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The Relationship Between Microbiota, Diet, and Energy Production in the Alpaca

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ABSTRACT

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The alpaca is a small South American camelid (SAC) that is an important production animal in Peru, especially among the highly impoverished communities of the high Andes, and raised for its fiber and meat. Alpacas are highly reliant on the microbes within their digestive tracts to digest the plant material they consume; volatile fatty acids (VFAs) are released as a byproduct of this microbial fermentation and used as a major source of energy by the alpaca. To explore optimal parameters for alpaca microbiome analysis, performed 16S rRNA gene surveys on alpaca C1 and fecal samples that had been extracted using one of three different DNA extraction methods (PowerFecal® DNA Isolation Kit (MO BIO); ZR Fecal DNA MiniPrep™ (Zymo); and a non-commercial extraction method called salting out) and amplified using one of two different polymerase enzyme mixes (AccuPrime™ *Pfx* SuperMix and 5 PRIME HotMasterMix). We found that choice of polymerase enzyme had a profound effect on the recovered microbiome, with the majority of 5 PRIME-amplified fecal samples failing to amplify. Extraction method had an effect on the recovered microbiome of fecal samples (but not C1 samples), with samples extracted using the MO BIO kit and the salting out method recovering different communities. The Zymo extraction kit returned microbial communities comparable to each of the other extraction methods. These results suggested that the AccuPrime enzyme and either the MO BIO or Zymo kits were optimal for alpaca gut microbiome analysis. We also performed two 16S rRNA gene surveys, the first from alpacas fed either a grass hay (GH) or alfalfa hay (AH) diet, and the second a C1 survey of alpacas fed two-week periods of mixed grass hay plus one of four supplements. We discovered body site and diet effects on the microbiota of alpacas fed either the GH or AH diet, with samples grouping by general body site (C1, small intestine, and distal intestine) and diet. However, we found no significant effect on the C1 microbiome of alpacas administered grain supplements. To study how energy extraction related to the microbiome, we correlated OTUs from GH/AH-fed alpaca with C1 VFA abundances. We discovered no significant correlations, and a 16S survey of low body condition (LBC) and good body condition (GBC) alpacas showed no difference in C1 microbial communities. We concluded that the microbiota of the alpaca digestive tract follow trends seen in microbiome studies of ruminants, but found no evidence of a relationship between body condition, energy extraction, and the C1 microbiome in alpacas.

Keywords: alpaca, gut microbiome, DNA extraction, DNA amplification, technical parameters, feces, C1, small intestine, large intestine, volatile fatty acids, body condition score

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The alpaca (*Vicugna pacos*) is a South American camelid (SAC) of great importance as a production animal in Peru and Bolivia. Alpacas are primarily raised for their luxurious fiber, or hair. Approximately 90% of the alpaca fiber used in textiles worldwide comes from Peru (Paredes et al., 2013). However, alpacas are arguably most important to impoverished families in the high Andes. They depend on their alpacas for both their fiber and their meat, which is highly nutritious, low in cholesterol, and an important source of protein, and their livelihoods depend on the size and productivity of their alpaca herds (Cristofanelli et al., 2005).

Despite their importance in certain areas of the world, alpacas are not widely studied. Scientific literature about alpacas and camelids in general lacks the breadth of study given to cattle and other ruminants; fewer search results are returned for PubMed searches of the terms "alpaca" (2,153), "llama" (2,316), "camel" (5,065), "camelid" (2,424), and "camelid microbiome" (5) than for the terms "cattle" (337,856), "ruminant" (456,331), and "ruminant microbiome" (818). While the need for a better understanding of camelids may be a daunting task, it is somewhat mediated by our better understanding of the highly-studied ruminants. Camelids share many similarities to ruminants such as cattle, and information about ruminants may be extrapolated to camelids in some purposes. However, this extrapolation may act as a roadblock to a true understanding of camelids, as extrapolation is not appropriate in all cases. For instance, camelids produce less methane than ruminant livestock, likely as a result of their lower relative food intake and superior feed efficiency (Dittmann et al., 2014). Prior to explicit studies of C1 methane production, estimates of camelid methane emissions were much too high because these estimates were extrapolated from ruminant data. In addition, camelids clearly have unique traits and health concerns. Some of the traits unique to camelids include their skin and their two-

digit, padded feet (Atlee et al., 1997; Fowler, 2010). They also have thin, elliptical erythrocytes that are very small and present in extremely high numbers (Jain and Keeton, 1974; Smith et al., 1979), conferring increased hemoglobin counts. Additionally, their metabolism of glucose may be unique (Fowler, 2010), and they differ from ruminants in that they have the ability to recycle urea (Hinderer and Engelhardt, 1975). They have unique reproductive physiology with high rates of infertility and a greater occurrence of congenital disease (Fowler, 2010). Camelids are also subject to different forms of disease. For instance, separate mycoplasma strains infect SACs and cattle, with *Mycoplasma haemolamae* specifically producing anemia in alpacas and llamas (Messick et al., 2002; Tornquist et al., 2010), and certain species of parasites uniquely parasitize camelids (Fayer et al., 1991; Starkey et al., 2007). Because of these unique traits and concerns, perhaps more study of the camelids is warranted, especially in areas for which camelid physiology is assumed to be similar to ruminant physiology and not explicitly researched.

