ($P < 0.01$) for BER. Total gas produced per g of incubated DM, and concentration of CH_4 per g of incubated DM were both lowest ($P < 0.01$) for BER. Overall, the inclusion of the studied enzymes improved important traits such as IVDMD, ADF digestibility, and production of VFA, however, these improvements were more evident for the 10x doses, suggesting that this level of inclusion would be more appropriate if these enzymes are used in creep feeds.

Key words: creep feeding, exogenous feed enzymes, in vitro.

INTRODUCTION

The inherent ability of ruminants to convert plant biomass into desirable products such as meat is largely dependent on the digestibility of plant cell walls (Jung and Allen, 1995; Phakachoed et al., 2013; Meale et al., 2014). From the plant perspective, the cell wall is a very important structure since it provides protection and structural support, however, from the animal nutrition standpoint, it constitutes a barrier that limits access to nutrients situated inside the cell. Although the ruminal microbial community is able to ferment the polysaccharides in plant cell walls, the resultant metabolizable energy is usually not enough to meet the requirements of high-producing animals (Jung and Allen, 1995; Beauchemin et al., 2003). Nevertheless, the inclusion of exogenous feed enzymes in ruminant diets has the potential to improve fiber digestion, which may result in improved animal performance, especially in situations where energy is the first-limiting nutrient in the diet (Beauchemin et al., 2003). Fortunately, due to recent improvements in their manufacturing process, feed enzymes have become more economically viable, which has allowed the development of commercial products that can lower the cost of body weight gain in beef cattle (Balci et al., 2007).

Beef calves in the suckling phase experience many physiological transformations including a rapid development of their rumen and its microbial population (Church, 1979). Because calves are usually introduced to forages at an early age, their forestomach is still not fully developed. Consequently, forage digestion is expected to be lower than what is observed in adult beef cattle. In light of this, we hypothesized that suckling beef calves would benefit from the inclusion of feed enzymes in their diets through an enhanced creep feed to improve fiber digestion. Thus, an in vitro experiment was carried

out to investigate this hypothesis, and the effects of adding different carbohydrases into a creep feed and their impacts on production of VFA, fermentation gas, fiber degradability, production of methane, and IVDMD were evaluated.

MATERIALS AND METHODS

The present study was completely performed in vitro, and it was the first portion of a multi-step approach. All procedures involving live animals were verified and approved by the University of Georgia's Office of Animal Care and Use (Animal Use Protocol #A2015 07–018-Y1-A0).

An in vitro batch culture technique was used in which several different treatments were incubated for 24 h using rumen fluid obtained from 6-month-old suckling calves. The treatments were formulated using a combination of bermudagrass (*cynodon dactylon*), a creep feed, and selected carbohydrases, which were added to the treatments both individually or in combination. The carbohydrases elected to be part of this study were: endo-1,4-β-xylanase (EC/IUB No. 3.2.1.8), α-amylase (EC/IUB No. 3.2.1.1), endo-1,3(4)-β-glucanase (EC/IUB No. 3.2.1.6), and endo-1,4-β-glucanase (EC/IUB No. 3.2.1.4). These carbohydrases were tested at 2 distinct levels of inclusion: at their labeled doses and at doses 10 times greater than those indicated in their labels (**10x**). The rationale for testing doses 10 times greater than normal in this experiment was that the label recommendations of these enzymes (except for α-amylase) assumed that their utilization would be in diets of monogastric animals such as swine and poultry. However, since beef calves normally have diets that are richer in fiber compared to monogastrics,

and since their forestomach is still undergoing several transformations, it is expected that the addition of a greater quantity of enzymes to their diets would have positive effects.

As explained below, the individual enzymes or the combinations of enzymes were added to a forage:concentrate mix composed of 75% bermudagrass and 25% creep feed. This ratio was set on a DM basis and intended to mimic the daily DMI of a beef calf supplemented with a moderate amount of creep feed. The forage used in this study was collected from a plot that was harvested at 4-week intervals, and the creep feed was composed mainly of corn and soybean meal. Their chemical compositions are listed in Table 3.1.

A total of 12 different treatments were evaluated in the present study, including the ones in which the tested enzymes were added at 10 times their label doses (**10x**). On a DM basis, the 12 treatments were:

1) 100% bermudagrass (**BER**);

2) a mixture of 75% bermudagrass and 25% CF (**BERCF**);

3) BERCF enhanced with endo-1,4-β-xylanase at 300,000 U/t of DM (**BERCF+XYL**);

4) BERCF enhanced with endo-1,4-β-xylanase at 3,000,000 U/t of DM (**BERCF+XYL 10x**);

5) BERCF enhanced with endo-1,3(4)-β-glucanase at 20,000 U/t of DM (**BERCF+BGLUC**);

6) BERCF enhanced with endo-1,3(4)-β-glucanase at 200,000 U/t of DM (**BERCF+BGLUC 10x**);

7) BERCF enhanced with α-amylase at 390,000 U/t of DM (**BERCF+AMYL**);

8) BERCF enhanced with α-amylase at 3,900,000 U/t of DM (**BERCF+AMYL 10x**);

9) BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 540,000, 140,000, and 160,000 U/t of DM, respectively (**BERCF+COMB1**);

10) BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 5,400,000, 1,400,000, and 1,600,000 U/t of DM, respectively (**BERCF+COMB1 10x**);

11) BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 540,000, 140,000, 160,000, and 390,000 U/t of DM, respectively (**BERCF+COMB2**); and

12) BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 5,400,000, 1,400,000, 1,600,000, and 3,900,000 U/t of DM, respectively (**BERCF+COMB2 10x**).

Substrate Preparation. Prior to being used, each feed ingredient was ground to pass a 2-mm screen using a Model 4 Wiley Mill (Thomas Scientific, Swedesboro, NJ). The ground ingredients were then combined accordingly to prepare the 12 treatments. Once all treatments were prepared, 1.4 g of each one was placed into 250-mL glass bottles, which were used to determine the production of gas and VFA during the 24 h of fermentation. A total of 5 bottles per treatment were used (5 replications). In addition to the glass bottles, a separate set of 125-mL plastic scintillation vials was also prepared for the incubations (Lourenco et al., 2016). The plastic bottles were used exclusively to determine IVDMD and fiber degradability. They contained half the amount of treatment used in the glass bottles – 0.7 g each. A total of 5 plastic scintillation vials were used for each treatment. In order to determine the blank contributions and make the proper

adjustments, an additional set of five 250-mL glass bottles and five 125-mL plastic vials were used in incubations.

Preparation of the Inoculum and Inoculation. The inoculum used in all incubations was prepared by mixing McDougall's buffer (McDougall, 1948) and rumen fluid in a 2:1 ratio. The rumen fluid was collected by esophageal tubing from 12 six-month-old beef calves (6 males and 6 females; 186 ± 7 days-old) which were still in the suckling phase and raised on a forage-based regimen. Approximately 600 mL of fluid were collected from each animal and immediately placed in a sealed thermos to be transported to the laboratory, where it was further processed. Processing began by individually straining the fluids from the 12 calves through a 500-micron nylon mesh to remove any feed particles and then combining them into one mixture. This mixture was placed in a water bath at 39°C and CO_2 was bubbled into it for 5 minutes. Then, each one of the previously prepared 250-mL glass bottles received 100 mL of the inoculum. The bottles were then gassed with CO_2, sealed with rubber stoppers, and placed in a water bath incubator at 39°C. Incubations lasted 24 hours. The 125-mL plastic vials were inoculated following the same protocol, but with 2 modifications: the amount of inoculum used was 50 mL, and their tops were sealed with rubber stoppers fitted with a 16-gauge needle to allow gas release.

Collection and Analysis of Gases. Collection of fermentation gases were conducted at 3-hour intervals on all of the 250-mL glass bottles using a water displacement method. Briefly, this method consisted of inserting one 22-gauge needle into the incubation bottle, and another one into a water-filled bottle that was connected to a 60-mL syringe by a 3-way valve. Gas production was measured by allowing the gas to

flow from the incubation bottle to the 60-mL syringe. In this process, gas pressure moved the syringe plunger until pressure was equilibrated. The incubation bottle was swirled to allow all the produced gas to escape. Once the plunger stopped moving, the syringe reading was recorded. The 3-way valve was then turned to direct the collected gas into the water-filled bottle. During this procedure, an extra 22-gauge needle was inserted into the water-filled bottle to allow displacement of water and capture of gas inside the bottle, which was upside down. The apparatus was then disconnected and the water bottle containing the gas was kept upside down until analysis of its gas content was carried out. Compositional analysis of the collected gas was performed for CH_4 by gas chromatography. Ten mL of gas was sampled from each bottle and injected in an Agilent 7820A gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a 0.25 mL sample loop and a flame ionization detector. A capillary column (Molsieve, 5A, 25 m x 0.32 mm, Varian CP7536) was used. The injector, oven, and detector temperatures were set at 80°C, 160°C, and 200°C, respectively. Total gas produced in 24 h, bottle headspace, and bottle volume were also considered in the calculations of CH_4 concentrations.

Analysis of VFA. Upon conclusion of the 24-h fermentation, the 250-mL glass bottles were opened and their contents were transferred to polypropylene tubes, which were immediately frozen at -20°C. One week later, these samples were thawed and centrifuged for 10 minutes at 10,000 x *g* at 4°C. Then, 2 mL of the supernatant was pipetted into centrifuge tubes along with 0.4 mL of a metaphosphoric acid:crotonic acid (internal standard) solution, and samples were frozen overnight. On the next day, samples were once again thawed and centrifuged for 10 minutes at 10,000 x *g* at 4°C. The

supernatant was transferred into vials and mixed with ethyl acetate in a 2:1 ratio of ethyl acetate to supernatant. After 5 minutes, a subsample of the supernatant was transferred to a screw-thread vial for analysis of VFA in an Agilent 7820A gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m x 0.53 mm, Varian CP7767). Sample injection volume was 0.50 μL. The column temperature was maintained at 110°C, and the injector and detector temperatures were 200°C and 220°C, respectively. Nitrogen was used as the carrier gas, set at a flow rate of 40 mL/min.

In Vitro Digestibility Analysis. Digestibility analyses were performed both for total DM and for the fiber portion of the treatments (NDF and ADF). Once the 24 h fermentation was concluded, all the 125-mL plastic scintillation vials were capped and placed in a freezer at -20°C. One week later, they were freeze-dried at -50°C for 36 h (FreeZone 6, Labconco Corporation, Kansas City, MO), and then placed in a drying oven for 16 h at 100°C (Blue M Electric Company, Blue Island, IL). The IVDMD was obtained by difference between initial and final incubated DM mass, corrected by blank contributions. Next, the dried samples were mashed and 0.5 g were placed into filter bags (F57 filter bags, Ankom Technology, Macedon, NY). These samples and the original treatments (prior to the 24 h fermentation) were both subjected to analysis of NDF and ADF, and results were compared to calculate the in vitro digestibility of these fiber portions. Similarly, the contents of the plastic scintillation vials incubated as blanks were analyzed for their NDF and ADF contents, and results were utilized in the adjustments of treatment NDF and ADF digestibilities.

Statistical Analysis. Analysis of variance was performed using treatment and replicate bottle (or vial) as factors. The 250-mL glass bottles or the 125-mL plastic scintillation vials were considered the experimental unit. Contrasts were compared using Tukey's honest significant difference test. Differences were considered significant at $P < 0.05$, and were treated as tendencies when $0.05 < P < 0.10$. All the statistical analyses were carried out using the software R (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS AND DISCUSSION

Production of Gas and Methane. Results from Table 3.2 show that the total volume of gas produced per g of incubated DM after 24 h of fermentation was lowest ($P < 0.01$) for BER, and there were no differences ($P > 0.05$) in gas production among all the other treatments. Gas measurement provides useful data on digestion kinetics of both soluble and insoluble fractions of feedstuffs, and for that reason, the gas measuring technique has been widely used in the evaluation of nutritive value of feeds (Getachew et al., 1998). In a recent study, Kholif et al. (2017) evaluated the effects of different forage:concentrate ratios on in vitro total gas production using rumen fluid from a cannulated cow. Their results showed that increasing the concentrate portion of the diet linearly increased the volume of gas produced per g of incubated DM, which is in agreement with our findings since all of our treatments, except for BER, had concentrate in their composition, which resulted in greater gas production after 24 h of incubation.

Among all treatments, total production of CH_4 in 24 h was lowest ($P < 0.01$) when BER was used as substrate. Likewise, production of CH_4 per g of incubated DM

was the least among all treatments for BER ($P < 0.01$). Giraldo et al. (2008) studied the effects of fibrolytic enzymes on in vitro fermentation of substrates with different forage:concentrate ratios. Their results showed that, regardless of the inclusion of fibrolytic enzymes, the forage:concentrate ratio has a significant effect on the quantity of CH_4 produced per g of incubated DM. Similarly, Kholif et al. (2017) reported that the ratio of forage:concentrate in the diet affects the proportional production of CH_4 per g of incubated DM in a quadratic manner, for incubations lasting 24 h. Although the current study did not evaluate more than 2 levels of inclusion of concentrate in the treatments (i.e. the percentage of concentrate was actually 0% in BER, and 25% in all the other treatments), these results agree with the findings of these authors since they clearly show that the presence or absence of concentrate markedly affected production of CH_4. With respect to the use of feed enzymes, despite the level of inclusion (label dose or 10x), our results did not show any differences in CH_4 production, which is in harmony with the findings from Giraldo et al. (2008). These authors tested the inclusion of xylanase from *Trichoderma viride*, and cellulase from *Aspergillus niger*, both at 2 doses – 40 and 80 enzymatic units per g of diet DM. Regardless of enzyme type or dose of inclusion, they found no significant variations in production of CH_4 after 24 h of incubation.

Digestibility of DM and Fiber Portions. As shown in Table 3.3, IVDMD was lowest ($P = 0.02$) for BER, and the only treatment that significantly improved this trait compared to BER was BERCF+XYL 10x, which increased it by 50.7% (27.39 versus 41.28%). Moreover, it can be noticed that the average percentage of IVDMD across all treatments was 35.5%, which is considerably lower than what is usually reported in the literature for batch culture incubations lasting 24 h. For instance, a recent study from our

group (Lourenco et al., 2016) used a similar in vitro technique and found IVDMD values ranging from 69.9 to 74.5%. Such appreciable differences may be partially explained by the composition of the treatments used in both studies: while the present study used forage-based diets, that study used diets that resemble the ones typically used in drylots, which are richer in concentrate feeds. However, the main factor behind the lower IVDMDs observed in the present study is probably the age of the rumen fluid donors. Whereas Lourenco et al. (2016) used rumen fluid obtained from mature steers, the present study used fluid collected from a group of 186-day-old calves which were still in their suckling phase. Because calves in that stage of their lives are still experiencing transformations in their forestomach, and since their associated microbial population is not fully developed, it seems reasonable that IVDMD values assessed using rumen fluid obtained from young calves would be inferior than what is normally seen when the fluid is obtained from mature animals. Segers et al. (2013) performed in vitro evaluations of diets that had 75% roughage in their composition, which is the same percentage used in most of our treatments. Still, their IVDMD values were markedly higher than the ones observed in our study: they fluctuated from 70.5 to 74.9% for all their tested diets. Not surprisingly, the rumen fluid used in their study was obtained from notorious DM digesters: lactating dairy cows. In another in vitro study performed with treatments containing the same roughage:concentrate ratios that were used in our research, Ramírez et al. (2015) found IVDMD values ranging from 52.5 to 61.6% for treatments with a 75:25 roughage:concentrate ratio, and 42.2 to 52.4% for treatments composed of 100% roughage, incubated for 24 h. In accordance with Segers et al. (2013) and Lourenco et al. (2016), Ramírez et al. (2015) also utilized rumen fluid from adult animals in their

incubations, which in their case was obtained from 3 Holstein Friesian cows. Therefore, the relatively low IVDMD values observed in the present study are presumably due to the young age of the 12 rumen fluid donors, which were only 186 ± 7 days-old when their fluid was collected.

In regard to the fiber portion of our treatments, no differences (P = 0.08) were detected in digestibility of NDF (Table 3.3). However, digestibility of ADF differed across treatments: it was lowest (P = 0.02) for BER and highest for BERCF+BGLUC 10x. Our results diverge from the ones reported by Giraldo et al. (2008), who evaluated the use of the 2 distinct cellulases and 1 xylanase on in vitro fermentations. When compared to their control treatment (no addition of feed enzyme), all the enzymes used in their research significantly increased ADF digestibility after 24 h of incubation. However, NDF digestibility at 24 h was only improved by the addition of xylanase obtained from *Trichoderma viride*, and the cellulase from *Trichoderma longibrachiatum*. In another study, He et al. (2014) used 4 adult cannulated heifers to evaluate the effects of xylanase and cellulase on in situ and in vivo digestion. Although they did not measure in situ ruminal disappearance of ADF, their data show that ruminal disappearance of the NDF fraction at 24 h was not affected by the addition of the fibrolytic enzymes. Likewise, they found no response of adding those enzymes on total tract digestibility of NDF or ADF, even when the dose of inclusion was doubled (i.e. from 16,150 to 32,300 Units/kg DM for xylanase; and from 500 to 1,000 Units/kg DM for cellulase).

Volatile Fatty Acids. Tables 3.4 and 3.5 show the results with regard to VFA production after 24 h of incubation. It can be noticed that total VFA concentration was lowest (P < 0.01) for BER, followed by BERCF, and it was maximized when

BERCF+COMB2 10x was used as the substrate for incubation. Because the in vitro technique used in the present study allows VFA to be produced but not absorbed as it happens in vivo (Carpenter et al., 2017), these results indicate that more fermentation (and consequently greater VFA production) occurred when the creep feed was added to the pure bermudagrass, moreover, they show that the rumen microorganisms were able to ferment even more compounds when the different enzymes were added to the treatments, especially when the second combination of enzymes were used at 10 times the label dose (i.e. BERCF+COMB2 10x). With respect to individual VFA, it can be noticed that although BER yielded the lowest ($P < 0.01$) production of acetate (54.70 m*M*), the proportion of this VFA when BER was incubated was actually the greatest ($P < 0.01$) among all treatments (66.69 mol/100mol). Concentration of propionate after 24 h of fermentation was lowest ($P < 0.01$) for BER and it did not differ between all the other treatments, but no differences were detected ($P = 0.25$) in molar proportion of this VFA. Among all treatments, BER had the lowest ($P < 0.01$) concentration and the smallest ($P < 0.01$) proportion of butyrate, however, both the concentration and proportion of this VFA were significantly improved by the addition of the creep feed and the enzymes, regardless of their type or rate of inclusion.

An adequate development of the ruminal epithelial tissue of calves is critical, for this tissue is responsible for absorption of VFA. Consumption of dry feeds and the resulting end products of microbial fermentation stimulate the development of the rumen epithelium, and no end product stimulates such development more than butyrate (NRC, 2001; Yáñez-Ruiz et al., 2015). Since the production of butyrate was increased in all

treatments in comparison to BER, we presume that these treatments will tend to cause a greater ruminal epithelial tissue development in calves.

In summary, there was a clear distinction between pure bermudagrass (BER) and all the other treatments for most of the traits analyzed in this study. The reduced digestibilities of fiber and DM, and decreased production of VFA observed for BER suggest that suckling beef calves receiving forage as their only source of dry feed may not have the same development as calves that receive supplementation. Moreover, the inclusion of the studied enzymes at the doses indicated in their labels may be effective for monogastric animals such as chicken and swine; however, this in vitro assessment using rumen fluid from suckling beef calves revealed that calves in that stage of their lives will likely not benefit from their inclusion at those rates. Instead, the effects caused by the enzymes at the label doses were not markedly different from the ones observed for the treatment containing the plain creep feed (BERCF). Furthermore, although the addition of the selected feed enzymes did not improve every trait measured in this study, some important features such as total production of VFA, IVDMD and ADF digestibility were augmented by their inclusion: BERCF+COMB2 10x yielded the greatest numerical concentration of total VFA among all treatments; BERCF+BGLUC 10x had the greatest numerical ADF digestibility; and the BERCF+XYL 10x treatment had the greatest numerical IVDMD. These results imply that the rate of inclusion of the studied enzymes should be greater than what is indicated in their labels. Lastly, further research (including animal feeding trials) should be conducted to confirm this assumption.