Like ruminants, alpacas and other camelids are foregut fermenters and rely heavily on the microbes within their gut to digest the plant materials they feed on; however, while ruminants possess a four-chambered stomach, the pseudoruminant camelids have a three-chambered stomach. The first compartment of the foregut, compartment 1 (C1), is the largest chamber and functions similarly to the ruminant rumen, reticulum, and omasum (Vallenas et al., 1971). The majority of microbial fermentation takes place in C1. Here, the bacteria digest cellulose, hemicellulose, and other plant cell wall components, releasing volatile fatty acids (VFAs) - primarily acetate, butyrate, and propionate- as a byproduct. These VFAs are then used by the host animal as a source of energy, supplying a large portion of ruminant and camelid energy needs (Bergman, 1990).

Although they are efficient production animals (Genin and Tichit, 1997; Rübsamen and von Engelhardt, 1979; San Martin and Bryant, 1989; Van Soest, 1994), alpaca herders face a number of problems. Alpaca fiber products can be quite expensive in developed countries, but alpaca herders themselves see very little of the profit. Herd numbers tend to remain low because of the alpaca's low reproductive rate. Additionally, a number of low body condition (LBC; i.e. very thin) animals tend to present in any alpaca herd, even if efforts are taken to treat all animals the same in regards to feed access, vaccination, and other factors.

Body condition has been linked to energy balance (Pryce et al., 2001). Proper energy balance is crucial to reproduction, production, and survival in ruminants and camelids (Pryce et al., 2001; Van Saun, 2008). On the other hand, LBC has been associated with low productivity in animals and may be associated with negative energy balance, where the animal is using more energy than is being produced (Kristjanson et al., 2007; Reyna, 2005). Because energy balance may be influenced by the gut microbiota (Bäckhed et al., 2005; Wang et al., 2012), we theorized that the C1 microbes may play a role in the differences observed between LBC and GBC (good body condition) alpacas by influencing how much and what type of VFAs are produced for host use.

Metagenomic analysis of the alpaca gut microbiome may be a step toward understanding and solving problems such as unexplained LBC status in alpacas. A microbiome survey reveals the taxonomic identity of the microbial community within an environmental sample, usually through amplicon or target gene sequencing of the 16S rRNA gene (Langille et al., 2013). To achieve this, the microbial DNA from each sample is isolated and special primers are used to amplify a particular gene or section of a gene from each strand of DNA during polymerase chain reaction (PCR). The amplified sections of DNA, or "amplicons," are then sequenced and

taxonomic identity is assigned by comparing the 16S amplicons to reference genes in a database (Zhou et al., 2011). Although community identity can be studied, 16S marker gene surveys typically do not tell anything about bacterial metabolism or function; however, programs such as PICRUSt can be used to predict metagenomes and subsequent gene function (Langille et al., 2013).

Little is known about the camelid gut microbiota, and most relevant studies focus on C1 microbiome analyses (Henderson et al., 2015; Pei et al., 2010; Pei et al., 2013). Ruminants are more well-studied, with microbiome analyses of the whole digestive tract existing (de Oliveira et al., 2013; Mao et al., 2015). Few studies have been performed on the microbiota of the camelid large intestine or feces (Espinosa et al., 2015), and we are unaware of any microbiome studies of the camelid small intestine. To fill this gap in alpaca gastrointestinal knowledge, we performed a number of 16S rRNA gene surveys on the alpaca gut microbiome with the ultimate goal of discovering how body condition relates to the gut microbiome in alpacas. Our first study involved assessing how three different DNA extraction methods (PowerFecal® DNA Isolation Kit from MO BIO Laboratories, Inc.; ZR Fecal DNA MiniPrep™ from Zymo Research; and a non-commercial extraction method called salting out) and two different polymerase enzyme mixes (AccuPrime™ *Pfx* SuperMix and 5 PRIME HotMasterMix) affected the recovery of microbial DNA from alpaca C1 and fecal samples. Here, we hoped to find the optimal combination of extraction method and enzyme mix for use in preparing alpaca microbiome data for sequencing. Our second study focused on how different dietary regimes influenced the alpaca gut microbiome at various sites throughout the digestive tract, how these diets correlated with VFA production, and whether the C1 microbiota of LBC and GBC alpacas differed. Ultimately, our goals were to 1) gain a comprehensive overview of the alpaca digestive tract microbiomes;

2) discover candidate operational taxonomic units (OTUs, i.e. microbial strains) that, due to their possible link to VFA abundance, may be used via prebiotic or probiotic treatment to ameliorate LBC status in alpacas, if body condition does appear to be linked to the C1 microbiome.

REVIEW OF LITERATURE

High-throughput sequencing technology has revolutionized metagenomics by improving our ability to quickly sequence large numbers of reads. By sequencing DNA extracted straight from a source, high-throughput or "next-generation" methods avoid the complications involved with cloning- high-throughput methods are much less time-consuming, and the culture-independent methods account for the wide range of microbes that cannot be grown on plates (Mardis, 2011).

There are four main methods of high-throughput sequencing- pyrosequencing, ion semiconductor sequencing, sequencing by ligation, and sequencing by synthesis. As such, there are a variety of sequencing platforms. Although each sequencing method differs in flow-through, read length, and accuracy, they all share a number of traits. First, high-throughput sequencing platforms process millions of reads in parallel rather than a single read at a time (Mardis, 2011). High-throughput sequencing library preparation can be achieved in various ways. However, each method of library preparation involves four general steps- the target DNA must be fragmented or "sized;" the target fragments must be converted to double-stranded DNA; oligonucleotides must be ligated to the ends of each fragment; and the library product must be quantitated (Head et al., 2014).

DNA Preparation Methods Affect Microbial Recovery

Due to the multiple steps involved in high-throughput sequencing and the vast number of options available to prepare samples, the steps involved in preparing samples for high-throughput sequencing can introduce bias or error into the dataset. Because of this, the sequenced reads do not accurately reflect the true microbial population of the samples they come from. Three common sources of bias and error in microbiome surveys include cell lysis bias, PCR bias, and sequence artifacts.

DNA extraction bias

Microbial cells must be lysed and the DNA purified and captured before the 16S rRNA gene can be PCR-amplified. Choice of DNA isolation method can affect the recovery of microbiota. In particular, the cell lysis method used in DNA extraction has a noticeable effect on which bacteria appear in next-generation reads. Cell lysis may be achieved through mechanical, chemical, and/or enzymatic forms of lysis. Each form of lysis can affect the abundance or presence of different microbes.

Mechanical lysis involves breaking cell membranes through bead beating. Methods that include a mechanical lysis step, such as bead beating, generally lead to greater recovery of gram-positive bacteria than methods without a mechanical lysis step. It has been hypothesized that the thick peptidoglycan layers of gram-positive bacteria, such as many species of the *Firmicutes* phylum, are difficult to lyse without mechanical lysis (Roose-Amsaleg et al., 2001). Studies by Burbach, et al. (2016) and Henderson, et al. (2013) have reported that an increased amount of time spent on bead beating led to larger DNA yields and a higher *Firmicutes*:*Bacteroidetes* ratio; meanwhile, less mechanical lysis favored the recovery of the gram-negative *Bacteroidetes*,

possibly due to lower DNA yields comprised mostly of the more readily lysed gram-negative bacteria (Henderson et al., 2013; Olson and Morrow, 2012).

Chemical lysis utilizes chemicals to break open cells, while enzymatic lysis uses enzymes. Most frequently, chemical detergents like SDS are used (Roose-Amsaleg et al., 2001). The enzyme most frequently used in cell lysis is lysozyme, which is responsible for neuropeptide hydrolysis (Roose-Amsaleg et al., 2001). However, proteases like proteinase K (Kong et al., 2010; Miller et al., 1988) and achromopeptidase (Ezaki and Suzuki, 1982; Tajima et al., 2001) have also been used (Roose-Amsaleg et al., 2001), and some protocols utilize mutanolysin (Kong et al., 2010). Unlike mechanical lysis, chemical and enzymatic forms of lysis are discriminatory in the types of cells they can break; however, they are gentler than mechanical forms of lysis and DNA extracted with these methods is more likely to be intact (Roose-Amsaleg et al., 2001).

Sequence Artifacts

Stretches of DNA that have been changed in any way from their original template during PCR are termed "sequence artifacts." These errors can arise due to chimera formation, heteroduplex formation, and polymerase error. Chimeras form when an incompletely extended primer anneals to another template with some homology (Pääbo et al., 1990; Shuldiner et al., 1989) or when templates switch (Odelberg et al., 1995). By only using as many cycles as necessary for sufficient amplification, chimera formation will be less likely to occur as the primer:incompletely extended product ratio will remain higher (Thompson et al., 2002). Odelberg et al. reduced chimera formation by physically separating the complementary template strands using streptavidin beads (Odelberg et al., 1995). Heteroduplexes are formed when heterologous sequences anneal together, and again may be prevented from forming by limiting

the number of PCR cycles used so more primer is available in the reaction, reducing the probability that heterologous sequences will bind together (Kanagawa, 2003); additionally, reconditioning PCR by applying PCR product diluted with fresh reaction mixture for three cycles can also help eliminate heteroduplexes (Thompson et al., 2002). Random events such as polymerase errors and misannealing of primers can be ameliorated by mixing replicate PCR amplifications together before sequencing (Wagner et al., 1994).