LITERATURE CITED

Balci, F., S. Dikmen, H. Gencoglu, A. Orman, I. I. Turkmen, and H. Biricik. 2007. The effect of fibrolytic exogenous enzyme on fattening performance of steers. Bulg. J. Vet. Med. 10:113−118.

Beauchemin, K. A., D. Colombatto, D. P. Morgavi, and W. Z. Yang. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. J. Anim. Sci. 81:E37–E47. doi: 10.2527/2003.8114_suppl_2E37x.

Carpenter, A. J., E. Binversie, M. Ruiz-Moreno, and M. D. Stern. 2017. Effect of dried fermentation biomass on microbial fermentation in continuous culture and *in vitro* intestinal digestibility. Anim. Feed Sci. Technol. 230:47–58. doi: 10.1016/j.anifeedsci.2017.05.020.

Church, D. C. 1979. Growth and development of the ruminant stomach. Pages 34–45 in Digestive physiology and nutrition of ruminants. Volume 1: Digestive Physiology. 2nd ed. Oxford Press Inc., Portland, OR.

Getachew, G., M. Blümmel, H. P. S. Makkar, and K. Becker. 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. Anim. Feed Sci. Technol. 72:261–281. doi: 10.1016/S0377-8401(97)00189-2.

Giraldo, L. A., M. L. Tejido, M. J. Ranilla, and M. D. Carro. 2008. Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage: concentrate ratios. Anim. Feed Sci. Technol. 141:306–325. doi: 10.1016/j.anifeedsci.2007.06.013.

He, Z. X., M. L. He, N. D. Walker, T. A. McAllister, and W. Z. Yang. 2014. Using a fibrolytic enzyme in barley-based diets containing wheat dried distillers grains

with solubles: Ruminal fermentation, digestibility, and growth performance of feedlot steers. J. Anim. Sci. 92:3978–3987. doi: 10.2527/jas.2014-7707.

Jung, H. G., and M. S. Allen. 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73:2774–2790. doi: 10.2527/1995.7392774x.

Kholif, A. E., M. M. Y. Elghandour, A. Z. M. Salem, A. Barbabosa, O. Márquez, and N. E. Odongo. 2017. The effects of three total mixed rations with different concentrate to maize silage ratios and different levels of microalgae Chlorella vulgaris on *in vitro* total gas, methane and carbon dioxide production. J. Agric. Sci. 155:494–507. doi: 10.1017/S0021859616000812.

Lourenco, J. M., N. DiLorenzo, A. M. Stelzleni, J. R. Segers, and R. L. Stewart, Jr. 2016. Use of by-product feeds to decrease feed cost while maintaining performance of developing beef bulls. Prof. Anim. Sci. 32:287–294. doi: 10.15232/pas.2015-01436.

McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J. 43:99–109.

Meale, S. J., K. A. Beauchemin, A. N. Hristov, A. V. Chaves, and T. A. McAllister. 2014. Board-Invited Review: opportunities and challenges in using exogenous enzymes to improve ruminant production. J. Anim. Sci. 92:427–442. doi: 10.2527/jas.2013-6869.

NRC. 2001. Nutrient requirements of dairy cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Phakachoed, N., W. Suksombat, D. Colombatto, and K. A. Beauchemin. 2013. Use of fibrolytic enzymes additives to enhance in vitro ruminal fermentation of corn silage. Livest. Sci. 157:100–112. doi: 10.1016/j.livsci.2013.06.020.

Ramírez, J., S. Posada, and R. Noguera. 2015. Effects of Kikuyu grass (*Pennisetum clandestinum*) age and different forage: concentrate ratios on methanogenesis. Rev. MVZ Cordoba 20:4726–4738.

Segers, J. R., A. M. Stelzleni, T. D. Pringle, M. A. Froetschel, C. L. Ross, and R. L. Stewart, Jr. 2013. Use of corn gluten feed and dried distillers grains plus solubles as a replacement for soybean meal and corn for supplementation in a corn silage-based stocker system. J. Anim. Sci. 91:950–956. doi: 10.2527/jas.2011-4392.

Yáñez-Ruiz, D. R., L. Abecia, and C. J. Newbold. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. Front. Microbiol. 6:1133. doi: 10.3389/fmicb.2015.01133.

Table 3.1. Chemical composition of 4-week-old bermudagrass and creep feed (dry matter basis)[1].

	Feedstuff	
Item	**Bermuda-grass**	**Creep feed[2]**
Crude protein	11.60	22.70
Neutral detergent fiber	66.96	9.51
Acid detergent fiber	38.70	4.50
Ether extract	2.19	2.53
Non-fiber carbohydrates	10.00	55.60
Net energy for maintenance (Mcal/kg)	1.21	1.83
Net energy for gain (Mcal/kg)	0.64	1.19
Calcium	0.43	2.34
Phosphorus	0.33	0.55
Magnesium	0.16	0.43
Potassium	1.84	1.18
Sodium	0.02	0.09

[1] Cumberland Valley Analytical Services (Hagerstown, MD).
[2] Creep feed composition (DM basis): 62% ground corn, 31.2% soybean meal, 4.8% limestone, 2% mineral mix.

Table 3.2. Total production of gas (mL/g of incubated DM), production of methane (m*M*), and production of methane per g of incubated DM (mmol/g of incubated DM) after 24 h of incubation of treatments.

	Feature		
Treatment[1]	**mL of Gas[2]**	**CH_4 (m*M* in 24h)**	**CH_4/g of DM**
BER	84.62[b]	6.69[b]	0.88[b]
BERCF[*]	111.4[a]	9.07[a]	1.44[a]
BERCF+XYL	111.6[a]	8.65[a]	1.38[a]
BERCF+XYL 10x	112.1[a]	8.91[a]	1.42[a]
BERCF+BGLUC	112.4[a]	9.24[a]	1.48[a]
BERCF+BGLUC 10x	113.7[a]	9.20[a]	1.49[a]
BERCF+AMYL	112.9[a]	9.23[a]	1.48[a]
BERCF+AMYL 10x	113.9[a]	9.47[a]	1.53[a]
BERCF+COMB1	112.8[a]	9.29[a]	1.50[a]
BERCF+COMB1 10x	110.7[a]	9.21[a]	1.46[a]
BERCF+COMB2	112.0[a]	9.11[a]	1.46[a]
BERCF+COMB2 10x	111.5[a]	9.21[a]	1.48[a]
SE	1.69	0.35	0.06
P-value	< 0.01	< 0.01	< 0.01

[a-b] Means within a column with different superscripts differ ($P \leq 0.05$).

[*] CF = Creep feed.

[1] BER = 100% bermudagrass; BERCF = 75% bermudagrass and 25% CF; BERCF+XYL = BERCF enhanced with endo-1,4-β-xylanase at 300,000 U/t of DM; BERCF+XYL 10x = BERCF enhanced with endo-1,4-β-xylanase at 3,000,000 U/t of DM; BERCF+BGLUC = BERCF enhanced with endo-1,3(4)-β-glucanase at 20,000 U/t of DM; BERCF+BGLUC 10x = BERCF enhanced with endo-1,3(4)-β-glucanase at 200,000 U/t of DM; BERCF+AMYL = BERCF enhanced with α-amylase at 390,000 U/t of DM; BERCF+AMYL 10x = BERCF enhanced with α-amylase at 3,900,000 U/t of DM; BERCF+COMB1 = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 540,000, 140,000, and 160,000 U/t of DM; BERCF+COMB1 10x = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 5,400,000, 1,400,000, and 1,600,000 U/t of DM; BERCF+COMB2 = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 540,000, 140,000, 160,000, and 390,000 U/t of DM; BERCF+COMB2 10x = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 5,400,000, 1,400,000, 1,600,000, and 3,900,000 U/t of DM.

[2] Total volume of gas (mL) produced per g of incubated DM after 24 h.

Table 3.3. In vitro digestibility of NDF and ADF, and IVDMD for the different treatments after 24 h of incubation of treatments.

	Feature		
Treatment[1]	**IVDMD, %**	**NDF Digestibility, %**	**ADF Digestibility, %**
BER	27.39[b]	51.10	48.82[b]
BERCF[*]	31.63[ab]	52.84	52.34[ab]
BERCF+XYL	38.94[ab]	52.79	53.78[ab]
BERCF+XYL 10x	41.28[a]	51.86	52.78[ab]
BERCF+BGLUC	37.88[ab]	53.45	52.56[ab]
BERCF+BGLUC 10x	35.38[ab]	56.05	56.32[a]
BERCF+AMYL	30.17[ab]	51.89	49.93[ab]
BERCF+AMYL 10x	37.02[ab]	51.50	51.55[ab]
BERCF+COMB1	36.15[ab]	50.97	49.55[ab]
BERCF+COMB1 10x	36.52[ab]	54.81	53.99[ab]
BERCF+COMB2	39.35[ab]	52.29	51.69[ab]
BERCF+COMB2 10x	34.44[ab]	56.25	55.08[ab]
SE	2.82	1.31	1.43
P-value	0.02	0.08	0.02

[a-b] Means within a column with different superscripts differ ($P \leq 0.05$).

[*] CF = Creep feed.

[1] BER = 100% bermudagrass; BERCF = 75% bermudagrass and 25% CF; BERCF+XYL = BERCF enhanced with endo-1,4-β-xylanase at 300,000 U/t of DM; BERCF+XYL 10x = BERCF enhanced with endo-1,4-β-xylanase at 3,000,000 U/t of DM; BERCF+BGLUC = BERCF enhanced with endo-1,3(4)-β-glucanase at 20,000 U/t of DM; BERCF+BGLUC 10x = BERCF enhanced with endo-1,3(4)-β-glucanase at 200,000 U/t of DM; BERCF+AMYL = BERCF enhanced with α-amylase at 390,000 U/t of DM; BERCF+AMYL 10x = BERCF enhanced with α-amylase at 3,900,000 U/t of DM; BERCF+COMB1 = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 540,000, 140,000, and 160,000 U/t of DM; BERCF+COMB1 10x = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 5,400,000, 1,400,000, and 1,600,000 U/t of DM; BERCF+COMB2 = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 540,000, 140,000, 160,000, and 390,000 U/t of DM; BERCF+COMB2 10x = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 5,400,000, 1,400,000, 1,600,000, and 3,900,000 U/t of DM.

Table 3.4. Concentrations of the main VFA (m*M*), acetate:propionate ratio, and Total VFA concentration (m*M*) after 24 h of incubation of treatments.

	Feature						
Treatment[1]	**Acetate**	**Propionate**	**Butyrate**	**Isovalerate**	**n-Valerate**	**Total VFA**	**A:P[2]**
BER	54.70^{c}	15.08^{b}	9.36^{b}	1.08^{c}	0.92^{d}	82.01^{c}	3.63^{a}
BERCF*	58.36^{bc}	17.50^{a}	11.81^{a}	1.38^{ab}	1.10^{abc}	91.28^{b}	3.33^{b}
BERCF+XYL	61.48^{ab}	17.66^{a}	12.01^{a}	1.42^{ab}	1.12^{ab}	94.76^{ab}	3.48^{ab}
BERCF+XYL 10x	61.39^{ab}	17.83^{a}	12.24^{a}	1.47^{a}	1.15^{a}	95.17^{ab}	3.45^{ab}
BERCF+BGLUC	61.95^{ab}	18.19^{a}	11.99^{a}	1.38^{ab}	1.11^{ab}	95.72^{ab}	3.41^{b}
BERCF+BGLUC 10x	61.74^{ab}	17.93^{a}	12.12^{a}	1.41^{ab}	1.12^{ab}	95.44^{ab}	3.44^{ab}
BERCF+AMYL	61.73^{ab}	17.82^{a}	11.98^{a}	1.41^{ab}	1.12^{ab}	95.19^{ab}	3.46^{ab}
BERCF+AMYL 10x	61.43^{ab}	17.88^{a}	11.92^{a}	1.40^{ab}	1.12^{ab}	94.86^{ab}	3.44^{ab}
BERCF+COMB1	60.99^{ab}	17.46^{a}	11.83^{a}	1.36^{b}	1.09^{bc}	93.72^{ab}	3.49^{ab}
BERCF+COMB1 10x	60.02^{ab}	17.45^{a}	12.04^{a}	1.41^{ab}	1.11^{ab}	93.16^{ab}	3.44^{ab}
BERCF+COMB2	61.21^{ab}	17.73^{a}	11.74^{a}	1.33^{b}	1.10^{abc}	94.20^{ab}	3.45^{ab}
BERCF+COMB2 10x	63.82^{a}	18.26^{a}	11.91^{a}	1.32^{b}	1.06^{c}	97.39^{a}	3.50^{ab}
SE	0.92	0.22	0.12	0.02	0.01	1.19	0.04
P-value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

[a-d] Means within a column with different superscripts differ ($P \leq 0.05$).

[*] CF = Creep feed.

[1] BER = 100% bermudagrass; BERCF = 75% bermudagrass and 25% CF; BERCF+XYL = BERCF enhanced with endo-1,4-β-xylanase at 300,000 U/t of DM; BERCF+XYL 10x = BERCF enhanced with endo-1,4-β-xylanase at 3,000,000 U/t of DM; BERCF+BGLUC = BERCF enhanced with endo-1,3(4)-β-glucanase at 20,000 U/t of DM; BERCF+BGLUC 10x = BERCF enhanced with endo-1,3(4)-β-glucanase at 200,000 U/t of DM; BERCF+AMYL = BERCF enhanced with α-amylase at 390,000 U/t of DM; BERCF+AMYL 10x = BERCF enhanced with α-amylase at 3,900,000 U/t of DM; BERCF+COMB1 = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 540,000, 140,000, and 160,000 U/t of DM; BERCF+COMB1 10x = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 5,400,000, 1,400,000, and 1,600,000 U/t of DM; BERCF+COMB2 = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 540,000, 140,000, 160,000, and 390,000 U/t of DM; BERCF+COMB2 10x = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 5,400,000, 1,400,000, 1,600,000, and 3,900,000 U/t of DM.

[2] A:P = acetate:propionate ratio.

Table 3.5. Molar proportions of VFA (mol/100 mol) after 24 h of incubation of treatments.

	VFA						
Treatment[1]	**Acetate**	**Propionate**	**Butyrate**	**Isobutyrate**	**Isovalerate**	**n-Valerate**	**Caproate**
BER	66.69^{a}	18.39	11.42^{c}	0.83^{cd}	1.31^{d}	1.12^{bc}	0.23
BERCF[*]	63.93^{c}	19.18	12.93^{a}	0.88^{ab}	1.51^{ab}	1.21^{a}	0.35
BERCF+XYL	64.88^{bc}	18.64	12.68^{ab}	0.87^{abc}	1.49^{ab}	1.18^{ab}	0.26
BERCF+XYL 10x	64.50^{bc}	18.73	12.86^{a}	0.90^{a}	1.55^{a}	1.21^{a}	0.26
BERCF+BGLUC	64.71^{bc}	19.01	12.52^{ab}	0.84^{bcd}	1.44^{abc}	1.16^{abc}	0.32
BERCF+BGLUC 10x	64.67^{bc}	18.79	12.70^{ab}	0.86^{abc}	1.48^{ab}	1.18^{ab}	0.32
BERCF+AMYL	64.84^{bc}	18.72	12.59^{ab}	0.86^{abc}	1.49^{ab}	1.17^{abc}	0.32
BERCF+AMYL 10x	64.75^{bc}	18.85	12.56^{ab}	0.85^{abcd}	1.47^{ab}	1.18^{ab}	0.33
BERCF+COMB1	65.06^{b}	18.63	12.63^{ab}	0.86^{abc}	1.45^{abc}	1.16^{abc}	0.19
BERCF+COMB1 10x	64.42^{bc}	18.73	12.93^{a}	0.89^{a}	1.52^{a}	1.19^{ab}	0.32
BERCF+COMB2	64.98^{bc}	18.82	12.47^{ab}	0.83^{cd}	1.41^{bcd}	1.17^{abc}	0.32
BERCF+COMB2 10x	65.52^{b}	18.75	12.24^{b}	0.82^{d}	1.36^{cd}	1.09^{c}	0.22
SE	0.23	0.16	0.098	0.009	0.022	0.017	0.039
P-value	< 0.01	0.25	< 0.01	< 0.01	< 0.01	< 0.01	0.09

[a-d] Means within a column with different superscripts differ ($P \leq 0.05$).

[*] CF = Creep feed.

[1] BER = 100% bermudagrass; BERCF = 75% bermudagrass and 25% CF; BERCF+XYL = BERCF enhanced with endo-1,4-β-xylanase at 300,000 U/t of DM; BERCF+XYL 10x = BERCF enhanced with endo-1,4-β-xylanase at 3,000,000 U/t of DM; BERCF+BGLUC = BERCF enhanced with endo-1,3(4)-β-glucanase at 20,000 U/t of DM; BERCF+BGLUC 10x = BERCF enhanced with endo-1,3(4)-β-glucanase at 200,000 U/t of DM; BERCF+AMYL = BERCF enhanced with α-amylase at 390,000 U/t of DM; BERCF+AMYL 10x = BERCF enhanced with α-amylase at 3,900,000 U/t of DM; BERCF+COMB1 = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 540,000, 140,000, and 160,000 U/t of DM; BERCF+COMB1 10x = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and endo-1,4-β-glucanase at 5,400,000, 1,400,000, and 1,600,000 U/t of DM; BERCF+COMB2 = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 540,000, 140,000, 160,000, and 390,000 U/t of DM; BERCF+COMB2 10x = BERCF enhanced with a combination of endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase and α-amylase at 5,400,000, 1,400,000, 1,600,000, and 3,900,000 U/t of DM.

CHAPTER 4

INCLUSION OF EXOGENOUS FEED ENZYMES AS A WAY TO ENHANCE CREEP FEEDS – ANIMAL PERFORMANCE EVALUATIONS[1]

---

[1]Lourenço, J. M., B. T. Campbell, J. R. Segers, and R. L. Stewart Jr. To be submitted to *Journal of Animal Science*.

ABSTRACT

In a two-year study, 130 cow-calf pairs (cows initial BW = 562 ± 80 kg; calves initial BW = 179 ± 33 kg) were split into 3 groups and assigned to different treatments, as follows: 1) Group with no supplementation of calves (**NO FEED**); 2) Group in which the calves were supplemented in a creep feeding system with a regular grain mix (**PLAIN FEED**); and 3) Group where the calves received the same supplement offered in PLAIN FEED but with the addition of the fibrolytic enzyme xylanase in their supplement (**ENZYME FEED**). Feed was offered in the supplemented groups 3 times per week, and orts were collected on a weekly basis. Groups received their respective treatments for 105 d in year 1, and 97 d in year 2, and performance of cows and calves were assessed 3 times during the experimental period. Assessments included weighing all cows and calves, and evaluation of BCS of cows. On the last day of the feeding period, rumen fluid was collected by esophageal tubing from 9 randomly selected calves from each treatment group, and all calves were weaned and placed in a common diet. Four weeks later, rumen fluid was sampled again to allow pre versus post-weaning comparisons. Analysis of variance was performed using treatment, year, sex, initial age, and farm as factors. Results showed that ADG was greatest (P = 0.03) in ENZYME FEED, followed by PLAIN FEED, with NO FEED having the lowest daily gains among all groups (1.19, 1.14, and 1.07 kg/d, respectively). Although not statistically significant, supplement DMI expressed in kg/day, or as a percentage of calves' BW were lower in ENZYME FEED, compared to PLAIN FEED. Supplement gain:feed ratio was numerically higher in ENZYME FEED compared to PLAIN FEED, however, both treatments had negative financial net returns due to low cattle prices compare to feed prices. No differences were

detected in total VFA concentration due to treatments, but molar proportions of butyrate were greater in the supplemented groups, notably in ENZYME FEED ($P < 0.001$). No effects of treatments ($P \geq 0.09$) were detectable on the cows during the study. Overall, because calves in ENZYME FEED had superior ADG, the greatest proportion of butyrate in their rumen fluid, and a numerically greater feed efficiency, we concluded that the inclusion of xylanase in beef cattle creep feeds is advantageous and should be encouraged. However, further research is needed to confirm if the level of inclusion of xylanase used in this study is optimum.

Key words: animal performance, butyric acid, creep feeding, exogenous feed enzymes, xylanase.

INTRODUCTION

Literature shows that supplementation of beef calves during their suckling phase (commonly known as creep feeding) can significantly improve calf weaning weight (Prichard et al. 1989; Tarr et al., 1994; Sampaio et al., 2002; Viñoles et al., 2013), and even their post-weaning performance (Brazle et al., 1992; Moriel and Arthington, 2013). Despite its benefits, historically, less than one third of the cow-calf operations in the southeast United States (where a remarkable number of cow-calf farms are located) use this type of supplementation (Parish and Rhinehart, 2009). The development of new supplements using technical knowledge may stimulate more producers to adopt this supplementation, especially if this supplement is able to improve calf performance in an economical way.

The use of exogenous feed enzymes is a common practice in the poultry and swine production industries, and the benefits associated with their use is well documented (Choct et al., 1999; Mathlouthi et al., 2002; Barrera et al., 2004; Wu et al., 2004). However, utilization of feed enzymes in ruminant diets is still an emerging field, and animal responses to their use have been extremely variable (Beauchemin et al., 2003; Adesogan et al., 2014; Meale et al., 2014). Furthermore, to our knowledge, there is no research on utilization of feed enzymes as a way to enhance beef cattle creep feeds. Therefore, the aim of this study was to evaluate the inclusion of a granulated endo-1,4-β-xylanase (or simply xylanase; EC number 3.2.1.8) in a creep feed that was formulated specifically for suckling beef calves. The justification for using this particular enzyme were the results of a recent in vitro study from our group (Lourenco, 2017). Findings from this in vitro study, along with some economic considerations, identified that the

inclusion of xylanase at a rate of 12,000,000 units per tonne of DM in creep feeds would maximize our chances of success in an eventual in vivo feeding trial.

MATERIALS AND METHODS

All procedures involving live animals were verified and approved by the University of Georgia's Office of Animal Care and Use (Animal Use Protocol #A2015 07–018-Y1-A0).

The present study was carried out using spring-born calves over 2 consecutive years (2016 and 2017) beginning at the end of May and ending in the first half of September. It was conducted at 2 different research stations: the first one located in Eatonton, GA (University of Georgia Eatonton Beef Research Unit; 33°24 N, 83°29 W), and the second in Alapaha, GA (University of Georgia Alapaha Range Grazing Unit; 31°21 N, 83°13 W). At the first station (Eatonton, GA), 36 steers (179 ± 28 kg) and 6 heifers (163 ± 21 kg) were used in year 1; and 21 steers (233 ± 23 kg) and 6 heifers (221 ± 8 kg) in year 2. Only steers were used at the second station (Alapaha, GA): 37 (172 ± 29 kg) in year 1; and 24 (167 ± 21 kg) in year 2. On both farms, the cow-calf herd was divided into 3 groups, namely: 1) Group with no supplementation of calves (**NO FEED**); 2) Group in which the calves were supplemented in a creep feeding system with a regular grain mix (**PLAIN FEED**); and 3) Group where the calves received the same supplement offered in PLAIN FEED but with the addition of the fibrolytic enzyme xylanase (EC number 3.2.1.8; RONOZYME® WX (CT), DSM Nutritional Products) in their supplement (**ENZYME FEED**).

The commercial enzyme used in ENZYME FEED was selected based on the results of a previous in vitro screening trial (Lourenco, 2017). In that trial, several enzymes and enzyme combinations were included in a 75:25% bermudagrass:creep feed mix and incubated for 24 h in rumen fluid collected from 6-month-old beef calves. Digestibilities of the fiber fractions and total DM were quantified, along with production of gas, CH_4, and VFA. This in vitro assessment indicated xylanase as one of the best candidates for use in beef cattle creep feeds. Moreover, it is normally less expensive than the other enzymes evaluated in that trial. Thus, it was decided that the supplement offered in the ENZYME FEED group would contain xylanase at a rate of 13,800,000 fungal xylanase units per tonne of creep feed DM (or 0.69% of commercial enzyme preparation in the creep feed DM). The commercial enzyme used in our feeding trial was a granulated, heat-stable endo-1,4-β-xylanase from *Thermomyces lanuginosus*, produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism. Its average particle size is approximately 600 μm, and the declared activity of the product is 2,000 fungal xylanase units per g.

In order to have uniformity, the 3 aforementioned cow-calf groups were formed taking into consideration age, sex, and live body weight of the calves. Once the 3 groups were formed they remained separated for the entire trial, which lasted 105 days in the first year, and 97 days in the second year. A total of 9 paddocks were used on each farm over the course of the 2 years. Within each farm, the forage composition of the experimental paddocks was approximately the same. On the farm located in Eatonton, GA, the research paddocks were composed of a combination of bermudagrass (*Cynodon dactylon*) and dallisgrass (*Paspalum dilatatum*), whereas in Alapaha, GA, animals were

grazing a combination of bermudagrass and bahiagrass (*Paspalum notatum*). One initial and one final forage sample was obtained from each individual paddock on both years.

Both the calves and their respective dams had their performance traits evaluated 3 times during the study: at the beginning, middle, and end of the trial. On these evaluation days, body condition scores were assessed for the cows, and live body weights were assessed for both cows and calves. Feed was offered in the supplemented groups 3 times per week, and the portion of the feed that was not consumed by calves was recorded on a weekly basis. Upon conclusion of the feeding period, rumen fluid was collected by esophageal tubing from 27 calves (9 from each treatment-group) for analysis of VFA and their associated rumen microbiome. Because the results concerning their microbiome are presented in another publication, they will not be shown here.

On the last day of the feeding trial, immediately after rumen fluid collection, all calves were weaned and placed in a common diet consisting of pasture (bermudagrass) plus 2.3 kg/day of a commercial feed formulated with cereal grains and by-products. Animals also had ad libitum access to water and a mineral mix (18% Ca, 8% P, 11% NaCl, 2% Mg, 1% S, 0.75% K, 3000 mg/kg Mn, 4000 mg/kg Zn, 1500 mg/kg Cu, 60 mg/kg Co, 72 mg/kg I, 26.5 mg/kg Se). Four weeks after being in this common diet, rumen fluid samples from the same animals previously selected were collected once again for comparisons of the pre- versus post-weaning timepoints.

Concentrations of VFA in calves' ruminal fluid were determined in a water-based solution using ethyl acetate extraction as described by Henry et al. (2015). Briefly, 5 mL of rumen fluid was centrifuged for 10 min at 10,000 x *g* at 4°C, and 2.5 mL of the supernatant was transferred to another centrifuge tube. Then, 1 mL of a metaphosphoric

acid:crotonic acid (internal standard) solution was added, and samples were vortexed and frozen overnight. On the following day, samples were thawed and centrifuged one more time for 10 min at 10,000 x *g* at 4°C. One mL of supernatant was transferred into a vial and mixed with 2mL of ethyl acetate, and the vial was vortexed. After 5 min, the ethyl acetate fraction separated and a subsample of 0.5 mL was transferred to another vial and analyzed by gas chromatography (Shimadzu GC-2010 Plus; Shimadzu Corporation, Kyoto, Japan) using a flame ionization detector and a capillary column (Zebron ZB-FFAP GC Cap. Column 30 m x 0.32 mm x 0.25 μm; Phenomenex Inc., Torrance, CA). Column temperature was initially set at 110°C and gradually increased to 200°C. Injector and detector temperatures were set at 250 and 350°C, respectively.

Statistical Analysis. Statistical analyses were done using the software R (R Foundation for Statistical Computing, Vienna, Austria). Analysis of variance was performed using treatment, year, sex, and farm as fixed effects. The model also included the interaction terms for these effects. Additionally, because there were some variations across farms and years regarding calf initial age, and since the accuracy of the model was significantly improved by its inclusion, calf initial age was classified into 6 sub-categories and included in the model as well. Orthogonal contrasts were tested using Tukey's honest significant difference test, and means were considered different at $P \leq 0.05$. All traits concerning just the groups that received supplementation (e.g. supplement gain:feed ratio for PLAIN FEED and ENZYME FEED) were analyzed using two-sample t tests.

For VFA concentrations and molar proportions, ANOVA were conducted using treatment and year as factors, as well as a treatment x year interaction. Contrasts were

calculated using Tukey's pairwise comparison test, and results were considered significant at $P \leq 0.05$. Comparisons of VFA in rumen fluid for samples collected on weaning day versus the ones collected 4 weeks later were accomplished through two-sample t tests, in which the 2 groups were: 1) at weaning; and 2) 4 weeks after weaning.

RESULTS AND DISCUSSION

Performance of calves over the 2 years is summarized in Table 4.2. Their initial BW were similar ($P = 0.67$) across treatment groups, and the same was observed for final BW ($P = 0.47$). However, calf ADG during the first half of the feeding trial was greatest ($P = 0.02$) for ENZYME FEED and lowest for NO FEED. No significant differences ($P = 0.06$) were observed on ADG during the second half of the trial, although the 2 groups that were supplemented tended to have greater gains. When compared for the entire trial, ADG was greatest ($P = 0.03$) in ENZYME FEED, followed by PLAIN FEED, with NO FEED having the lowest gains among all groups. These results are completely aligned with the IVDMD values assessed in a previous in vitro study conducted by our lab (Lourenco, 2017). In that study, the IVDMD of pure bermudagrass (which would be similar to NO FEED in this study) was the lowest among all treatments: 27.39%. In contrast, the treatment from that study that had the greatest IVDMD (41.28%) was the one containing xylanase at a rate of 12,000,000 units/t of creep feed DM (which is comparable to ENZYME FEED in the present study). Lastly, their in vitro treatment composed only by a combination of forage and creep feed on a 75:25 ratio, which would correspond to PLAIN FEED in this study, had an intermediate IVDMD: 31.63%.

Therefore, as can be noticed, the IVDMD values of the analogous treatments from that trial are in agreement with the overall ADG observed in the present study.

As previously mentioned, there are no data in the scientific literature describing the use of feed enzymes in beef cattle during their suckling phase. However, 2 particular studies (Beauchemin et al., 1995 and McAllister et al., 1999) were performed using fibrolytic enzymes in recently-weaned beef cattle, and their results may be in some way comparable to those from the current study. McAllister et al. (1999) conducted a feeding trial using recently-weaned steers weighing 265 kg on average and fed them a 60:40% roughage:concentrate diet with 3 different levels of inclusion of cellulase and xylanase for 120 days. Their results showed no differences in overall ADG (day 0 to 120) due to enzyme inclusion, regardless of the dose of inclusion. Moreover, no differences were detected on their overall feed efficiency. In another study using a combination of xylanase and cellulase, Beauchemin et al. (1995) fed steers that were slightly older (and consequently heavier: average BW = 289 kg). Although they tested incremental levels of enzymes on different diets, one particular treatment was composed of Timothy grass (*Phleum pratense*) hay and approximately 0.8 kg of concentrate. Steers fed this treatment had greater ADG when the tested enzymes were included in their diets, and this increase in gain was even more pronounced when the enzymes were used at their greatest dosage (10,920,000 units of xylanase plus 436,800 units of cellulase/t of DM). In addition to increased gains, authors also observed improved feed efficiency and greater ADF digestibility at this dosage.

Daily intake of supplement and intake of supplement as a percentage of calves' BW were numerically higher in PLAIN FEED, compared to ENZYME FEED, which

resulted in a numerically lower monthly supplementation cost for ENZYME FEED (Table 4.3). Supplement gain:feed ratio was numerically higher in ENZYME FEED. Finally, a comparison of the net return due to supplementation in each group showed that this trait was numerically improved in the ENZYME FEED group, compared to PLAIN FEED group. However, due to low cattle prices compared to feed prices, under our experimental conditions both treatments had negative financial returns.

Results from Table 4.4 show that performance of cows during the creep feeding trial was practically not affected. Cows' BW gain (or loss) did not change ($P \geq 0.09$) in any of the assessed periods, regardless of calves' feed regimen. Similarly, cows' BCS was not influenced ($P \geq 0.32$) by any of the calf treatments. Given that this feeding trial began when calves were on average 133-days-old, the lack of effects on cows' BW and BCS are not surprising. It is well stablished that beef cows reach peak milk production 55 to 65 days postpartum, and that this stage is the most critical in their entire productive cycle (NRC, 2000). Consequently, regardless if cows gained or lost weight and BCS during that high-demand phase of their cycle, any effect related had already past when this experiment started. Thus, since the cows already had their conditions well established when supplementation of calves started, the influence of supplementation was minimal on them. In the same way, considering that both research stations have well stablished breeding seasons (which last less than 90 days) reproductive traits such as cow conception rates were likely not affected as well, although none of them were evaluated in this study.

Concentrations of the main VFA are shown in Table 4.5. An effect of treatment was observed for both isobutyric and isovaleric acids ($P \leq 0.02$). In both cases,

concentrations were highest in rumen fluid obtained from calves in ENZYME FEED. A year effect was also observed for these 2 VFA, and for acetate, total VFA concentration, and acetate:propionate ratio ($P \leq 0.04$). While animals in the first year of the study (2016) had greater concentration of branched chain VFA in their rumen fluid, the opposite was found regarding concentrations of acetate and total VFA. Likewise, the acetate:propionate ratio was higher ($P = 0.004$) in the rumen fluid of animals that participated in the trial in 2017, compared to the ones that participated in 2016. These differences may be explained, at least in part, by the age of the calves, since animals in the second year of the study were on average 11 days older than animals in the first year.

Another aspect presented in Table 4.5 demonstrates the singularity of the present study. Concentrations of VFA detected in the rumen fluid of developing calves is significantly lower than what is normally reported for adult ruminants. For instance, while the total VFA concentration ranged from 28.76 to 35.91 m*M* in the present study, a work by Lewis et al., (1996) using mature beef steers reported values ranging from 84.3 to 182.0 m*M*. Likewise, He et al. (2014) found total VFA concentrations varying from 139.6 to 146.5 m*M* in adult heifers. In a study working with yearling steers, McGinn et al. (2004) found total VFA concentrations in the 68.1 to 82.0 m*M* range.

Regarding the molar proportions of the analyzed VFA (Table 4.6), an effect of year ($P \leq 0.04$) was detected for all of them, except for butyrate ($P = 0.38$). Molar proportion of acetate was highest ($P = 0.001$) in NO FEED and lowest in ENZYME FEED, with PLAIN FEED being intermediate. However, proportions of isobutyric, butyric, and isovaleric acids were all greatest ($P \leq 0.004$) in ENZYME FEED, compared to the other treatments. A greater proportion of butyric acid may have important

biological consequences in the developing rumen of a calf, since this VFA is recognized as having the greatest stimulant effect on the development of ruminal epithelial tissue (NRC, 2001; Yáñez-Ruiz et al., 2015). Consequently, although not directly measured, ENZYME FEED likely had a greater effect on papillae development in the rumen than the other treatments, which may have contributed to the greater gains observed for animals in this treatment.

Figure 4.1 shows the comparisons between samples collected at the end of the feeding trial (i.e. the day calves were weaned) versus the ones collected 4 weeks later, after calves had been weaned and fed a common diet. It can be noticed that total VFA concentration went up for all the 3 treatments after calves were weaned, however, this difference was statistically significant only in ENZYME FEED ($P = 0.04$). Changes were also observed in molar proportions of the major VFA. Acetate proportions were reduced ($P \leq 0.03$) in the rumen fluid of calves from all treatments 4 weeks after weaning, and the most significant reduction was observed in NO FEED ($P < 0.001$). Conversely, molar proportions of propionate and butyrate increased ($P \leq 0.04$) in all treatment groups as calves aged, except for propionate proportion in the rumen fluid of PLAIN FEED calves ($P = 0.24$).

In conclusion, although no changes were observed in their dams, calves in the ENZYME FEED group experienced greater ADG, a numerical reduction in supplement intake, and numerically greater feed efficiency. In addition, calves in this group had the greatest proportion of butyrate in their rumen fluid, which is the VFA with the greatest stimulatory effect on rumen papillae development in young calves. Therefore, taken together, these results demonstrate that the inclusion of xylanase in beef cattle creep feeds

is advantageous and should be encouraged. However, further research is needed to explore inclusion levels in creep feeds other than 13,800,000 fungal xylanase units/t of creep feed DM.

LITERATURE CITED

Adesogan, A. T., Z. X. Ma, J. J. Romero, and K. G. Arriola. 2014. RUMINANT NUTRITION SYMPOSIUM: Improving cell wall digestion and animal performance with fibrolytic enzymes. J. Anim. Sci. 92:1317–1330. doi: 10.2527/jas.2013-7273.

Barrera, M., M. Cervantes, W. C. Sauer, A. B. Araiza, N. Torrentera, and M. Cervantes. 2004. Ileal amino acid digestibility and performance of growing pigs fed wheat-based diets supplemented with xylanase. J. Anim. Sci. 82:1997–2003. doi: 10.2527/2004.8271997x.

Beauchemin, K. A., L. M. Rode, and V. J. H. Sewalt. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75:641–644. doi: 10.4141/cjas95-096.

Beauchemin, K. A., D. Colombatto, D. P. Morgavi, and W. Z. Yang. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. J. Anim. Sci. 81:E37–E47. doi: 10.2527/2003.8114_suppl_2E37x.

Brazle, F. K., G. L. Kuhl, C. E. Binns, K. O. Zoellner, L. R. Corah, and R. R. Schalles. 1992. The influence of limited creep feed on pre- and post-weaning performance of spring born calves. Prof. Anim. Sci. 8:55–57. doi: 10.15232/S1080-7446(15)32139-2.

Choct, M., R. J. Hughes, and M. R. Bedford. 1999. Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. Br. Poult. Sci. 40:419–422. doi: 10.1080/00071669987548.

He, Z. X., M. L. He, N. D. Walker, T. A. McAllister, and W. Z. Yang. 2014. Using a fibrolytic enzyme in barley-based diets containing wheat dried distillers grains with solubles: ruminal fermentation, digestibility, and growth performance of feedlot steers. J. Anim. Sci. 92:3978–3987. doi: 10.2527/jas.2014-7707.

Henry, D. D., M. Ruiz-Moreno, F. M. Ciriaco, M. Kohmann, V. R. G. Mercadante, G. C. Lamb, and N. DiLorenzo. 2015. Effects of chitosan on nutrient digestibility, methane emissions, and in vitro fermentation in beef cattle. J. Anim. Sci. 93:3539–3550. doi: 10.2527/jas.2014-8844.

Lewis, G. E., C. W. Hunt, W. K. Sanchez, R. Treacher, G. T. Pritchard, and P. Feng. 1996. Effect of direct-fed fibrolytic enzymes on the digestive characteristics of a forage-based diet fed to beef steers. J. Anim. Sci. 74:3020–3028. doi: 10.2527/1996.74123020x.

Lourenco, J. M. 2017. Utilization of Exogenous Feed Enzymes as a Way to Enhance Creep Feeds – In Vitro, In Vivo, and Ruminal Microbiome Evaluations. PhD Diss. Univ. of Georgia, Athens.

Mathlouthi, N., J. P. Lallès, P. Lepercq, C. Juste, and M. Larbier. 2002. Xylanase and β-glucanase supplementation improve conjugated bile acid fraction in intestinal contents and increase villus size of small intestine wall in broiler chickens fed a rye-based diet. J. Anim. Sci. 80:2773–2779. doi: 10.2527/2002.80112773x.

McAllister, T. A., S. J. Oosting, J. D. Popp, Z. Mir, L. J. Yanke, A. N. Hristov, R. J. Treacher, and K-J. Cheng. 1999. Effect of exogenous enzymes on digestibility of barley silage and growth performance of feedlot cattle. Can. J. Anim. Sci. 79:353–360. doi: 10.4141/A98-099.

McGinn, S. M., K. A. Beauchemin, T. Coates, and D. Colombatto. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. J. Anim. Sci. 82:3346–3356. doi: 10.2527/2004.82113346x.

Meale, S. J., K. A. Beauchemin, A. N. Hristov, A. V. Chaves, and T. A. McAllister. 2014. Board-Invited Review: opportunities and challenges in using exogenous enzymes to improve ruminant production. J. Anim. Sci. 92:427–442. doi: 10.2527/jas.2013-6869.

Moriel, P., and J. D. Arthington. 2013. Effects of trace mineral-fortified, limit-fed preweaning supplements on performance of pre- and postweaned beef calves. J. Anim. Sci. 91:1371–1380. doi: 10.2527/jas.2012-5469.