PCR Bias

Unlike sequence artifacts, PCR bias describes an incorrect distribution of PCR products that results when the template DNA is not amplified equally (Acinas et al., 2005). Differences in primer binding energies can create PCR bias. Often, this takes the form of primer mismatch. When a primer has a mismatch with the target, the primer binding energy will be low and the target is less likely to be amplified (Ishii and Fukui, 2001). Biases due to dissociating energy can be the result of G/C content. Sequences with high G+C contents dissociate less readily from their templates, so some templates may be preferentially amplified due to low G+C content (Dutton et al., 1993). Additionally, the reannealing of products can prevent some sequences from being amplified (Suzuki and Giovannoni, 1996). Finally, organisms can have different 16S copy numbers and genome sizes, resulting in different product:template ratios (Farrelly et al., 1995). PCR bias may be reduced by performing replicate PCR reactions and mixing the products (Wagner et al., 1994); lowering annealing temperatures to decrease bias due to primer binding energy differences (Ishii and Fukui, 2001); and using the least number of PCR cycles required to obtain enough product (Suzuki and Giovannoni, 1996).

DNA Extraction Methods Used

The PowerFecal® DNA Isolation Kit from MO BIO Laboratories, Inc. (Carlsbad, CA) is a field standard used in mammalian gut microbiome studies. Cell lysis is achieved with a combination of both mechanical and chemical lysis, specifically via bead beating with garnet beads and an SDS solution. Additionally, the PowerFecal kit provides for inhibitor removal and a silica spin column is used to capture the DNA. A study by Janabi, et al. (Janabi et al., 2016) revealed that this kit returned greater *Bacteroidetes* : *Firmicutes* ratio than a phenol-based extraction method, although the *Bacteroidetes* genus *Barnesiella* was present in lower proportions.

The ZR Fecal DNA MiniPrep™ kit from Zymo Research (Irvine, CA) is another commercial kit used to extract DNA from gut microbiome samples. This kit uses mechanical lysis via bead beating to lyse microbial cells. DNA is also isolated on a silica spin filter. In a study by Mackenzie, et al. (Mackenzie et al., 2015), DNA extracted using the Zymo kit had the lowest mean yield and quality measurements when compared to four other extraction methods, but the highest diversity measurements and observed number of OTUs. It also extracted the lowest proportion of *Bacteroidetes* and the highest of *Firmicutes*, along with high levels of *Bifidobacterium adolescentis*, and was noted as the only method that detected *Acidobacteria*, *Thermi*, and *Chlorobi*.

Salting out is a non-commercial method of DNA extraction that does not require the use of dangerous organic compounds like phenol or chloroform. First described by Miller, et al. (1988), this method of DNA extraction involves using high molar concentrations of sodium chloride (NaCl) to dehydrate and precipitate the cell digests and “salt out” the cellular proteins. The DNA left in the supernatant is then precipitated using ethanol. Different variations of the

salting out protocol have been used to isolate DNA from various sources. Rivero (2006) used ammonium acetate for protein precipitation and isopropanol for DNA precipitation to isolate DNA from formalin-fixed, paraffin-embedded tissues. D'Angelo (2007) precipitated DNA extracted from milk somatic cells using isopropanol. A protocol used by Noguera (2000) to isolate DNA from blood utilized a phenol/chloroform step before ethanol precipitation to remove inhibitors. The protocol we used in our experiment was derived from one used to isolate DNA from insects (Cenis et al., 1993) and was chosen because it had been used previously in our lab. Our protocol involved protein precipitation using 3M sodium acetate (NaOAC) and DNA precipitation using isopropanol.

PCR Enzymes Used

The 5 PRIME HotMasterMix (5 PRIME) is an enzyme mix used in PCR amplification. It consists of a *Taq* polymerase enzyme, a polymerase inhibitor, and a buffer. The inhibitor prevents amplification at certain temperatures by blocking the substrate binding site of the polymerase at temperatures lower than 40°C. As the temperature rises between 40°C and 55°C, the inhibitor's binding affinity for the polymerase lowers and the binding affinity of the DNA increases, competing with the inhibitor for access to the polymerase binding sites. Above 55°C, inhibitors are completely dissociated from the polymerase in favor of template DNA-polymerase complexes. This mix is notable for the polymerase's ability to be deactivated after a high-temperature step due to the inhibitor activity. Additionally, the inhibitor is viable through multiple temperature cycles. The 5 PRIME HotMasterMix buffer adjusts the Mg^{2+} concentration in the reaction through weak chelation of Mg^{2+} ions, which are released when Mg^{2+} concentrations are low but bound when they are high. For optimized amplification, the initial

denaturation step should take place at 94°C for two minutes (it is a "hot start" polymerase), and primer elongation should be performed at 65°C (60 - 70°C).