NRC. 2000. Nutrient Requirements of Beef Cattle: Update 2000. 7th rev. ed. Natl. Acad. Press, Washington, DC.

NRC. 2001. Nutrient requirements of dairy cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Parish, J. A., and J. D. Rhinehart. 2009. Creep feeding beef calves. Mississippi State University Extension Service. Publication 2524.

Prichard, D. L., D. D. Hargrove, T. A. Olson, and T. T. Marshall. 1989. Effects of creep feeding, zeranol implants and breed type on beef production: I. Calf and cow performance. J. Anim. Sci. 67:609–616. doi: 10.2527/jas1989.673609x.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Sampaio, A. A. M., R. M. de Brito, G. M. da Cruz, M. M. de Alencar, P. F. Barbosa, and R. T. Barbosa. 2002. Use of NaCl in Supplement as an Alternative to Viabilize the Calf Creep-Feeding System. R. Bras. Zootec. 31:164–172. doi: 10.1590/S1516-35982002000100019.

Tarr, S. L., D. B. Faulkner, D. D. Buskirk, F. A. Ireland, D. F. Parrett, and L. L. Berger. 1994. The value of creep feeding during the last 84, 56, or 28 days prior to weaning on growth performance of nursing calves grazing endophyte-infected tall fescue. J. Anim. Sci. 72:1084–1094. doi: 10.2527/1994.7251084x.

Viñoles, C., M. Jaurena, I. De Barbieri, M. Do Carmo, and F. Montossi. 2013. Effect of creep feeding and stocking rate on the productivity of beef cattle grazing grasslands. New Zeal. J. Agr. Res. 56:279–287. doi: 10.1080/00288233.2013.840320.

Wu, Y. B., V. Ravindran, D. G. Thomas, M. J. Birtles, and W. H. Hendriks. 2004. Influence of phytase and xylanase, individually or in combination, on performance, apparent metabolisable energy, digestive tract measurements and gut morphology in broilers fed wheat-based diets containing adequate level of phosphorus. Br. Poult. Sci. 45:76–84. doi: 10.1080/00071660410001668897.

Yáñez-Ruiz, D. R., L. Abecia, and C. J. Newbold. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. Front. Microbiol. 6:1133. doi: 10.3389/fmicb.2015.01133.

Table 4.1. Ingredients used in the creep feed offered in PLAIN FEED and ENZYME FEED[1], and its analyzed chemical composition.

Ingredient	**% Inclusion (DM basis)**
Corn	60.23
Soybean meal	25.01
Salt	8.77
Limestone	3.95
Molasses	1.14
Dicalcium phosphate	0.74
Trace mineral concentrate	0.11
Vitamin ADE	0.05
Analyzed chemical composition[2]	
Nutrient	**% DM**
Crude protein	18.85
Neutral detergent fiber	7.60
Acid detergent fiber	3.70
Ether extract	2.34
Net energy for maintenance (Mcal/kg)	1.61
Net energy for gain (Mcal/kg)	0.99
Calcium	2.32
Phosphorus	0.51
Magnesium	0.17
Sodium	4.16

[1] The feed offered in the PLAIN FEED and ENZYME FEED groups were the same, except for the addition of xylanase at a rate of 0.69% of DM (RONOZYME® WX; manufactured by DSM Nutritional Products).

[2] Cumberland Valley Analytical Services (Waynesboro, PA).

Table 4.2. Performance of calves during the creep feeding trial at 2 experimental stations over 2 years.

	Treatment[1]				
Item	**NO FEED**	**PLAIN FEED**	**ENZYME FEED**	**SE[2]**	***P*-value**
Number of animals	44	43	43	-	-
Number of pens	6	6	6	-	-
Initial Age, days	131	136	133	3.14	0.48
Initial BW, kg	182.1	176.9	178.3	4.79	0.67
Final BW, kg	290.1	292.4	298.7	6.01	0.47
ADG day 1 to 51, kg	1.18[b]	1.24[ab]	1.32[a]	0.033	0.02
ADG day 51 to 101, kg	0.96	1.03	1.06	0.036	0.06
ADG day 1 to 101, kg	1.07[b]	1.14[ab]	1.19[a]	0.026	0.03

[a-b] Means within a row with different superscripts differ ($P \leq 0.05$).

[1] NO FEED = conventional cow-calf system without supplementation of calves. PLAIN FEED = calves were creep fed. ENZYME FEED = calves were creep fed with an enhanced feed containing xylanase.

[2] Standard error of main-effect means.

Table 4.3. Feed consumption and productivity parameters of calves during the creep feeding trial at 2 experimental stations over 2 years.

	Treatment[1]				
Item	**NO FEED**	**PLAIN FEED**	**ENZYME FEED**	**SE[2]**	***P*-value**
Average supplement DMI, kg/d	-	0.97	0.92	0.105	0.78
Average supplement DMI, % BW	-	0.40	0.38	0.047	0.75
Supplement gain:feed ratio	-	0.054	0.099	0.0305	0.32
Additional gain per month due to supplementation, kg	-	1.21	2.77	0.88	0.24
Monthly return due to additional gain, $/month[3]	-	3.45	7.38	2.3	0.26
Cost to supplementing each calf, $/month[4]	-	12.94	12.36	1.3	0.76
Net monthly return with supplementation, $/calf/month	-	-9.49	-4.98	2.7	0.27

[1] NO FEED = conventional cow-calf system without supplementation of calves. PLAIN FEED = calves were creep fed. ENZYME FEED = calves were creep fed with an enhanced feed containing xylanase.

[2] Standard error of main-effect means.

[3] Calf prices were $2.47/kg in 2016 and 2.91/kg in 2017.

[4] Feed costs were $385.35 in 2016 and $399.04 in 2017.

Table 4.4. Performance of cows during the creep feeding trial at 2 experimental stations over 2 years.

	Treatment[1]				
Item	**NO FEED**	**PLAIN FEED**	**ENZYME FEED**	**SE[2]**	***P*-value**
Cow initial BW, kg	570.5	564.4	552.1	12.1	-
Cow ADG from day 1 to 51, kg/d	0.27	0.35	0.31	0.07	0.67
Cow ADG from day 51 to 101, kg/d	0.00	-0.14	0.03	0.06	0.09
Cow ADG from day 1 to 101, kg/d	0.14	0.12	0.17	0.04	0.65
Cow avg BCS on day 1	5.92	5.70	5.91	0.20	0.54
Cow avg BCS on day 51	5.53	5.60	5.53	0.21	0.94
Cow avg BCS on day 101	6.33	6.09	6.22	0.25	0.67
BCS Change day 1 to 51	-0.39	-0.10	-0.39	0.19	0.32
BCS Change day 51 to 101	0.81	0.49	0.69	0.20	0.33
BCS Change day 1 to 101	0.42	0.39	0.31	0.18	0.83

[1] NO FEED = conventional cow-calf system without supplementation of calves. PLAIN FEED = calves were creep fed. ENZYME FEED = calves were creep fed with an enhanced feed containing xylanase.

[2] Standard error of main-effect means.

Table 4.5. Concentrations of VFA (m*M*), acetate:propionate ratio, and Total VFA concentration (m*M*) in the rumen fluid of beef calves fed different treatments[1] for 101 days.

							***P*-value**		
	Year 1[2]			**Year 2[3]**					
Item	**NO FEED**	**PLAIN FEED**	**ENZYME FEED**	**NO FEED**	**PLAIN FEED**	**ENZYME FEED**	**Treat.**	**Year**	**Treatment x Year**
Acetate	21.28	18.36	18.11	22.23	23.66	23.19	0.72	0.002	0.22
Propionate	5.60	5.15	4.98	5.45	6.06	5.88	0.91	0.11	0.35
Isobutyrate	0.46	0.41	0.51	0.40	0.37	0.45	0.01	0.04	0.91
Butyrate	4.08	3.77	4.01	3.60	4.56	5.23	0.14	0.11	0.08
Isovalerate	0.69	0.57	0.73	0.55	0.51	0.64	0.02	0.02	0.70
Valerate	0.35	0.30	0.32	0.28	0.32	0.36	0.62	0.92	0.13
Caproate	0.26	0.20	0.17	0.16	0.13	0.16	0.36	0.054	0.49
Total VFA	32.72	28.76	28.83	32.67	35.61	35.91	0.97	0.01	0.20
A:P ratio[4]	3.83	3.61	3.68	4.07	3.91	3.95	0.20	0.004	0.95

[1] NO FEED = conventional cow-calf system without supplementation of calves. PLAIN FEED = calves were creep fed. ENZYME FEED = calves were creep fed with an enhanced feed containing xylanase.

[2] Year 1: 2016. [3]Year 2: 2017.

[4]A:P ratio: Acetate to Propionate ratio.

Table 4.6. Molar proportions of VFA (mol/100 mol) in the rumen fluid of beef calves fed different treatments[1] for 101 days.

							P-value		
	Year 1[2]			Year 2[3]			Treat.	Year	Treatment x Year
Item	NO FEED	PLAIN FEED	ENZYME FEED	NO FEED	PLAIN FEED	ENZYME FEED			
Acetate	65.19	64.01	62.93	67.96	66.44	64.67	0.001	< 0.001	0.76
Propionate	17.03	17.84	17.19	16.73	17.06	16.38	0.17	0.04	0.75
Isobutyrate	1.41	1.47	1.79	1.23	1.06	1.28	0.003	< 0.001	0.11
Butyrate	12.35	12.99	13.84	11.04	12.75	14.38	< 0.001	0.38	0.16
Isovalerate	2.13	2.01	2.57	1.69	1.44	1.81	0.004	< 0.001	0.46
Valerate	1.06	1.04	1.09	0.85	0.90	1.01	0.22	0.004	0.52
Caproate	0.82	0.64	0.59	0.49	0.36	0.48	0.33	0.009	0.57

[1] NO FEED = conventional cow-calf system without supplementation of calves. PLAIN FEED = calves were creep fed. ENZYME FEED = calves were creep fed with an enhanced feed containing xylanase.

[2] Year 1: 2016. [3]Year 2: 2017.

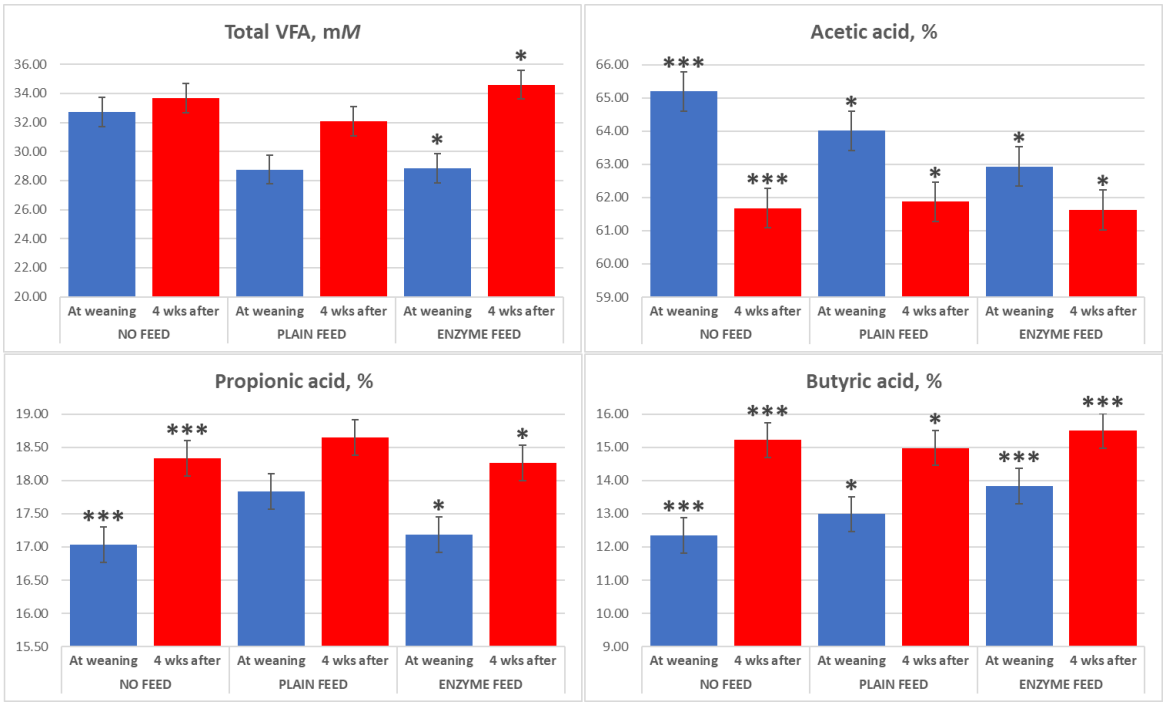

Figure 4.1. Comparisons of samples collected on weaning day versus samples collected 4 weeks later regarding total VFA concentration and molar proportions of the 3 main VFA in rumen fluid of calves (* = $P < 0.05$; and *** = $P < 0.001$).

CHAPTER 5

ANALYSIS OF THE GASTROINTESTINAL TRACT-ASSOCIATED MICROBIOME OF CALVES SUPPLEMENTED DURING THE SUCKLING PHASE[1]

---

[1]Lourenço, J. M., T. J. Kieran, J. C. McCann, T. C. Glenn, and R. L. Stewart Jr. To be submitted to *Journal of Animal Science*.

ABSTRACT

Forty-two suckling calves along with their respective mothers were divided into 3 groups, and distinct treatments were offered to each group for 14 weeks, as follows: 1) No supplementation of calves (**NO FEED**); 2) Creep feeding supplementation of calves with a mixture of grains (**PLAIN FEED**); or 3) Creep feeding supplementation of calves with the same mixture offered in PLAIN FEED, but enriched with the enzyme xylanase (**ENZYME FEED**). Upon conclusion of the 14 weeks, the contents of the forestomach of selected calves from each group was collected by esophageal tubing and the calves were weaned, grouped together, and offered a common diet. Four weeks later, a second collection procedure was conducted on the calves, and all the resulting samples were subjected to microbiome analysis using 16S rRNA gene paired-end sequencing. Overall, the number of reads per sample decreased ($P = 0.001$) 4 weeks after weaning, compared to samples collected on weaning day. In addition, regardless of treatment and collection day, the phyla *Bacteroidetes* and *Firmicutes* comprised 75 to 80% of the bacterial abundance. For samples collected on calves' weaning day, the phylogenetic diversity of whole tree was lower ($P \leq 0.02$) in PLAIN FEED and ENZYME FEED, compared to NO FEED, and no differences ($P \geq 0.23$) were detected for this trait in samples collected 4 weeks post-weaning. A two-way ANOVA with treatment and collection day as factors revealed changes due to treatment ($P \leq 0.02$) for *Bacteroidetes*, TM7, and *Spirochaetes*, and an effect of collection day ($P < 0.01$) for *Bacteroidetes*, *Firmicutes*, *Tenericutes*, and TM7. At the genus level, of the genera with relative abundances greater than 1%, a treatment effect ($P \leq 0.02$) was observed for *Prevotella* and *Treponema*, and a day effect ($P < 0.01$) for *Prevotella*, *Ruminococcus*, *Anaeroplasma*, and *Succiniclasticum*. These

results demonstrate that both treatment and collection day had some effects on the calves' rumen microbiome, however, the changes were not as great as some findings commonly reported in the literature, and consequently, they may not fully explain the differences observed in body weight gain that the calves experienced when offered the 3 treatments. Further research is necessary to establish a solid relationship between young beef calf's diet and their rumen microbiome.

Key words: 16S rRNA, creep feeding supplementation, exogenous feed enzymes, rumen microbiome, xylanase.

INTRODUCTION

At birth, the reticulo-rumen portion of the stomach of beef calves represents less than 40% of the total tissue weight; however, this segment of their stomach quickly becomes the dominant part, and around their weaning age, it represents nearly 2/3 of this organ (Church, 1979). This substantial change takes place at the same time the calves' forestomach is being colonized by a myriad of microorganisms, and such transformations are largely influenced by the nature of their diet (Yáñez-Ruiz et al., 2015). Moreover, given that several bacteria in cattle's forestomach utilize specific substrates, changing the composition of their diet should have a significant impact on the composition of their rumen microbiome (Petri et al., 2013; Henderson et al., 2015).

New approaches such as next-generation DNA sequencing are facilitating our comprehension of the rumen microbiome, and the combination of these new techniques with animal performance parameters has produced significant advances in our knowledge of functional microflora. For instance, associations between specific bacterial phylotypes and animal performance traits such as feed efficiency have been identified (Hernandez-Sanabria et al., 2012; Myer et al., 2015). However, information is limited on the ruminal microbiome profile of suckling beef calves and its correlation with calf performance. Therefore, the aim of this study was to examine if beef calves subjected to 3 different treatments during their suckling stage would develop distinct ruminal microbiomes. In addition, we wanted to investigate if the potential diet-driven changes in their rumen microbiome would persist after they were grouped together and offered the same diet for 4 weeks.

MATERIALS AND METHODS

All procedures involving live animals were verified and approved by the University of Georgia's Office of Animal Care and Use (Animal Use Protocol #A2015 07–018-Y1-A0). The cow-calf pairs used in this study were located at the University of Georgia Beef Research Unit in Tifton, GA.

***Animals and Sample Collection*.** A group of 42 suckling calves averaging 127 days-old were weighed and divided into 3 groups in which the calves had similar weights. The calves in these groups and their respective mothers were then used in a feeding trial that lasted 14 weeks. During this trial, the 3 cow-calf groups were assigned to 1 of 3 treatments, as follows: 1) Group with no supplementation of calves (**NO FEED**); 2) Group in which the calves were supplemented in a creep feeding system with a typical feed composed mainly of corn and soybean meal (**PLAIN FEED**); and 3) Group with the same feed regimen as PLAIN FEED but with the addition of the fibrolytic enzyme xylanase (EC number 3.2.1.8) in the supplement (**ENZYME FEED**). Both the cows and the calves had their performances evaluated during the 14-week feeding trial, however, although supplementary tables 5.10S to 5.18S display some of the important findings from this trial, the totality of the results is presented in a separate publication (Lourenco, 2017). Upon conclusion of the 14 weeks, 25 male calves were randomly selected (8 or 9 from each group) for collection of contents of their forestomach. Ruminal contents were collected from each calf by esophageal tubing and all calves were weaned immediately after this procedure. The weaned calves were placed in a common diet consisting of pasture plus 2.3 kg/day of a commercial mixture made of grains and by-product feeds. Four weeks after being in this common diet, sampling of their forestomach

contents was performed again, to detect if the changes caused by the treatments during their suckling phase would persist even after calves are placed in a common diet for 4 weeks.

As stated before, 2 collections of rumen contents were performed – one on calves' weaning day, and another 4 weeks later. The procedure was the same on each collection day. Briefly, about 200 mL of ruminal contents were individually collected from each calf by esophageal tubing using an electric pump, and as soon as the samples were obtained, they were transferred to 15-mL sterile tubes and flash frozen by immersion in liquid nitrogen. Once completely frozen, they were transferred to a thermal box filled with ice and transported to the laboratory, where they were placed in a freezer at -20 °C. Six weeks later, samples were thawed, homogenized, and 0.5 mL of their liquid fraction was pipetted into plastic bead tubes. The samples were then further processed to isolate their DNA content.

***DNA Extraction and Amplification*.** DNA extraction was accomplished using a MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). A MoBio vortex adaptor was used, and PowerBead tubes containing the rumen samples were attached to it and vortexed for 20 min. After this bead beating step, 500µL of samples were taken for the subsequent processes following manufacturer's protocol (PowerSoil DNA isolation kit, Version 11212013). At the end of the protocol, 100µl of molecular grade water was used to elute DNA from Spin Filter membranes, and 30µL of DNA were transferred to a 96-well PCR plate.

PCR libraries were generated using the S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') forward and S-D-Bact-0785-a-A-21 (5'-

GACTACHVGGGTATCTAATCC-3') reverse primer pair (Klindworth et al., 2013) for which we added a modification following previous studies (Wang et al., 2016; Kieran et al., 2017). On the 5' end, we added Illumina TruSeq Read 1 to the forward and Illumina TruSeq Read 2 to the reverse primer. We synthesized 8 forward and 12 reverse fusion primers, each with a unique variable length (5-8bp) index sequence between the 16S and TruSeq sequences, which are called iTru-16S fusions.

DNA from each sample was amplified using two rounds of PCR. The first PCR used the iTru-16S fusions primers in 12.5-µl reactions using the KAPA HiFi Hotstart PCR kit (KAPA Biosystems, Wilmington, MA) using 2.5µL of 5x Buffer, 0.375µL of 10mM dNTPs, 0.25µL HotStart, 3.4µL molecular grade water, 1µL of 5µM forward primer, 1µL 5µM reverse primer, and 4µL of DNA. Thermocycler conditions were as follows: 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final elongation step of 72°C for 5 min. PCR was performed using a T100 Thermal Cycler (BioRad, Hercules, CA) and amplicons were visualized using 1.5% gel electrophoresis. We pooled 3µL of each PCR product and purified with SPRI-beads (Thermo-Scientific, Waltham, MA) using a 0.92:1 ratio of beads to product pool.

The second-round PCR primers consisted of Illumina TruSeqHT compatible 8 nucleotide indexed primers, iTru primers (Glenn et al., 2016). We used 25 µL reaction of KAPA HiFi HotStart Kits using 5µL of 5x Buffer, 0.75µL of 10mM dNTPs, 0.5µL HotStart, 3.75µL molecular grade water, 2.5µL of 5µM forward primer, 2.5µL 5µM reverse primer, and 10µL of purified iTru-16S amplicon pool. The following thermocycler conditions were used: 98°C for 2 min, followed by 10 cycles at 98°C for 30s, 60°C for 30s, 72°C for 30s and a final extension at 72°C for 5 min. Library product

was purified and primers were removed with SPRI-beads (1:1 ratio) and pooled with other uniquely indexed samples prior to sequencing.

***16S rRNA Gene Sequencing*.** All libraries were sent to the Georgia Genomics Facility (http://dna.uga.edu) for sequencing on an Illumina MiSeq using a v3 600 cycle kit (Illumina, San Diego, CA).

***Sequence Read Analysis*.** Sequencing data were demultiplexed according to outer iTru indexes using bcl2fastq (Illumina, v1.8.4) to identify the sample pool. The iTru-16S amplicon pool was demultiplexed by internal barcodes to identify individual samples and primers removed using Mr. Demuxy v1.2.0 (https://pypi.python.org/pypi/Mr_Demuxy/1.2.0). Paired-end sequencing reads were imported into Geneious v10.0.9 (Biomatters Limited, NJ), set as paired-reads with an expected insert size of 400 bp, and trimmed to remove low quality bases using default settings and a quality score of 0.001. Paired-end sequencing reads were then merged using the FLASH v1.2.9 plugin (Magoc and Salzberg, 2011). Data were exported from Geneious as FASTA files for further analysis using software package QIIME v1.9.1 (Caporaso et al., 2010).

Statistical Analysis. Statistical analyses were performed using QIIME scripts (QIIME pipeline v1.9.1; Caporaso et al., 2010) and the software R (R Foundation for Statistical Computing, Vienna, Austria). Results were considered significant at $P \leq 0.05$, and were treated as trends when their associated *P*-values were between 0.05 and 0.10.

Alpha diversity indexes were computed using QIIME's "alpha_rarefaction.py" script, which generates rarefied OTU tables and computes measures of alpha diversity for

each rarefied OTU table. Comparisons were performed between treatment groups using a two-sample nonparametric t-test. The computed indexes were: Shannon diversity index, Simpson's diversity index, Chao1, Strong's dominance index, Phylogenetic diversity whole tree, and Observed OTUs.

Beta diversity between all pairs of samples was calculated using QIIME's "beta_diversity_through_plots.py" script. This script rarefies OTU tables to remove sampling depth heterogeneity, generates a beta diversity matrix with the dissimilarity (or "distance") between every pair of samples, and runs Principal Coordinates Analysis (PCoA, including the generation of plots). The differences in beta diversity between groups were accessed by permutational multivariate analysis of variance (PERMANOVA). Initially, the metrics tested were Weighted and Unweighted UniFrac distances, Bray-Curtis, Morisita-Horn, and Euclidean distances. However, because the UniFrac metrics use phylogenetic information (Lozupone and Knight, 2005), they yield results that can be more useful (Hamady and Knight, 2009). Therefore, a two-way PERMANOVA using Akutils' script (Krohn, 2016) was performed using the unweighted UniFrac distance matrix, with treatment and day of sampling as factors.

Comparison of OTU frequencies across sample groups was done in 2 different ways: through a two-way ANOVA where treatment and collection day were the factors; or using the "group_significance.py" script from QIIME, which was carried out separately for each collection day. Briefly, the "group_significance.py" script constructs a OTU x Sample contingency table and tests whether each OTU is differentially represented in the groups. The script yields 3 distinct *P*-values: one generated by the Kruskal-Wallis test, another generated by the Benjamini-Hochberg False Discovery Rate

procedure, and a third one originated by the Bonferroni correction procedure for multiple comparisons. In the present study, although the 3 *P*-values are listed, significance was determined based on the *P*-values from the Kruskal-Wallis procedure. The nature of this test (nonparametric ANOVA) allows the comparison of whether there are significant differences between groups, even when the data are not normally distributed.

RESULTS AND DISCUSSION

As previously mentioned, each of the 25 calves used in this study was sampled twice – at weaning, and 4 weeks after. Thus, after quality control and chimera removal, the samples obtained on weaning day yielded a total of 306,414 cleaned reads, resulting in an average of 12,256.56 reads per sample. The total cleaned reads represented individual samples ranging from 6,040 to 17,627 reads. On the samples collected 4 weeks after weaning, sequencing yielded a total of 224,445 cleaned reads with an average of 8,977.80 reads per sample. The total cleaned reads represented individual samples ranging from 3,484 to 13,683 reads. Comparison of the samples collected before and after weaning revealed that the number of reads per sample was greater ($P = 0.001$; Table 5.1) for samples collected on the day calves were weaned.

Alpha and Beta Diversities. Two distinct sampling depths were used in the analyses: 6,040 and 3,484 sequences per sample. The former value was used in the analysis of the samples collected on weaning day, whereas the latter for the samples collected 4 weeks after weaning. As can be seen in Table 5.2, for the samples obtained on weaning day, there was a distinction between non-supplemented and supplemented

calves. Overall, non-supplemented calves (i.e. NO FEED group) had greater richness and diversity. For instance, phylogenetic diversity of whole tree was greater ($P \leq 0.02$) for calves in NO FEED, compared to both PLAIN FEED and ENZYME FEED. Moreover, except for Chao 1, all the other alpha diversity indexes were different when NO FEED was compared to PLAIN FEED ($P \leq 0.03$). The number of observed OTUs also differed between these 2 groups ($P = 0.01$) with animals in NO FEED having greater OTU counts than animals in PLAIN FEED. Interestingly, no differences in any of the alpha diversity indexes were detected when comparing PLAIN FEED to ENZYME FEED ($P = 0.99$). Such similarities between these 2 groups suggest that the inclusion of xylanase in one of the supplements offered to suckling calves did not generate significant diversity within samples. Results summarized in Table 5.3 show no dissimilarities across treatments for richness and diversity in samples collected 4 weeks after weaning, as none of the alpha diversity indexes or the number of observed OTUs were significantly altered by treatments ($P \geq 0.23$). These findings show that although some dissimilarities were present in the rumen of the calves at weaning, placing them in the same feed regimen for a period of 4 weeks was enough to equalize such initial dissimilarities in alpha diversity.

Regarding beta diversity, results from the two-way PERMANOVA performed using the unweighted UniFrac distance matrix revealed that collection day had a significant effect ($P < 0.001$) on this trait whereas only a trend ($P = 0.09$) was detected for treatment. In addition, a treatment x collection day interaction was observed ($P = 0.04$). The graphical results from the principal coordinate analysis of beta diversity using the unweighted UniFrac distance matrix are shown in Figures 5.1 thru 5.4.

Bacterial Abundance. Regardless of treatment group or collection day, the most abundant phylum observed in the rumen fluid samples was *Bacteroidetes*, with a relative abundance ranging from 47.1 to 52.3%. The second most abundant phylum was *Firmicutes*, with relative abundances varying from 26.8 to 32.2%. These results are in alignment with the findings of McCann et al. (2014), Rosewarne et al., (2014), and Myer et al. (2015), which reported a predominance of *Bacteroidetes* in their samples. In contrast, part of the results reported by Brulc et al. (2009) and McCann et al. (2016) reveals the exact opposite – a predominance of *Firmicutes* over *Bacteroidetes*. However, both studies were performed analyzing the liquid and the solid fractions of the collected ruminal contents, whereas, in the present study, only the liquid fraction of the ruminal contents was analyzed. In fact, the results reported by McCann et al. (2016) concerning the liquid fraction of their samples are very similar to our findings regarding the 2 predominant phyla. Their divergent results were the ones dealing with the solid fractions. Therefore, there is evidence to suggest that the portion of the rumen digesta (liquid or solid) that is utilized in microbiome analysis plays a significant role in the bacterial abundances detected.

Individual tables were constructed for bacterial relative abundances for both collection days, at several taxonomic levels (i.e. phylum, class, order, family, and genus), however, results are presented only for phylum and genus (Tables 5.4 – 5.7). For information on the other taxonomic levels, refer to supplemental tables 5.13S to 5.18S. Table 5.4 shows the effect of treatments on bacterial abundance at the phylum level for samples collected on calves' weaning day. As previously explained, although 3 distinct *P*-values are shown, results were considered significant based on the probabilities

resulting from the Kruskal-Wallis test. In light of this, the phyla *Bacteroidetes*, *Lentisphaerae*, *Cyanobacteria*, SR1, and *Euryarchaeota* were significantly affected ($P \leq 0.05$) by the different treatments. However, 4 weeks after weaning (Table 5.5) differences were observed ($P = 0.02$) only for the phylum TM7. Likewise, Tables 5.6 and 5.7 show that at the genus level, the effect of treatment ($P \leq 0.05$) was observed for 8 genera immediately before calves were weaned: *Prevotella*, an unidentified genus from the family *Paraprevotellaceae*, an unidentified genus from the order YS2, an unidentified genus from the family *Victivallaceae*, vadinCA11, BF311, an unidentified genus from the family S24-7, and *Clostridium*. Whereas, in samples collected 4 weeks after weaning, differences were detected ($P = 0.02$) only for 2 genera: YRC22, and an unidentified genus of the family F16. These findings demonstrate that most of the differences caused by the treatments during the feeding trial were evened up after animals were weaned and stayed in a common diet for 4 weeks.

A two-way ANOVA using treatment and collection day as factors was performed on all phyla with relative abundances greater than 2%, and results are shown in Table 5.8. A treatment effect ($P \leq 0.02$) was observed for the phyla *Bacteroidetes*, TM7, and *Spirochaetes*. An effect of collection day ($P < 0.01$) was detected for *Bacteroidetes*, *Firmicutes*, *Tenericutes*, and TM7. Moreover, a treatment x day interaction ($P = 0.01$) was observed for the phylum TM7. Comparison of the samples collected after weaning with the ones obtained on weaning day revealed that this phylum was increased by 54% in NO FEED, and 79% in ENZYME FEED, however, its relative abundance went up 167% for PLAIN FEED in the same period. As a consequence, PLAIN FEED became the

group with the greatest abundance of TM7 (4.08%) 4 weeks after the calves were weaned.

At the genus level, two-way ANOVA was performed only in genera that were identified on the 2 collection days, and that had relative abundances greater than 0.5%. Additionally, all unidentified genera were removed from the pre- versus post-weaning comparisons. Of the genera meeting these criteria, *Prevotella* was the genus with greatest relative abundance (ranging from 15.38% to 22.15%; Table 5.9) and NO FEED had the lowest ($P < 0.01$) abundance of this genus both before and after weaning. Besides *Prevotella*, a treatment effect ($P \leq 0.03$) was observed for *Treponema* and *Methanobrevibacter*, with *Treponema* having a greater presence in NO FEED than in the other groups. A day effect ($P \leq 0.02$) was observed for the genera *Prevotella*, *Ruminococcus*, *Anaeroplasma*, *Succiniclasticum*, *Butyrivibrio*, *Coprococcus*, and *Clostridium*. A treatment x day interaction ($P = 0.01$) was observed for only one genus: YRC22. While abundance of this genus was numerically lower in ENZYME FEED at weaning day, it was the greatest among all treatment groups 4 weeks later.

Several studies have linked *Prevotella* abundance to animal performance. Carberry et al. (2012) studied the effect of residual feed intake (**RFI**) and forage content in the diet on the rumen microbial community of beef cattle. They found greater relative abundance of *Prevotella* in high-RFI (i.e. less efficient) animals compared to low-RFI (more efficient). In addition, an effect of diet was reported: authors observed lower abundance of *Prevotella* in the rumen fluid of animals receiving a high-forage diet. This finding is in alignment with our results since the NO FEED group, which received only forage as solid feed, had the lowest relative abundance of *Prevotella*. Likewise, Bekele et

al. (2010) fed 2 distinct diets to fistulated sheep – one composed of 91% of forage, and another containing only 33% of forage. Their results showed that animals receiving the diet with higher percentage of forage had lower percentage of *Prevotella* in their rumen fluid. In contrast, a recent study by Kljak et al. (2017) found that heifers consuming greater amount of starch had decreased relative abundance of *Prevotella*. Moreover, the authors found that this decrease occurred in a linear manner as the level of starch was increased from 3.5 to 31.7% of the diet DM.

Significance of the Observed Bacterial Abundances. Tables 5.4 and 5.6 show the relative abundances of bacteria at the phylum and genus level for samples collected on the day calves were weaned. At that time, animals had been treated differently for 14 weeks, therefore, it is reasonable to assume that any effect of treatments would have been more detectable in samples collected on that day. Still, although some of the shifts in bacterial abundance were statistically significant, none of them was strikingly different, especially the ones observed in the bacterial groups with greater abundance, which implies that the observed shifts may not have great biological significance. As a comparison, Myer et al. (2015) reported microbial shifts of up to 10-fold at the genus level, whereas, in the present study, most the shifts were less than 2-fold. The lower magnitudes observed in our study may be due to the amount of supplement consumed by calves, which was estimated to be around 20% of their daily dry matter intake. This relatively low quantity of extra feed may have not been enough to produce severe modifications in their rumen microbial populations.

Another aspect that deserves consideration in microbiome studies is the statistical method. Depending on which statistical procedure is used, the observed results may not

be significant at a $P \leq 0.05$ level. This effect is illustrated by an examination of the 3 *P*-values reported in the comparisons of I frequencies across treatments. As mentioned before, the first *P*-value was generated by the Kruskal-Wallis procedure, the second by the Benjamini-Hochberg procedure, and the third by the Bonferroni correction procedure. These *P*-values are listed in order from the least conservative (Kruskal-Wallis) to the most conservative (Bonferroni). As it can be noticed at the phylum level (Table 5.4), differences in abundance of *Lentisphaerae* across treatment groups were significant ($P = 0.01$) in the Kruskal-Wallis test, however, they would be classified as just a trend ($P = 0.09$) using the Benjamini-Hochberg procedure, and would not be considered significant ($P = 0.19$) when compared using the Bonferroni procedure. Indeed, some authors have concluded that the Bonferroni procedure is too conservative if the number of tests is large (Perneger, 1998; Bender and Lange, 1999) which is usually the case of microbiome analyses, so it does not seem to be the most appropriate method for this type of study. On the other hand, the Kruskal-Wallis test is considered less conservative and is the default method for comparing I frequencies across groups in the "group_significance.py" script from the software QIIME. Additionally, the Kruskall-Wallis test contrasts the treatment medians and not the means. Consequently, its results are less affected by outliers. For those reasons, the use of *P*-values originated from the Kruskal-Wallis procedure seems to be adequate in determining significances in microbiome analysis, however, as discussed in the next section, statistical significance does not necessarily mean biological significance. Thus, even when the proper statistical procedure is utilized, caution must be exercised when interpreting the outcomes.

Rumen Microbiome and Calf Growth. Results from the 14-week feeding trial showed that calves in the different treatment groups had different performances. For instance, their average daily gains were 1.06, 1.10, and 1.21 kg per day for NO FEED, PLAIN FEED, and ENZYME FEED, respectively. However, given the extent of the differences detected in their rumen microbiomes, attributing those differences observed in their growth rates exclusively to rumen microbiome alterations may be misleading. Instead, those variations in growth were likely due to a combination of factors, in which, in spite of the participation of the ruminal bacterial community, other factors such as the amount of energy consumed by calves was probably more important. While calves in NO FEED had to rely on the milk from their mothers and on grass as their main feed sources, calves in PLAIN FEED and ENZYME FEED received an extra load of calories through their creep feed supplements. Moreover, bromatological analysis showed that the creep feed supplement used in the trial had more digestible energy than the grass present in the experimental paddocks: 3.04 versus 2.47 Mcal of DE per kg of dry matter. So, presumably, the increased energy intake experienced by the supplemented calves was probably a major factor affecting the observed differences in calf growth. Furthermore, in the case of ENZYME FEED, the presence of xylanase – which increases energy availability in feeds (Choct et al., 1999; Beauchemin et al., 2003) was probably of great significance as well.

In conclusion, although some shifts were observed in the rumen microbiome of calves due to creep feeding supplementation (e.g. increased relative abundance of *Prevotella* at the genus level), these microbial fluctuations were not of great magnitude. Therefore, factors such as an increased amount of energy intake due to supplementation,

and increased metabolizable energy due to addition of xylanase, were probably more important than any shifts observed in the microbial community. Consequently, in the present study, we presume that these factors were more influential than the shifts identified in the microbial community, and for that reason, we infer that they had a greater contribution on the observed differences in calf growth. Additional research is needed to establish a reliable relationship between suckling beef calf's diet and their rumen microbiome.

LITERATURE CITED

Beauchemin, K. A., D. Colombatto, D. P. Morgavi, and W. Z. Yang. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. J. Anim. Sci. 81:E37–E47. Doi: 10.2527/2003.8114_suppl_2E37x.

Bekele, A. Z., S. Koike, and Y. Kobayashi. 2010. Genetic diversity and diet specificity of ruminal *Prevotella* revealed by 16S rRNA gene-based analysis. FEMS Microbiol. Lett. 305:49–57. Doi: 10.1111/j.1574-6968.2010.01911.x.

Bender, R., and S. Lange. 1999. Multiple test procedures other than Bonferroni's deserve wider use. BMJ 318:600–601.

Brulc, J. M., D. A. Antonopoulos, M. E. B. Miller, M. K. Wilson, A. C. Yannarell, E. A. Dinsdale, R. E. Edwards, E. D. Frank, J. B. Emerson, P. Wacklin, P. M. Coutinho, B. Henrissat, K. E. Nelson, and B. A. White. 2009. Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside

hydrolases. Proc. Natl. Acad. Sci. U.S.A. 106:1948–1953. Doi: 10.1073/pnas.0806191105.

Carberry, C. A., D. A. Kenny, S. Han, M. S. McCabe, and S. M. Waters. 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. Appl. Environ. Microbiol. 78:4949–4958. Doi: 10.1128/AEM.07759-11.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335–336. Doi: 10.1038/nmeth.f.303.

Choct, M., R. J. Hughes, and M. R. Bedford. 1999. Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. Br. Poult. Sci. 40:419–422. Doi: 10.1080/00071669987548.

Church, D. C. 1979. Growth and development of the ruminant stomach. Digestive physiology and nutrition of ruminants. Volume 1: Digestive Physiology. 2nd ed. Oxford Press Inc., Portland, OR.

Glenn, T. C., R. Nilsen, T. J. Kieran, J. W. Finger, T. W. Pierson, K. E. Bentley, S. Hoffberg, S. Louha, F. J. Garcia-De-Leon, M. A. Portilla, K. Reed, J. L.

Anderson, J. K. Meece, S. Aggery, R. Rekaya, M. Alabady, M. Belanger, K. Winker, and B. C. Faircloth. 2016. Adapterama I: Universal stubs and primers for thousands of dual-indexed Illumina libraries (iTru & iNext). BioRxivorg. Doi: 10.1101/049114.

Hamady, M., and R. Knight. 2009. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. Genome Res. 19:1141–1152. Doi: 10.1101/gr.085464.108.