The AccuPrime™ *Pfx* SuperMix (Invitrogen™; Carlsbad, CA) is another "hot start" enzyme mix used in PCR amplification. The DNA polymerase used in the mix has a 3' to 5' exonuclease ability for proofreading. It is inactive at room temperature but reactivated after the initial denaturation step at 94°C. Additionally, proteins in the mix work to improve formation of specific primer-template complexes.

Different methods of DNA extraction and PCR amplification affect which microbes are recovered from high-throughput sequencing. Due to biased extraction and amplification, identical samples can appear to have different microbial communities upon sequencing. For our first experiment, we studied the MO BIO, Zymo, and salting out methods of DNA extraction to see how communities extracted using two less expensive methods (the Zymo kit and the salting out method) compared to the microbiota extracted using the MO BIO kit, which is a standard in ruminant microbiome studies. We also studied the effect of two recommended polymerase mixes, the AccuPrime enzyme (recommended by (Kozich et al., 2013)) and the 5 PRIME enzyme (recommended by the Earth Microbiome Project (http://www.earthmicrobiome.org/)), to see whether they gave comparable results. Overall, we hoped to discover an inexpensive, effective protocol for optimization of alpaca gut microbiome library preparation that would recover a community comparable to that found using a field standard.

CHAPTER ONE

DNA extraction and amplification protocols influence the microbiota recovered from the alpaca digestive tract

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ABSTRACT

The numerous technical parameters involved in preparing metagenomics samples for next-generation sequencing can affect which microbes are recovered from a sample. Biased extraction or amplification of microbial DNA may inaccurately reflect true populations, making taxon correlations and comparisons with other datasets inaccurate in return. The present study used three different DNA extraction methods (PowerFecal® DNA Isolation Kit from MO BIO Laboratories, Inc (Carlsbad, CA).; ZR Fecal DNA MiniPrep™ from Zymo Research (Irvine, CA); and a method called salting out) and two Taq polymerase enzymes (AccuPrime™ *Pfx* SuperMix (Carlsbad, CA) and 5 PRIME HotMasterMix) to determine how different enzyme-extraction method combinations affected microbial community recovery from the forestomach (C1) and feces of alpacas. We detected significant enzyme effects on all samples, but only detected a significant kit effect in AccuPrime-amplified fecal samples. A number of taxa displayed differential abundance as a result of enzyme or extraction method, including increased abundances of *Bacteroidales* in AccuPrime-amplified, MO BIO-extracted samples; *Euryarchaeota* and gram-positive bacteria in samples extracted through salting out; and *Clostridiales* in AccuPrime-amplified samples. Our results suggest that the Zymo kit and the AccuPrime enzyme are a comparable and cost-effective alternative to the field standards used in gut microbiome studies. Further research using mock communities and samples from a range of

ruminants and camelids may prove how accurate each method is at reflecting the microbiome, and show whether the Zymo extraction method and AccuPrime enzyme produce similar results in other foregut-fermenters.

INTRODUCTION

Next-generation sequencing technology has driven rapid progress in our abilities to analyze complex microbial communities. Previous methods of sequencing that depended on culturing and cloning were time-consuming and costly, and adequate sequencing depth was unreasonable to attain. In contrast, massively parallel next-generation sequencing enables a vast amount of sequences to be obtained at a much lower cost by extracting, amplifying, and sequencing microbial DNA straight from uncultured environmental samples (Mardis, 2008). As a result, we are better able to assess the makeup of microbial populations. Although next-generation sequencing gives us a better estimate of the true makeup of microbial populations, a number of technical parameters can affect which microbes in a community ultimately end up sequenced and may give a skewed image of any microbiome.

The effects of various technical parameters on the microbial communities recovered from different environments have been widely studied. Previous studies have looked at technical parameters such as extraction method (Espinosa et al., 2015; Fliegerova et al., 2014; Fouhy et al., 2016; Hart et al., 2015; Henderson et al., 2013; Kashinskaya et al., 2017; Lazarevic et al., 2013; Peng et al., 2013; Wagner Mackenzie et al., 2015; Yuan et al., 2012), use of cryoprotectants and storage temperature (Fliegerova et al., 2014), sequencing platform and primer choice (Fouhy et al., 2016), sampling technique and sample fractionation (Henderson et al., 2013), and polymerase (Schirmer et al., 2015); and samples from environments such as feces (Espinosa et al., 2015;

Hart et al., 2015; Peng et al., 2013; Wagner Mackenzie et al., 2015), rumen digesta (Fliegerova et al., 2014; Henderson et al., 2013), intestines (Kashinskaya et al., 2017), saliva (Lazarevic et al., 2013), and even mock communities (Fouhy et al., 2016; Schirmer et al., 2015). However, we are unaware of any studies on how technical parameters affect the recovered bacteria from different alpaca microbiomes.