Henderson, G., F. Cox, S. Ganesh, A. Jonker, W. Young, Global Rumen Census Collaborators, and P. H. Janssen. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci. Rep. 5:14567. Doi: 10.1038/srep14567.

Hernandez-Sanabria, E., L. A. Goonewardene, Z. Wang, O. N. Durunna, S. S. Moore, and L. L. Guan. 2012. Impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle. Appl. Environ. Microbiol. 78:1203–1214. Doi: 10.1128/AEM.05114-11.

Kieran, T. J., N. L. Gottdenker, C. P. Varian, A. Saldaña, N. Means, D. Owens, J. E. Calzada, T. C. Glenn. 2017. Bloodmeal Source Characterization Using Illumina Sequencing in the Chagas Disease Vector Rhodnius pallescens (Hemiptera: Reduviidae) in Panamá. J. Med. Entomol.

Klindworth, A., E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, F. O. Glockner. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical

and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41:e1. Doi: 10.1093/nar/gks808.

Kljak, K., F. Pino, K. J. Harvatine, and A. J. Heinrichs. 2017. Analysis of selected rumen microbial populations in dairy heifers limit fed diets varying in trace mineral form and starch content. Livest. Sci. 198:93–96. Doi: 10.1016/j.livsci.2017.02.012.

Krohn, A. 2016. Akutils-v1.2: Facilitating analyses of microbial communities through QIIME. Zenodo. doi: 10.5281/zenodo.61581.

Lourenco, J. M. 2017. Utilization of Exogenous Feed Enzymes as a Way to Enhance Creep Feeds – In Vitro, In Vivo, and Ruminal Microbiome Evaluations. PhD Diss. Univ. of Georgia, Athens.

Lozupone, C., and R. Knight. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71:8228–8235. Doi: 10.1128/AEM.71.12.8228-8235.2005.

Magoc, T., and S. L. Salzberg. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27:2957–2963. Doi: 10.1093/bioinformatics/btr507.

McCann, J. C., L. M. Wiley, T. D. Forbes, F. M. Rouquette Jr., and L. O. Tedeschi. 2014. Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures. PloS One. 9:e91864. Doi: 10.1371/journal.pone.0091864.

McCann, J. C., S. Luan, F. C. Cardoso, H. Derakhshani, E. Khafipour, and J. J. Loor. 2016. Induction of subacute ruminal acidosis affects the ruminal microbiome and epithelium. Front. Microbiol. 7:701. Doi: 10.3389/fmicb.2016.00701.

Myer, P. R., T. P. L. Smith, J. E. Wells, L. A. Kuehn, and H. C. Freetly. 2015. Rumen microbiome from steers differing in feed efficiency. PloS One. 10:e0129174. Doi: 10.1371/journal.pone.0129174.

Perneger, T. V. 1998. What's wrong with Bonferroni adjustments. BMJ 316:1236–1238.

Petri, R. M., T. Schwaiger, G. B. Penner, K. A. Beauchemin, R. J. Forster, J. J. McKinnon, and T. A. McAllister. 2013. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. PloS One 8:e83424. Doi: 10.1371/journal.pone.0083424.

Rosewarne, C. P., P. B. Pope, J. L. Cheung, and M. Morrison. 2014. Analysis of the bovine rumen microbiome reveals a diversity of Sus-like polysaccharide utilization loci from the bacterial phylum *Bacteroidetes*. J. Ind. Microbiol. Biotechnol. 41:601–606. Doi: 10.1007/s10295-013-1395-y.

Wang, J., L. Tang, T. C. Glenn, J. S. Wang. 2016. Alfatoxin B1 induced compositional changes in gut microbial communities of male F344 Rats. Toxicol. Sci. 150:54–63. Doi: 10.1093/toxsci/kfv259.

Yáñez-Ruiz, D. R., L. Abecia, and C. J. Newbold. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. Front. Microbiol. 6:1133. Doi: 10.3389/fmicb.2015.01133.

Table 5.1. Number of cleaned reads for samples collected from calves on weaning day or 4 weeks later.

Item	On Weaning Day	4 Weeks After Weaning	*P*-value
Total reads	306,414	224,445	N/A
Sample with lowest reads	6,040	3,484	N/A
Sample with highest reads	17,627	13,683	N/A
Average reads per sample	12,256.56	8,977.80	0.001

Table 5.2. Effect of treatment on I richness and alpha diversity at 97% similarity after rarefaction to 6,040 sequences per sample for samples collected on weaning day.

				Contrasts[2]					
	Treatment[1]			NF vs. PF		NF vs. EF		PF vs. EF	
Item	**No feed**	**Plain feed**	**Enzyme feed**	**SEM***	***P*-value**	**SEM***	***P*-value**	**SEM***	***P*-value**
Shannon index	10.37	10.15	10.17	0.01	0.01	0.02	0.13	0.02	0.99
Simpson index	0.9981	0.9976	0.9976	0.00	0.03	0.00	0.26	0.00	0.99
Chao1	7,082.1	6,729.2	6,626.2	31.2	0.10	39.7	0.11	40.4	0.99
Strong's dominance index	0.411	0.434	0.431	0.001	0.01	0.002	0.14	0.002	0.99
PD whole tree[3]	159.0	151.8	150.1	0.45	0.01	0.51	0.02	0.63	0.99
Observed OTUs	2,571.6	2,421.5	2,424.2	8.1	0.01	11.6	0.11	13.1	0.99

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Contrasts: NF vs. PF = No feed versus Plain feed; NF vs. EF = No feed versus Enzyme feed; PF vs. EF = Plain feed versus Enzyme feed.

[3] PD whole tree = Phylogenetic diversity of whole tree.

* Pooled SE of main-effect means; n = 8 for No feed, n = 9 for Plain feed, and n = 8 for Enzyme feed.

Table 5.3. Effect of treatment on I richness and alpha diversity at 97% similarity after rarefaction to 3,484 sequences per sample for samples collected 4 weeks after weaning.

				Contrasts[2]					
	Treatment[1]			NF vs. PF		NF vs. EF		PF vs. EF	
Item	**No feed**	**Plain feed**	**Enzyme feed**	**SEM***	***P*-value**	**SEM***	***P*-value**	**SEM***	***P*-value**
Shannon index	9.80	9.84	9.74	0.02	0.99	0.02	0.99	0.02	0.71
Simpson index	0.9973	0.9974	0.9972	0.000	0.99	0.000	0.99	0.000	0.99
Chao1	4,316.1	4,238.6	4,108.4	36.2	0.99	23.2	0.30	32.4	0.99
Strong's dominance index	0.383	0.376	0.390	0.002	0.99	0.003	0.99	0.002	0.59
PD whole tree[3]	104.3	103.5	100.7	0.49	0.99	0.40	0.23	0.40	0.54
Observed OTUs	1,592.4	1,609.1	1,555.5	9.9	0.99	9.8	0.99	8.6	0.62

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Contrasts: NF vs. PF = No feed versus Plain feed; NF vs. EF = No feed versus Enzyme feed; PF vs. EF = Plain feed versus Enzyme feed.

[3] PD whole tree = Phylogenetic diversity of whole tree.

* Pooled SE of main-effect means; n = 8 for No feed, n = 9 for Plain feed, and n = 8 for Enzyme feed.

Table 5.4. Effect of treatment on relative abundance of bacteria phyla for samples collected on weaning day.

	Treatment[1]			Test *P*-value[2]		
Phyla	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Bacteroidetes[##]	48.8	51.7	52.3	0.01	0.09	0.28
Firmicutes	26.8	28.4	27.8	0.25	0.37	1.00
Spirochaetes	2.7	2.0	1.7	0.16	0.31	1.00
Tenericutes	2.4	2.0	2.6	0.11	0.23	1.00
Verrucomicrobia	2.2	2.1	2.0	0.97	0.97	1.00
TM7	1.8	1.5	1.4	0.07	0.20	1.00
Proteobacteria	1.8	1.7	1.5	0.22	0.35	1.00
Lentisphaerae[##]	1.8	1.2	0.7	0.01	0.09	0.19
Cyanobacteria[##]	1.7	0.9	0.5	0.02	0.10	0.41
SR1[##]	1.4	0.8	1.0	0.02	0.11	0.56
Euryarchaeota[##]	1.3	1.1	1.9	0.05	0.17	1.00
Other phyla	1.5	1.4	1.2	≥ 0.01	≥ 0.09	≥ 0.13
Unassigned OTUs[##]	5.8	5.1	5.2	0.04	0.17	1.00

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

Table 5.5. Effect of treatment on relative abundance of bacteria phyla for samples collected 4 weeks after weaning.

	Treatment[1]			Test *P*-value[2]		
Phyla	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Bacteroidetes	47.1	47.3	50.3	0.12	0.53	1.00
Firmicutes	31.0	32.2	30.2	0.47	0.85	1.00
Tenericutes	4.0	3.3	3.8	0.44	0.85	1.00
TM7[##]	2.8	4.1	2.5	0.02	0.28	0.56
Spirochaetes	2.1	1.5	1.7	0.16	0.53	1.00
Verrucomicrobia	2.1	1.6	1.4	0.23	0.67	1.00
Proteobacteria	1.8	1.5	1.6	0.61	0.85	1.00
Euryarchaeota	1.1	1.0	1.2	0.57	0.85	1.00
SR1	0.9	1.1	1.3	0.65	0.85	1.00
Cyanobacteria	0.6	0.6	0.6	0.94	0.98	1.00
Fibrobacteres	0.6	0.5	0.4	0.14	0.53	1.00
Lentisphaerae	0.6	0.5	0.7	0.67	0.85	1.00
Other phyla	0.9	1.0	1.0	≥ 0.05	≥ 0.4	≥ 0.99
Unassigned OTUs[##]	4.4	3.8	3.6	0.01	0.17	0.17

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

Table 5.6. Effect of treatment on relative abundance of bacteria genera for samples collected on weaning day.

	Treatment[1]			Test *P*-value[2]		
Genera	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Unidentified genus 1[A]	17.4	17.6	16.8	0.94	1.00	1.00
Prevotella[##]	15.4	18.1	20.1	0.01	0.28	1.00
Unidentified genus 2[B]	7.7	8.0	8.2	0.26	0.65	1.00
Unidentified genus 3[C]	6.8	6.8	6.2	0.28	0.67	1.00
Unidentified genus 4[D]	5.3	4.3	4.8	0.49	0.76	1.00
Unidentified genus 5[E]	3.1	3.3	3.2	0.94	1.00	1.00
CF231	3.0	3.3	2.7	0.09	0.43	1.00
RFN20	2.7	2.3	2.5	0.78	0.90	1.00
Treponema	2.4	1.8	1.6	0.16	0.52	1.00
Unidentified genus 6[F]	2.4	3.0	2.9	0.06	0.36	1.00
Unidentified genus 7[G]	1.9	1.7	1.7	0.92	0.99	1.00
Unidentified genus 8[H]	1.8	1.5	1.4	0.07	0.40	1.00
Unidentified genus 9[I ##]	1.7	1.6	1.1	0.02	0.28	1.00
Unidentified genus 10[J ##]	1.7	0.9	0.5	0.02	0.28	1.00
Unidentified genus 11[K ##]	1.6	1.1	0.7	0.01	0.27	1.00
YRC22	1.3	1.6	1.2	0.21	0.56	1.00
Pseudobutyrivibrio	1.1	1.2	1.1	0.75	0.88	1.00
Ruminococcus	1.1	1.1	0.9	0.39	0.70	1.00
Unidentified genus 12[L]	0.9	0.6	0.9	0.07	0.40	1.00
Anaeroplasma	0.8	0.8	1.0	0.68	0.85	1.00
vadinCA11[##]	0.7	0.4	0.5	0.05	0.36	1.00
BF311[##]	0.7	0.6	0.4	0.02	0.28	1.00

Unidentified genus 13[M]	0.7	0.8	0.9	0.34	0.70	1.00
Succiniclasticum	0.6	0.9	0.9	0.26	0.65	1.00
Unidentified genus 14[N]	0.6	0.4	0.6	0.39	0.70	1.00
Methanobrevibacter	0.5	0.6	1.3	0.16	0.52	1.00
Unidentified genus 15[O ##]	0.5	0.7	1.2	0.02	0.28	1.00
Butyrivibrio	0.5	0.7	0.6	0.18	0.53	1.00
Coprococcus	0.4	0.5	0.5	0.37	0.70	1.00
Unidentified genus 16[P]	0.4	0.4	0.5	0.66	0.83	1.00
Clostridium[##]	0.4	0.5	0.3	0.002	0.22	0.34
Other genera	6.7	6.7	6.3	≥ 0.004	≥ 0.22	≥ 0.81
Unassigned OTUs[##]	7.2	5.9	6.2	0.04	0.35	1.00

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

[A]Order *Bacteroidales*; [B]Family *Ruminococcaceae;* [C]Order *Clostridiales;* [D]Family BS11; [E]Family RF16; [F]Family *Lachnospiraceae;* [G]Family RFP12; [H]Family F16; [I]Family *Paraprevotellaceae*; [J]Order YS2; [K]Family *Victivallaceae*; [L]Order RF39; [M]Family *Mogibacteriaceae*; [N]Order *Rickettsiales*; [O]Family S24-7; [P]Family *Veillonellaceae.*

Table 5.7. Effect of treatment on relative abundance of bacteria genera for samples collected 4 weeks after weaning.

	Treatment[1]			Test *P*-value[2]		
Genera	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Prevotella	18.3	22.1	21.6	0.11	0.74	1.00
Unidentified genus 1[A]	15.3	13.1	14.8	0.11	0.74	1.00
Unidentified genus 2[B]	8.8	9.1	7.9	0.08	0.74	1.00
Unidentified genus 3[C]	7.5	8.2	8.2	0.60	0.81	1.00
Unidentified genus 4[D]	4.1	3.0	3.3	0.10	0.74	1.00
RFN20	3.1	2.2	2.3	0.45	0.81	1.00
Unidentified genus 5[E]	3.0	3.1	2.6	0.16	0.74	1.00
CF231	2.9	2.4	2.9	0.49	0.81	1.00
Unidentified genus 6[F ##]	2.8	4.1	2.5	0.02	0.70	1.00
Unidentified genus 7[G]	2.6	2.4	3.2	0.47	0.81	1.00
Anaeroplasma	2.2	1.5	1.8	0.22	0.81	1.00
Treponema	2.0	1.5	1.6	0.19	0.79	1.00
Unidentified genus 8[H]	1.8	1.2	1.0	0.18	0.79	1.00
Ruminococcus	1.6	1.7	1.3	0.17	0.74	1.00
YRC22[##]	1.1	1.3	1.8	0.02	0.69	1.00
Succiniclasticum	1.1	1.3	1.2	0.22	0.81	1.00
Unidentified genus 9[I]	1.1	1.0	0.8	0.23	0.81	1.00
Pseudobutyrivibrio	1.1	1.1	1.5	0.47	0.81	1.00
Unidentified genus 10[J]	1.0	1.2	1.3	0.43	0.81	1.00
Unidentified genus 11[K]	0.9	1.1	1.3	0.65	0.82	1.00
Unidentified genus 12[L]	0.9	1.2	1.3	0.34	0.81	1.00
Butyrivibrio	0.8	0.8	0.7	0.38	0.81	1.00
Unidentified genus 13[M]	0.7	0.8	0.8	0.61	0.81	1.00

Coprococcus	0.7	0.8	0.7	0.56	0.81	1.00
Methanobrevibacter	0.7	0.7	0.8	0.53	0.81	1.00
Unidentified genus 14[N]	0.6	0.6	0.6	0.93	0.96	1.00
Fibrobacter	0.6	0.5	0.4	0.14	0.74	1.00
Unidentified genus 15[O]	0.4	0.5	0.5	0.40	0.81	1.00
Clostridium	0.4	0.5	0.5	0.36	0.81	1.00
Unidentified genus 16[P]	0.5	0.5	0.7	0.63	0.81	1.00
Other genera	6.9	6.8	7.0	≥ 0.01	≥ 0.66	≥ 0.99
Unassigned OTUs[##]	4.4	3.8	3.6	0.01	0.66	1.00

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

[A]Order *Bacteroidales*; [B]Order *Clostridiales*; [C]Family *Ruminococcaceae*; [D]Family RF16; [E]Family *Lachnospiraceae*; [F]Family F16; [G]Family BS11; [H]Family RFP12; [I]Family S24-7; [J]Family *Paraprevotellaceae*; [K]Phylum SR1; [L]Order RF39; [M]Family *Mogibacteriaceae*; [N]Order YS2; [O]Family *Veillonellaceae*; [P]Family *Victivallaceae*.

Table 5.8. Effect of treatment on relative abundance of the main phyla.[1]

	At weaning[2]			After weaning[3]			*P*-value[4]		
Phyla	**No feed**	**Plain feed**	**Enzyme feed**	**No feed**	**Plain feed**	**Enzyme feed**	**Treat-ment**	**Day**	**Treatment x Day**
Bacteroidetes	48.78	51.72	52.30	47.11	47.31	50.25	0.01	< 0.01	0.32
Firmicutes	26.80	28.40	27.83	31.03	32.18	30.18	0.34	< 0.01	0.67
Tenericutes	2.44	1.99	2.62	3.96	3.32	3.77	0.15	< 0.01	0.85
TM7	1.84	1.53	1.38	2.83	4.08	2.46	0.02	< 0.01	0.01
Spirochaetes	2.69	1.99	1.75	2.13	1.55	1.69	0.02	0.10	0.62
Verrucomicrobia	2.21	2.12	2.01	2.06	1.63	1.36	0.36	0.09	0.73

[1] Treatments were: No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase. Phyla with relative abundances greater than 2%.

[2] At weaning: ruminal samples were collected on weaning day.

[3] After weaning: ruminal samples were collected 4 weeks after weaning.

[4] *P*-values were calculated for the treatment effect, collection day (at weaning or 4 weeks after), and for the interaction between treatment and collection day.

Table 5.9. Effect of treatment on relative abundance of the main genera.[1]

	At weaning[2]			After weaning[3]			*P*-value[4]		
Genera	**No feed**	**Plain feed**	**Enzyme feed**	**No feed**	**Plain feed**	**Enzyme feed**	**Treatment**	**Day**	**Treatment x Day**
Prevotella	15.38	18.14	20.07	18.35	22.15	21.61	< 0.01	< 0.01	0.54
CF231	3.00	3.28	2.75	2.91	2.44	2.91	0.87	0.17	0.10
RFN20	2.66	2.34	2.51	3.08	2.22	2.27	0.36	0.96	0.74
Treponema	2.45	1.84	1.57	2.02	1.48	1.55	0.02	0.19	0.71
YRC22	1.31	1.60	1.24	1.07	1.32	1.76	0.11	0.91	0.01
Pseudobutyrivibrio	1.15	1.20	1.13	1.06	1.06	1.46	0.39	0.82	0.22
Ruminococcus	1.05	1.09	0.91	1.58	1.67	1.29	0.09	< 0.01	0.72
Anaeroplasma	0.80	0.79	1.03	2.25	1.47	1.79	0.27	< 0.01	0.26
Succiniclasticum	0.64	0.94	0.93	1.07	1.33	1.15	0.13	< 0.01	0.74
Methanobrevibacter	0.53	0.62	1.29	0.70	0.67	0.84	0.03	0.65	0.20
Butyrivibrio	0.48	0.68	0.63	0.79	0.78	0.69	0.42	0.02	0.21
Coprococcus	0.42	0.51	0.48	0.71	0.79	0.65	0.21	< 0.01	0.43
Clostridium	0.39	0.48	0.31	0.45	0.52	0.50	0.08	0.02	0.22

[1] Treatments were: No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase. Genera with relative abundances greater than 0.5% that were identified both at weaning and 4 weeks after weaning.

[2] At weaning: ruminal samples were collected on weaning day.

[3] After weaning: ruminal samples were collected 4 weeks after weaning.

[4] *P*-values were calculated for the treatment effect, collection day (at weaning or 4 weeks after), and for the interaction between treatment and collection day.

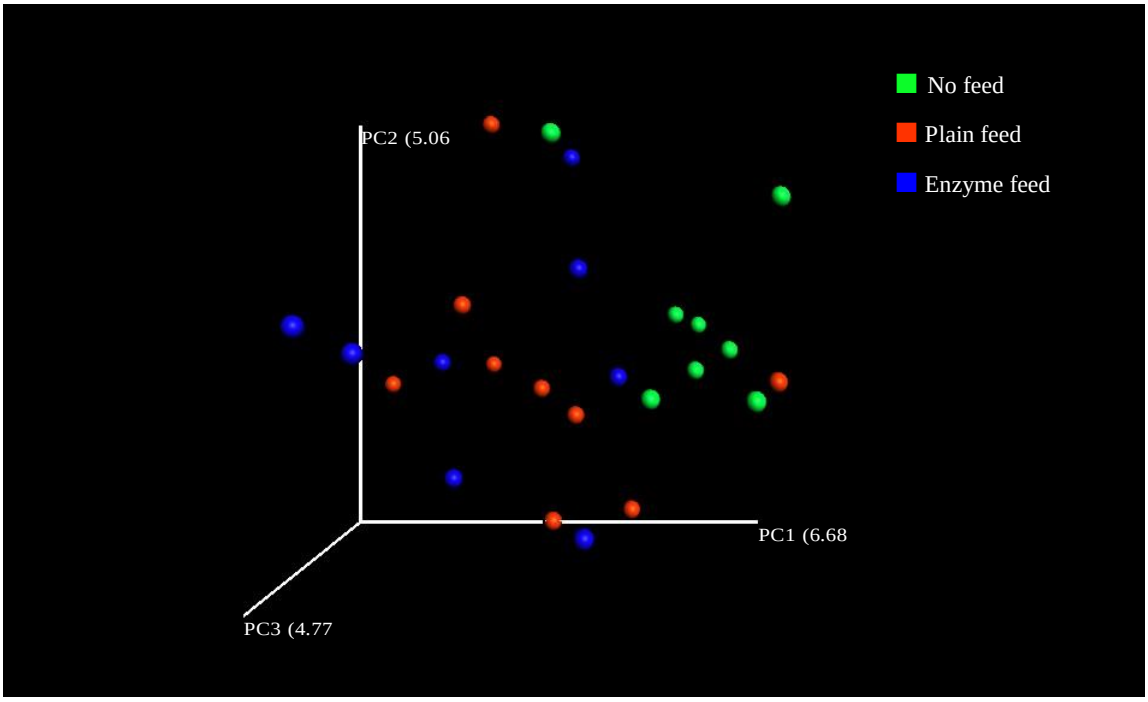

Figure 5.1. Principal coordinate analysis (PcoA) of beta diversity using the unweighted UniFrac distance matrix for samples collected on weaning day.

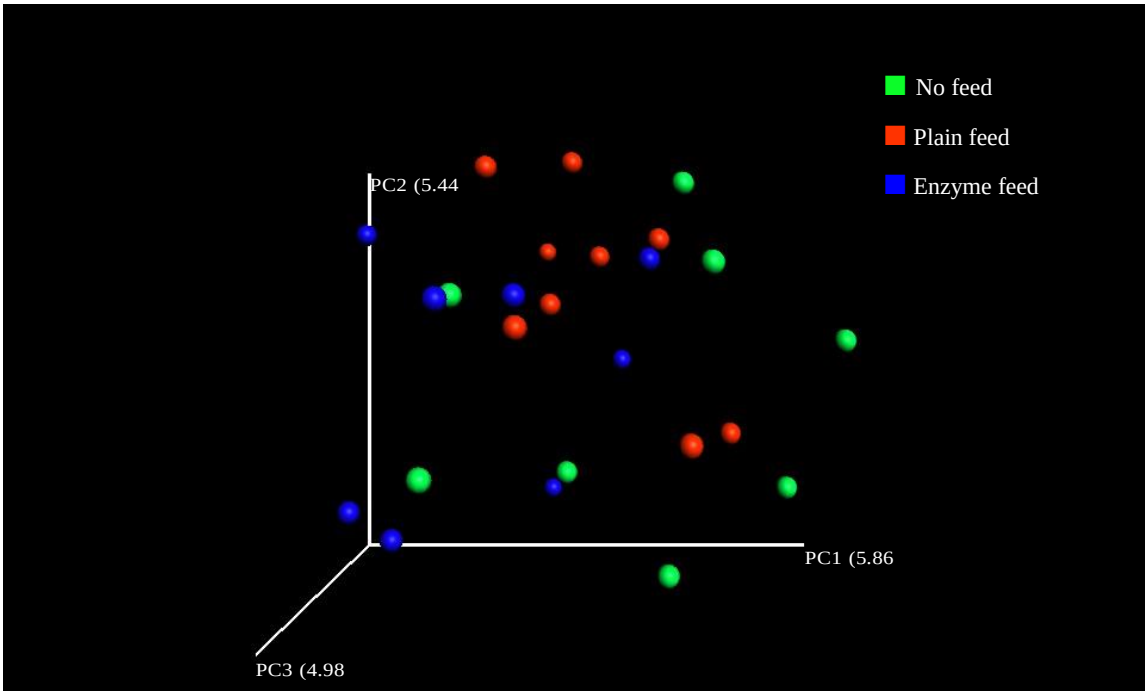

Figure 5.2. Principal coordinate analysis (PcoA) of beta diversity using the unweighted UniFrac distance matrix for samples collected on 4 weeks after weaning.

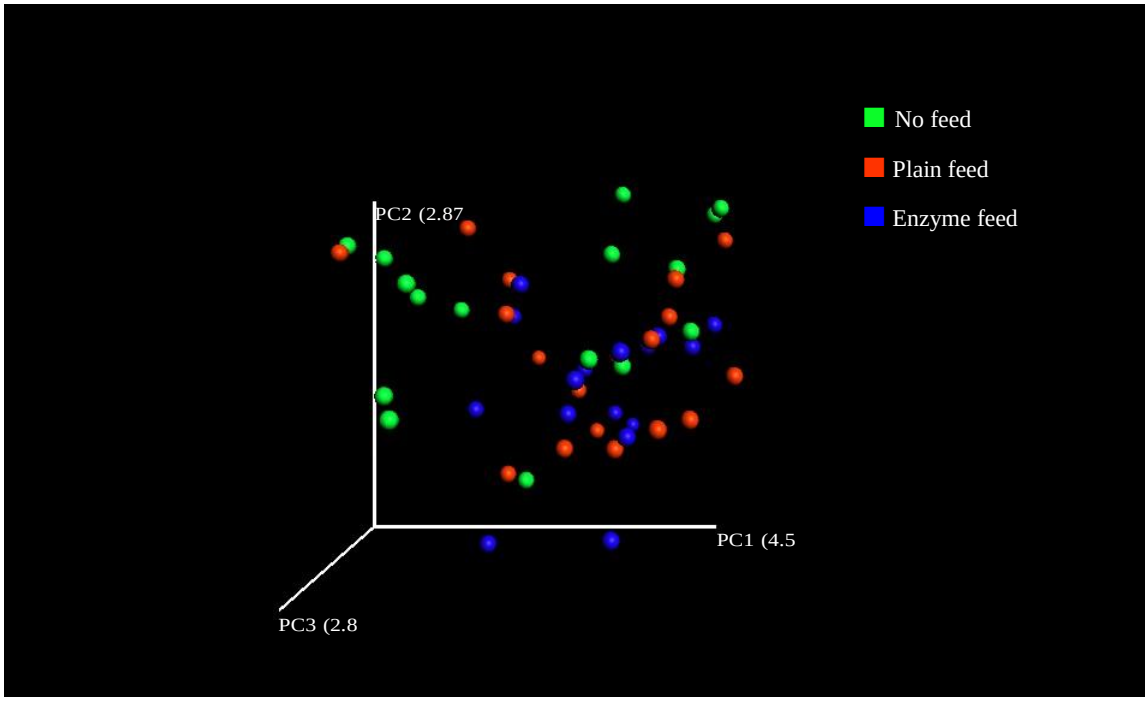

Figure 5.3. Principal coordinate analysis (PcoA) of beta diversity using the unweighted UniFrac distance matrix for all samples collected during the experiment (both collection days; shown by treatment).

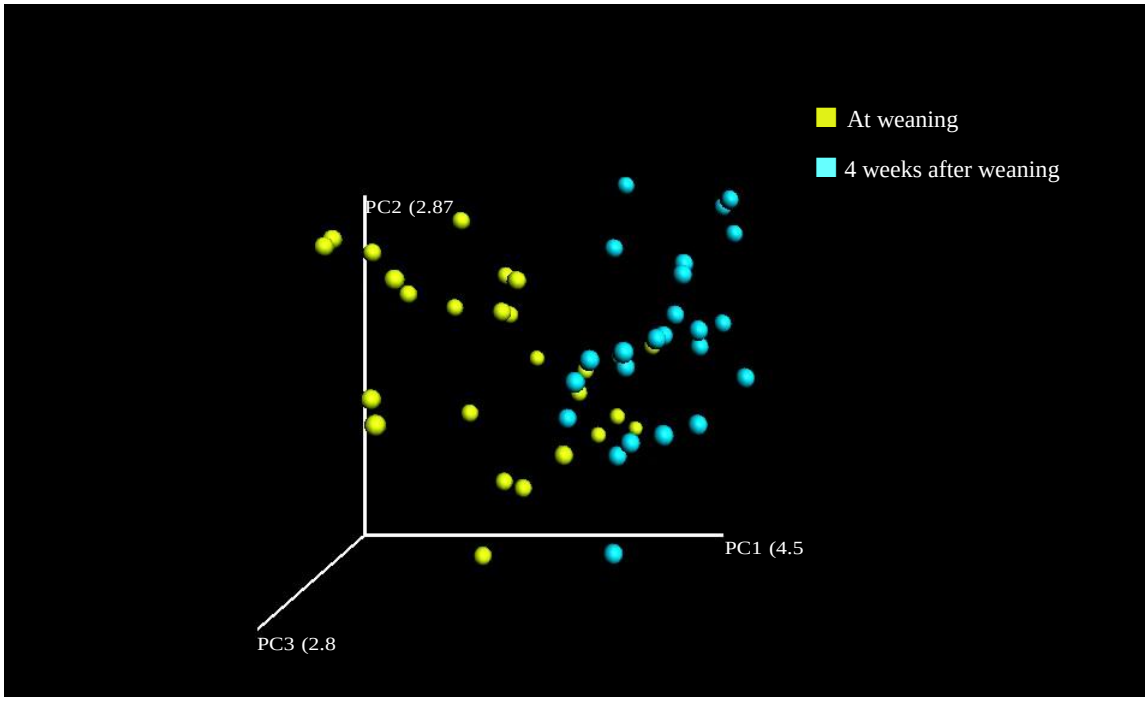

Figure 5.4. Principal coordinate analysis (PcoA) of beta diversity using the unweighted UniFrac distance matrix for all samples collected during the experiment (both collection days; shown by day).

Table 5.10S. Analyzed nutrient content of the grass and supplement offered to calves.

Analyzed Nutrient Content (dry matter basis)	GRASS	SUPPLEMENT
TDN, %	56.0	69.0
Crude Protein, %	12.0	18.9
Neutral Detergent Fiber, %	73.39	7.66
Acid Detergent Fiber, %	36.40	3.70
Calcium, %	0.50	2.32
Phosphorus, %	0.36	0.51
Magnesium, %	0.16	0.17
Digestible Energy, Mcal/kg	2.47	3.04

Table 5.11S. Performance of calves during the 14-week feeding trial.

	Treatment[1]		
Item	NO FEED	PLAIN FEED	ENZYME FEED
Initial Age, days	125	127	129
Initial BW, kg	174.2	174.4	175.6
Final BW, kg	285.7	288.6	302.5
Average daily gain d0 to 100, kg/day	1.06	1.10	1.21
Average daily gain d0 to 50, kg/day	1.15	1.18	1.31
Average daily gain d50 to 100, kg/day	0.98	0.99	1.12

[1] NO CREEP = conventional cow-calf system without supplementation of calves. PLAIN CREEP = calves were creep fed. ENZYME CREEP = calves were creep fed with an enhanced feed containing xylanase.

Table 5.12S. Performance of dams during the 14-week feeding trial.

	Treatment[1]		
Item	NO FEED	PLAIN FEED	ENZYME FEED
Dam Average daily gain d0 to 100, kg/day	0.33	0.35	0.38
Dam Average daily gain d0 to 50, kg/day	0.64	0.62	0.74
Dam Average daily gain d50 to 100, kg/day	0.05	0.10	0.04
Dam Body condition score d0	5.56	5.71	5.39
Dam Body condition score d50	5.06	5.23	4.90
Dam Body condition score d100	5.09	5.17	5.02
Body condition score Change d0 to 100	-0.46	-0.54	-0.37
Body condition score Change d0 to 50	-0.50	-0.48	-0.48
Body condition score Change d50 to 100	0.04	-0.06	0.12

[1] NO CREEP = conventional cow-calf system without supplementation of calves. PLAIN CREEP = calves were creep fed. ENZYME CREEP = calves were creep fed with an enhanced feed containing xylanase.

Table 5.13S. Effect of treatment on relative abundance of bacteria classes for samples collected on weaning day.

	Treatment[1]			Test *P*-value[2]		
Classes	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Bacteroidia[##]	48.8	51.7	52.3	0.01	0.17	0.50
Clostridia	23.9	25.7	25.0	0.38	0.58	1.00
Erysipelotrichi	2.9	2.5	2.7	0.84	0.88	1.00
Spirochaetes	2.6	2.0	1.7	0.17	0.34	1.00
Verruco-5	2.1	2.0	1.9	0.99	0.99	1.00
Mollicutes	2.0	1.7	2.4	0.13	0.27	1.00
TM7-3	1.8	1.5	1.4	0.07	0.25	1.00
Lentisphaeria[##]	1.8	1.2	0.7	0.01	0.17	0.33
4C0d-2[##]	1.7	0.9	0.5	0.02	0.20	0.99
Alphaproteobacteria	0.8	0.6	0.7	0.23	0.40	1.00
Thermoplasmata[##]	0.7	0.4	0.5	0.05	0.21	1.00
Methanobacteria	0.6	0.7	1.4	0.29	0.47	1.00
Gammaproteobacteria	0.5	0.6	0.3	0.10	0.25	1.00
Others classes	2.7	2.6	2.2	≥ 0.01	≥ 0.17	≥ 0.25
Unassigned OTUs[##]	7.2	5.9	6.2	0.04	0.21	1.00

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

Table 5.14S. Effect of treatment on relative abundance of bacteria classes for samples collected 4 weeks after weaning.

	Treatment[1]			Test *P*-value[2]		
Classes	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Bacteroidia	47.1	47.3	50.2	0.12	0.60	1.00
Clostridia	27.6	29.6	27.5	0.24	0.83	1.00
Mollicutes	3.6	3.1	3.4	0.63	0.96	1.00
Erysipelotrichi	3.3	2.4	2.5	0.43	0.96	1.00
TM7-3[##]	2.8	4.1	2.5	0.02	0.33	0.99
Spirochaetes	2.1	1.5	1.6	0.15	0.60	1.00
Verruco-5	2.0	1.6	1.3	0.30	0.83	1.00
Unidentified class 1[A]	0.9	1.1	1.3	0.65	0.96	1.00
Methanobacteria	0.8	0.8	0.9	0.67	0.96	1.00
Gammaproteobacteria	0.6	0.5	0.5	0.53	0.96	1.00
4C0d-2	0.6	0.6	0.6	0.93	0.98	1.00
Alphaproteobacteria	0.6	0.6	0.6	0.89	0.98	1.00
Fibrobacteria	0.6	0.5	0.4	0.14	0.60	1.00
Lentisphaeria	0.6	0.5	0.7	0.67	0.96	1.00
Deltaproteobacteria	0.5	0.4	0.4	0.81	0.98	1.00
Other classes	1.8	1.6	1.9	≥ 0.01	≥ 0.24	≥ 0.48
Unassigned OTUs[##]	4.4	3.8	3.6	0.01	0.24	0.30

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

[A] Phylum SR1.

Table 5.15S. Effect of treatment on relative abundance of bacteria orders for samples collected on weaning day.

	Treatment[1]			Test *P*-value[2]		
Orders	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Bacteroidales[##]	48.8	51.7	52.3	0.01	0.21	0.84
Clostridiales	23.9	25.7	25.0	0.38	0.67	1.00
Erysipelotrichales	2.9	2.5	2.7	0.84	0.92	1.00
Spirochaetales	2.5	1.9	1.6	0.15	0.39	1.00
WCHB1-41	2.0	1.9	1.8	0.96	0.98	1.00
CW040	1.8	1.5	1.4	0.07	0.30	1.00
YS2[##]	1.7	0.9	0.5	0.02	0.25	1.00
Victivallales[##]	1.6	1.1	0.7	0.01	0.21	0.62
Anaeroplasmatales	1.0	0.9	1.2	0.76	0.86	1.00
RF39	0.9	0.6	0.9	0.07	0.30	1.00
E2[##]	0.7	0.4	0.5	0.05	0.28	1.00
Methanobacteriales	0.6	0.7	1.4	0.29	0.61	1.00
Rickettsiales	0.6	0.4	0.6	0.39	0.67	1.00
Aeromonadales	0.5	0.5	0.2	0.19	0.46	1.00
Other orders	3.5	3.2	3.0	≥ 0.01	≥ 0.21	≥ 0.41
Unassigned OTUs[##]	7.2	5.9	6.2	0.04	0.28	1.00

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

Table 5.16S. Effect of treatment on relative abundance of bacteria orders for samples collected 4 weeks after weaning.

	Treatment[1]			Test *P*-value[2]		
Orders	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Bacteroidales	47.1	47.3	50.2	0.12	0.69	1.00
Clostridiales	27.6	29.6	27.5	0.24	0.78	1.00
Erysipelotrichales	3.3	2.4	2.5	0.43	0.87	1.00
CW040[##]	2.8	4.1	2.5	0.02	0.41	1.00
Anaeroplasmatales	2.5	1.7	2.0	0.24	0.78	1.00
Spirochaetales	2.0	1.5	1.6	0.18	0.70	1.00
WCHB1-41	1.9	1.5	1.2	0.32	0.83	1.00
Unidentified order 1[A]	0.9	1.1	1.3	0.65	0.95	1.00
RF39	0.9	1.2	1.3	0.34	0.86	1.00
Methanobacteriales	0.8	0.8	0.9	0.67	0.95	1.00
YS2	0.6	0.6	0.6	0.93	0.99	1.00
Fibrobacterales	0.6	0.5	0.4	0.14	0.70	1.00
Victivallales	0.5	0.5	0.7	0.63	0.95	1.00
Other orders	4.0	3.5	3.8	≥ 0.01	≥ 0.27	≥ 0.80
Unassigned OTUs[##]	4.4	3.8	3.6	0.01	0.27	0.50

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

[A] Phylum SR1.

Table 5.17S. Effect of treatment on relative abundance of bacteria families for samples collected on weaning day.

	Treatment[1]			Test *P*-value[2]		
Families	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Unidentified family 1[A]	17.4	17.6	16.8	0.94	0.98	1.00
Prevotellaceae[##]	15.5	18.3	20.2	0.01	0.20	1.00
Ruminococcaceae	8.9	9.2	9.3	0.37	0.64	1.00
Unidentified family 2[B]	6.8	6.8	6.2	0.28	0.60	1.00
Paraprevotellaceae[##]	6.0	6.5	5.1	0.001	0.17	0.17
BS11	5.3	4.3	4.8	0.49	0.70	1.00
Lachnospiraceae	4.8	5.9	5.6	0.14	0.40	1.00
RF16	3.1	3.3	3.2	0.94	0.98	1.00
Erysipelotrichaceae	2.9	2.5	2.7	0.84	0.93	1.00
Spirochaetaceae	2.5	1.9	1.6	0.15	0.41	1.00
RFP12	1.9	1.7	1.7	0.92	0.97	1.00
F16	1.8	1.5	1.4	0.07	0.31	1.00
Unidentified family 3[C ##]	1.7	0.9	0.5	0.02	0.25	1.00
Victivallaceae[##]	1.6	1.1	0.7	0.01	0.20	0.98
Veillonellaceae	1.6	1.9	1.9	0.32	0.63	1.00
Anaeroplasmataceae	1.0	0.9	1.2	0.76	0.88	1.00
Unidentified family 4[D]	0.9	0.6	0.9	0.07	0.31	1.00
Mogibacteriaceae	0.8	0.9	1.0	0.34	0.63	1.00
Bacteroidaceae[##]	0.8	0.8	0.5	0.01	0.20	1.00
Methanomassiliicoccaceae[##]	0.7	0.4	0.5	0.05	0.30	1.00
Methanobacteriaceae	0.6	0.7	1.4	0.29	0.60	1.00
Unidentified family 5[E]	0.6	0.4	0.6	0.39	0.64	1.00
Clostridiaceae[##]	0.5	0.7	0.5	0.02	0.25	1.00

S24-7[##]	0.5	0.7	1.2	0.02	0.25	1.00
Succinivibrionaceae	0.5	0.5	0.2	0.19	0.46	1.00
Other families	4.2	3.9	4.0	≥ 0.01	≥ 0.20	≥ 0.66
Unassigned OTUs[##]	7.2	5.9	6.2	0.04	0.29	1.00

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

[A]Order *Bacteroidales.*

[B]Order *Clostridiales.*

[C]Order YS2.