The alpaca (*Vicugna pacos*) is a South American camelid raised for its hair (fiber) and meat. Unlike true ruminants which have a four-chambered forestomach, the pseudoruminant camelids possess a three-chambered forestomach with the first compartment (C1) analogous to the rumen (Vallenas et al., 1971). This forestomach acts as a fermenting chamber, housing microbes that degrade the indigestible plant material these animals consume. As a byproduct of fermentation, the microbes release volatile fatty acids (VFAs) which are used by ruminants and camelids as a major source of energy. Camelids are more efficient at fiber degradation than ruminants, especially when fed low-quality, low-protein forages (Clemens and Stevens, 1980; Genin and Tichit, 1997; Rübsamen and von Engelhardt, 1979), although this efficiency may be attributable to an increased microbial yield, the presence of glandular saccules in C1, and/or greater feed retention time than ruminants (San Martin and Bryant, 1989). This digestive efficiency, along with the unique digestive physiology of camelids and their status as production animals, made the alpaca of particular interest for our studies.

We sought to determine how three different extraction methods (PowerFecal® DNA Isolation Kit from MO BIO Laboratories, Inc.; ZR Fecal DNA MiniPrep™ from Zymo Research; and a method called salting out) and two different Taq polymerase enzymes (AccuPrime™ *Pfx* SuperMix and 5 PRIME HotMasterMix) influenced the recovery of microbes from alpaca C1 and fecal samples.

MATERIALS AND METHODS

Sample Collection

The experiment was designed in accordance with animal care and use guidelines and with approval of The Camelid Center Animal Use Committee. Five alpaca individuals were used for the experiment. All animals were treated equally in regards to environment and feed administration. Upon completion of the experiment, the animals were sacrificed at a commercial slaughtering facility and digesta samples were taken from the C1 and feces of each alpaca. All samples were taken at the same time and from the same location within C1.

DNA Extraction and PCR

Three aliquots of each sample were prepared and each aliquot was extracted using a different method (PowerFecal® DNA Isolation Kit from MO BIO Laboratories, Inc. (Carlsbad, CA); Fecal DNA MiniPrep™ from Zymo Research (Irvine, CA); and a non-commercial extraction method used on flies called salting out). The salting out extraction was performed as described by Cenis, et al. (Cenis et al., 1993). Each 5-mg fecal sample was mixed with 180 uL lysis buffer and 20 mg/ml lysozyme, briefly vortexed, and incubated for 1 hour at 37°C. Glass beads were added to the samples and shaken for 5 minutes using a Disruptor Genie. Subsequently, 20 uL 10X extraction buffer and 10 uL proteinase K were added to each sample and the samples were incubated for 1 hour at 55°C. Following incubation, 100 uL of 3M NaOAc were added to each sample, incubated at -20 °C for 10 minutes, and centrifuged. The supernatant was transferred to new collection tubes, mixed with 300 uL cold isopropanol, and centrifuged for 30 minutes. The remaining supernatant was removed and the pellet was rinsed with 500 uL cold EtOH and allowed to air dry for 2 hours. After drying, the pellet was resuspended in sterile TE

buffer and incubated for 30 minutes at 55°C. The MO BIO and Zymo extractions were performed according to the manufacturers' instructions. All extractions were stored at -20°C.

Two PCR reactions were run on each extraction using a different Taq polymerase enzyme (AccuPrime *Pfx* SuperMix™ (Invitrogen, cat. no. 12344040) and 5 PRIME HotMasterMix (Quanta Biosciences, cat. no. 10052-240)) to amplify the V4 region of the 16S rRNA genes in each sample. Each sample was barcoded with a different pair of indexes as described by Kozich, et al. (Kozich et al., 2013) and amplified on the C1000 Touch™ thermal cycler from Bio-Rad (Hercules, CA) using unique pairs of A/B5 primers and A7 primers. All combinations from two body sites, three extraction methods, and two Taq polymerases were created.

A subset of samples were run on a gel to confirm amplification, and the samples were then normalized with the SequalPrep™ Normalization kit (Applied Biosystems, Foster City, CA) and sequenced on a single lane on the Illumina MiSeq 2x250 platform.

OTU Picking

Sample reads were quality filtered using default parameters in QIIME (Caporaso et al., 2010). Open-reference OTU picking was then performed in QIIME at 97% similarity using uclust (Edgar, 2010), and PyNAST (Caporaso et al., 2010) was used to align the reads to the GreenGenes Core reference alignment (DeSantis et al., 2006). RDP Classifier 2.2 (Wang et al., 2007) and a GreenGenes reference base (McDonald et al., 2012; Werner et al., 2012) were used to assign reads to the GreenGenes taxonomy, and FastTree 2.1.3 (Li and Durbin, 2009) was used to build a phylogenetic tree. To enable comparisons to be made between samples, the OTU table was subsampled to 4,410 reads. Due to subsampling, a number of samples that failed to amplify adequately were removed from further analyses; for the fecal samples, the 5 PRIME enzyme

failed to amplify all MO BIO-extracted and four salting out-extracted samples, so the fecal 5 PRIME-amplified MO BIO-extracted and salting out-extracted samples were excluded from subsequent analysis. A single AccuPrime-amplified fecal sample extracted through salting was also precluded from further analysis as it failed to meet the 4,410-read cutoff.

Statistical Analysis

Alpha and beta diversities were analyzed using QIIME's core diversity analysis function, which also supplied distance matrices, taxa summaries, rarified OTU tables and principal coordinate analysis plots. To determine whether groups of samples differed from one another, adonis was run on enzyme*extraction method*body site groups using unweighted Unifrac distance matrices (Lozupone and Knight, 2005). If a significant value returned ($p < 0.05$), these distance matrices were then used to create linear models in R (distance ~ enzyme*extraction method*body site); ANOVA was performed on each model to discover which groups had different microbiomes. Using R's multcomp package, a Tukey-corrected general linear hypothesis was used to test each linear model for multiple corrections. Significance was determined at false discovery rate (FDR)-corrected values of $p < 0.05$.

Microbial abundance comparisons were run separately for C1 and fecal samples and were made using rarified OTU tables and R's multcomp package. At each taxonomic level (phylum – species), read counts for each extraction method-enzyme combination were summed; taxa with abundant read counts ($\geq$ 500 reads in C1 samples; $\geq$ 317 reads in fecal samples) were compared using linear models (read count ~ extraction method * enzyme) and generalized linear hypotheses to determine whether the abundance of each taxon differed between groups. Resultant p-values were FDR-corrected. Compact letter displays (level = 0.05) were created

using linear models for taxa with significant ($p < 0.05$) FDR-corrected p-values to determine to determine which Taq polymerase or extraction method led to an increased abundance of that taxon; ANOVA was performed on separate linear models for taxa that displayed differential abundance by extraction method (read count ~ extraction method) or enzyme (read count ~ enzyme). Differential abundance by extraction method was compared separately for AccuPrime-amplified and 5 PRIME-amplified C1 samples, and only compared for AccuPrime-amplified fecal samples. C1 differential abundance by enzyme was compared separately for the MO BIO, salting, and Zymo methods of extraction; for the fecal samples, differential abundance by enzyme was only compared for the Zymo-extracted samples.

RESULTS

Effects of DNA Extraction and Amplification Methods on Community Recovery

To test how technical differences in alpaca sample preparation and sequencing influence the detected sequences, we compared Illumina reads obtained by three DNA extraction procedures and two Taq polymerase enzymes from each of ten alpaca GI samples (a C1 and a fecal sample from each of 5 alpacas). A total of 2,117,417 reads were obtained and subsampled to a depth of 4,410 reads per sample to compare the microbiome compositions of samples prepared with different DNA-extraction methods and enzymes using unweighted Unifrac analysis.

We compared the Unifrac distances of the C1 and fecal samples to determine whether the extraction methods and enzymes influenced the microbiota recovered from the each sample type. Principal coordinate analysis (PCoA; Fig. 1) revealed samples clustered by Taq polymerase (driven by principal coordinate 2; Fig. 1A and 1B), confirmed as significant differences in

unweighted Unifrac distance between the samples by ANOVA. Principal coordinates 1 and 3 did not appear to drive differences between polymerase enzymes (Fig. 1C). PCoA and ANOVA confirmed that there were significant differences between the Unifrac distances of salting out and MO BIO kit samples for feces ($p < 1e\text{-}5$; Fig. 2A), but there were no kit-specific effects on the C1 microbiome composition (Fig. 2B).