[D]Order RF39.

[E]*Order Rickettsiales.*

Table 5.18S. Effect of treatment on relative abundance of bacteria families for samples collected 4 weeks after weaning.

	Treatment[1]			Test *P*-value[2]		
Families	**No feed**	**Plain feed**	**Enzyme feed**	**Kruskal-Wallis**	**Benjamini-Hochberg**	**Bonferroni**
Prevotellaceae	18.5	22.3	21.7	0.11	0.65	1.00
Unidentified family 1[A]	15.3	13.1	14.8	0.11	0.65	1.00
Ruminococcaceae	9.2	10.0	9.5	0.61	0.84	1.00
Unidentified family 2[B]	8.8	9.1	7.9	0.08	0.65	1.00
Lachnospiraceae	6.0	6.3	5.9	0.42	0.84	1.00
Paraprevotellaceae	5.0	5.0	5.9	0.24	0.81	1.00
RF16	4.1	3.0	3.3	0.10	0.65	1.00
Erysipelotrichaceae	3.3	2.4	2.5	0.43	0.84	1.00
F16[##]	2.8	4.1	2.5	0.02	0.63	1.00
BS11	2.6	2.4	3.2	0.47	0.84	1.00
Anaeroplasmataceae	2.5	1.7	2.0	0.24	0.81	1.00
Spirochaetaceae	2.0	1.5	1.6	0.18	0.74	1.00
RFP12	1.8	1.2	1.0	0.18	0.74	1.00
Veillonellaceae	1.7	2.3	2.1	0.11	0.65	1.00
S24-7	1.1	1.0	0.8	0.23	0.81	1.00
Unidentified family 3[C]	0.9	1.1	1.3	0.65	0.84	1.00
Unidentified family 4[D]	0.9	1.2	1.3	0.34	0.84	1.00
Mogibacteriaceae	0.8	0.9	1.0	0.39	0.84	1.00
Methanobacteriaceae	0.8	0.8	0.9	0.67	0.84	1.00
Unidentified family 5[E]	0.6	0.6	0.6	0.93	0.97	1.00
Clostridiaceae	0.6	0.7	0.7	0.32	0.84	1.00
Fibrobacteraceae	0.6	0.5	0.4	0.14	0.72	1.00
Victivallaceae	0.5	0.5	0.7	0.63	0.84	1.00
Other families	5.1	4.7	5.0	≥ 0.01	≥ 0.42	≥ 0.99

Unassigned OTUs[##]	4.4	3.8	3.6	0.01	0.42	0.77

[1] No feed = conventional cow-calf system without supplementation of calves. Plain feed = calves were creep fed. Enzyme feed = calves were creep fed with an enhanced feed containing xylanase.

[2] Test *P*-value based on the statistical procedure: Kruskal-Wallis distribution-free test; Benjamini–Hochberg false discovery rate test; Bonferroni corrected test.

[##] At least one treatment is different ($P \leq 0.05$) according to the Kruskal-Wallis procedure.

[A]Order *Bacteroidales.*

[B]Order *Clostridiales.*

[C]Phylum SR1.

[D]Order RF39.

[E]Order YS2.

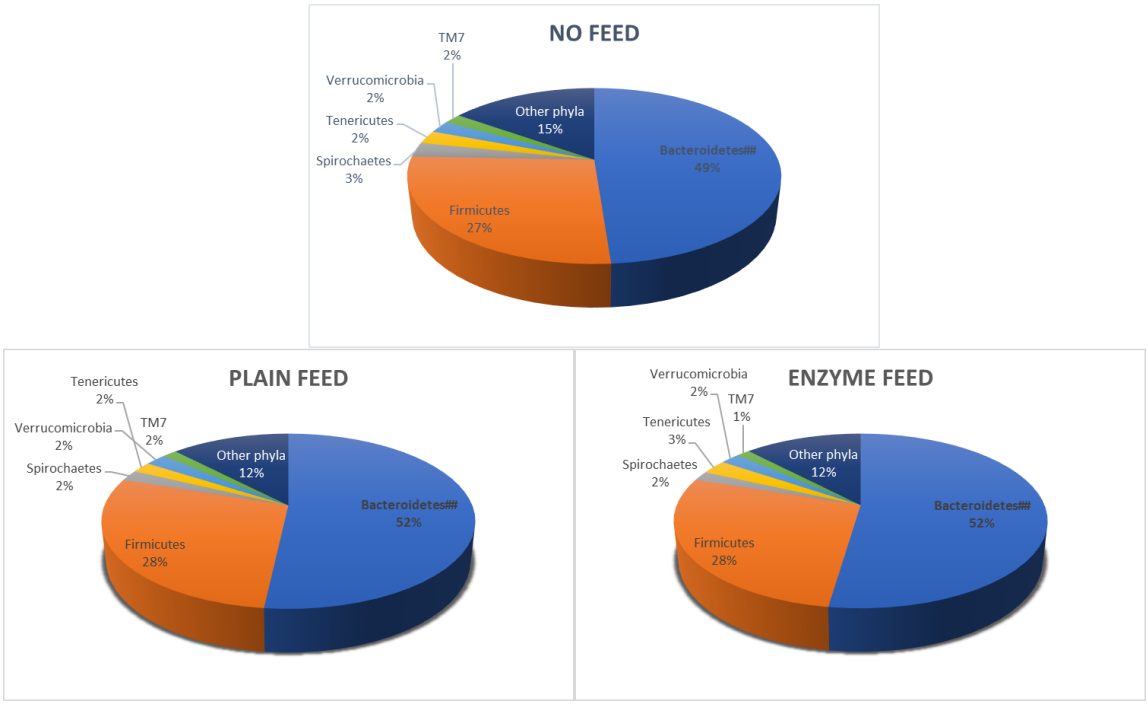

Figure 5.5S. Relative abundance of bacteria phyla for samples collected on weaning day (## = $P \leq 0.05$).

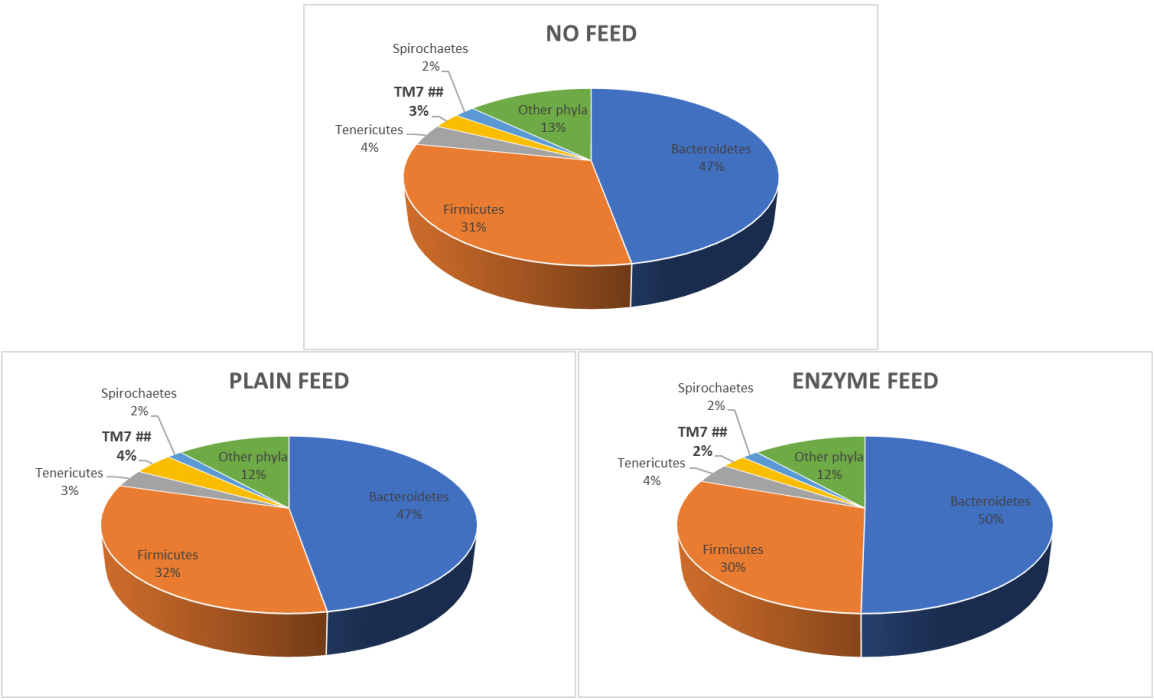

Figure 5.6S. Relative abundance of bacteria phyla for samples collected 4 weeks after weaning (## = $P \leq 0.05$).

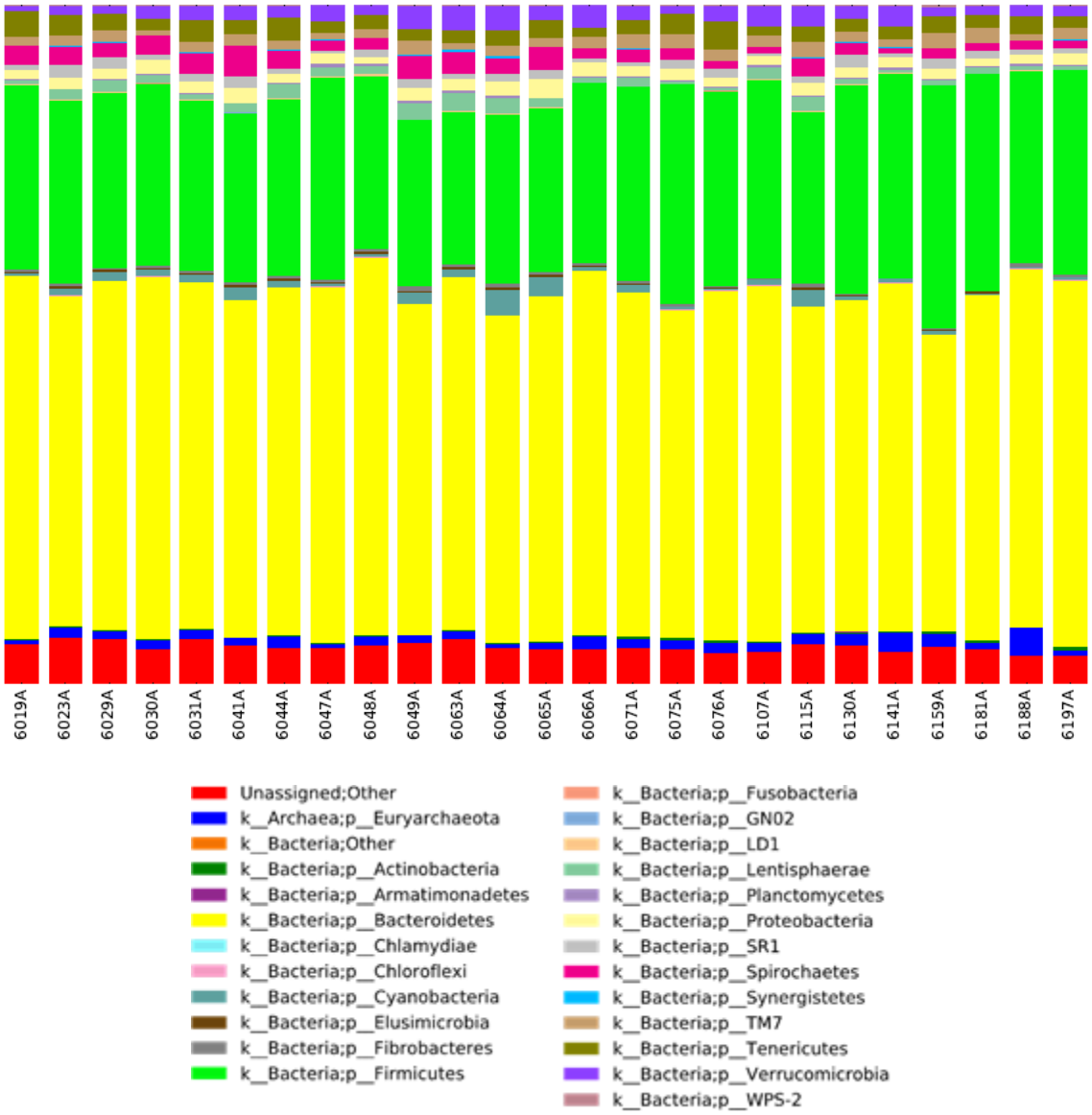

Figure 5.7S. Relative abundance of bacteria phyla by animal ID on weaning day.

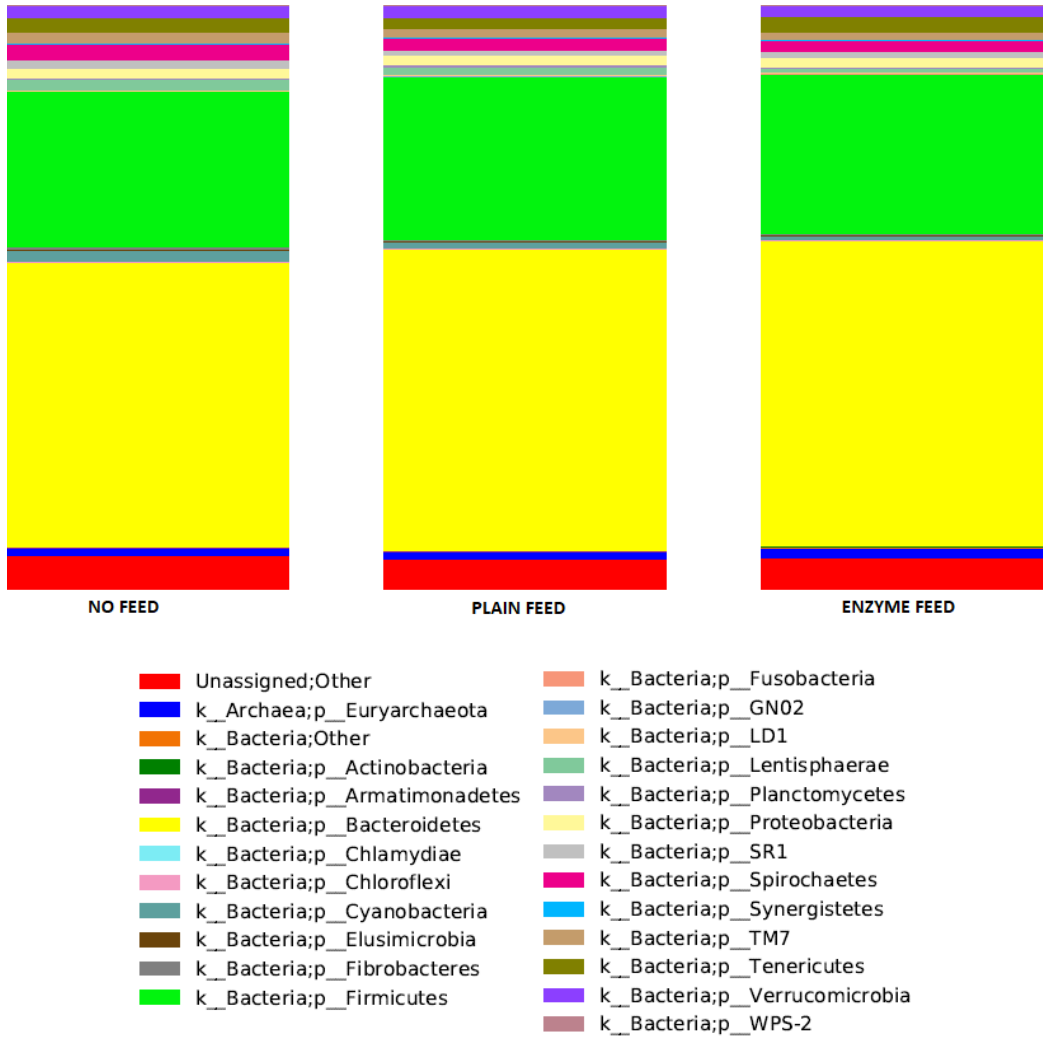

Figure 5.8S. Relative abundance of bacteria phyla by diet on weaning day.

CHAPTER 6

CONCLUSIONS

In an effort to reproduce the ruminal digestion of suckling beef calves in a proper way, the first step of this research (the in vitro batch culture study) was performed using rumen fluid collected from 6-month-old beef calves. Our results showed that, for the majority of the traits measured, there was a clear distinction between incubations using pure bermudagrass (**BER**) and all the other treatments (which were made of bermudagrass plus supplement with or without enzymes). A comparison of all treatments showed that BER had lower digestibility of ADF, reduced IVDMD, diminished production of total VFA in 24 h, and the lowest concentration of butyrate, which is the most important VFA concerning rumen papillae development in young calves. These findings indicate that suckling beef calves that rely exclusively on forages as their only source of nutrients (in addition to milk) may not have the same development as calves that are supplemented. Regarding the tested enzymes, although the rates of inclusion indicated in their labels produce satisfactory results in chicken and swine diets, our results showed that their inclusion in creep feeds for beef cattle will likely have to be adjusted (i.e. increased). Our in vitro findings indicate that the effects caused by them when they were used at their label doses were not markedly different from the ones observed in the treatment containing only plain creep feed (**BERCF**). Furthermore, although the addition of the selected feed enzymes did not improve every single trait measured in this study, some important features such as total production of VFA,

IVDMD and ADF digestibility were significantly increased by their inclusion at doses 10 times greater than what is prescribed in their labels. Therefore, our in vitro results indicate that this rate of inclusion (**10x**) would be more adequate for several of the enzymes tested, if they are to be used in beef cattle creep feeds.

Results from the second experiment (in vivo feeding trial) showed no effects of treatments on any of the traits analyzed in the cows, however, some important changes were detected on the calves: animals receiving the creep feed enhanced with xylanase (**ENZYME FEED**) experienced greater ADG, a numerical reduction in supplement intake, and improved feed efficiency. In addition, calves in the ENZYME FEED group had the greatest proportion of butyrate in their rumen fluid, which is in alignment with our findings from the in vitro assessment. Overall, results from this feeding trial confirmed that the inclusion of xylanase in beef cattle creep feeds is advantageous and should be encouraged. However, in order to identify the optimum rate of inclusion of this enzyme, an additional trial should be conducted to explore inclusion levels in creep feeds other than 13,800,000 fungal xylanase units/t of creep feed DM.

Lastly, the microbiome portion of this research showed that some shifts occurred in the rumen microbiome of calves due to creep feeding supplementation. For instance, an increased relative abundance of *Prevotella*, and a decreased abundance of *Treponema* were detected at the genus level in supplemented calves. However, these microbial fluctuations were not as great in magnitude as some reports usually found in the literature. Hence, other factors such as an increased amount of energy intake due to supplementation, and an increased availability of metabolizable energy due to the addition of xylanase, were probably more important than any shifts observed in the

microbial community. Therefore, we assume that these factors had a greater contribution on the observed differences in calf growth than any of the microbial shifts detected.

Taken together, our findings demonstrate that the inclusion of the enzyme endo-1,4-β-xylanase (or simply xylanase, EC number 3.2.1.8) at a rate of 13,800,000 fungal xylanase units per tonne of creep feed DM is effective. Even though further research with this enzyme in suckling beef cattle is encouraged, our results clearly revealed that xylanase is an excellent enhancer of creep feeds formulated for beef calves.