To identify bacterial taxa that were significantly influenced by the sample preparation methods, we compared the abundances of every taxonomic level from phylum to species. Amplification with the AccuPrime enzyme led to increased *Firmicutes* abundance, particularly in the *Clostridiales,* relative to the 5 PRIME enzyme (Table 1). The 5 PRIME enzyme led to greater amplification of *Spirochaetes* in MO BIO- and salting out-extracted C1 samples. There were also extraction method-specific effects on various microbes. Although extraction method did not significantly influence community composition in the C1, there were significant differences in the abundances of certain taxa: salting out extractions led to higher levels of *Euryarchaeota*, *Firmicutes*, and *Chloroflexi*, whereas the MO BIO kit samples yielded more *Bacteroidetes* reads when amplified with AccuPrime and more *Proteobacteria* reads with the 5 PRIME enzyme, and the Zymo kit increased extraction of the spirochaete *Treponema* (Table 2). The 5 PRIME enzyme also yielded greater amplification of *Spirochaetes* in MO BIO- and salting out-extracted C1 samples. Among the fecal samples, the salting out method led to significantly more *Actinobacteria*, *Euryarchaeota*, and *Firmicutes* reads than the MO BIO kit, which reported increases in *Bacteroidetes* and *Spirochaetes* (Table 3). Overall, the salting out method overwhelmingly extracted more gram-positive bacteria, while the MoBio kit tended to extract more gram-negative bacteria.

There were no enzyme- or extraction method- dependent differences in Alpha diversity (Chao1, Shannon, and Simpson; $p > 0.05$), suggesting that the different parameters tended to influence the abundances, but not necessarily presence, of various types of bacteria.

DISCUSSION

Our goal has been to investigate the influence of different DNA extraction methods and amplification enzymes on the detected microbiome composition of C1 and fecal samples from alpacas. The enzyme used led to detection of different microbial communities in both the C1 and feces, suggesting more reliable results with the AccuPrime enzyme as 5 PRIME led to a majority of failed PCR reactions on fecal samples. In contrast, DNA extraction method only influenced the detected microbial communities of fecal samples, with different phyla favored by either the MO BIO kit or salting out method. Taken together, these findings reveal microbiomes vary with body site and diet, and confirm the importance of controlling sample preparation method. This also suggests that care must be taken when comparing microbiome samples to other samples in genomic databases. comparisons between digesta samples of different species that have been amplified with different Taq polymerase enzymes will likely give inaccurate results, as observed differences may be the result of either species or enzyme; however, samples amplified with the same enzyme and extracted with either the MO BIO or Zymo kits can probably be compared. There were significant body site, kit, and extraction method effects, demonstrating influence of each factor, and our analysis revealed that the Zymo extraction kit (which is both less expensive than and gives results comparable to the MO BIO kit, a standard in the field) and the AccuPrime polymerase are ideal for future analyses. Further research with other camelids and ruminants may address whether the present findings apply to all foregut fermenters. . Additionally, studies

using mock communities of known microbial composition may indicate the accuracy of each extraction method and enzyme at representing a true community (Willner et al., 2012).

Our results showed some similarities to other studies on technical parameters and microbial recovery. The Zymo kit returned fewer reads assigned to *Bacteroidales* and a higher abundance of *Clostridiales*, mirroring the increase in *Firmicutes* and decrease in *Bacteroidetes* seen with the Zymo kit in a number of studies (Henderson et al., 2013; Janabi et al., 2016; Wagner Mackenzie et al., 2015). This may be attributed to the longer bead beating period in the Zymo protocol, as mechanical lysis is preferable for lysing gram-positive bacteria, which is presumed to be difficult to lyse (Nesme et al., 1995; Roose-Amsaleg et al., 2001). Increased bead beating time has been observed to increase both total DNA extraction and the *Firmicutes:Bacteroidetes* ratio (Burbach et al., 2016; Henderson et al., 2013); a shorter period of mechanical lysis would extract less DNA, with a greater proportion made up of the more easily lysed gram-negative bacteria (Henderson et al., 2013; Olson and Morrow, 2012). Additionally, two of the studies reported that the Zymo kit returned the highest abundance of *Spirochaetes* (Henderson et al., 2013; Janabi et al., 2016). While our results had some similarities with previous studies in regards to overall microbial community and differentially recovered taxa, we also had novel findings including the increase in *Euryarchaeota* found with the salting out procedure.

The salting out procedure yielded more gram-positive bacteria in comparison to the other two DNA extraction methods, particularly the MO BIO kit. Unlike the Zymo and MO BIO extraction methods, which both used mechanical and chemical lysis, the salting out protocol included an enzymatic lysis step using lysozyme. Because lysozyme breaks the glycosidic bonds in peptidoglycan, the peptidoglycan-rich cell walls of gram-positive bacteria are readily lysed

(Shehadul Islam et al., 2017). This likely explains the greater abundance of gram-positive bacteria in the samples extracted by salting.

This work identified kit- and enzyme-specific effects on the abundance of detected microbes, consistent with the expectation that variation in technical parameters influences microbiome composition (Aird et al., 2011; McOrist et al., 2002). The superior performance of the AccuPrime *Pfx* SuperMix relative to 5 PRIME enzyme caused us to focus most of our attention on the former enzyme. Although there were numerous differences in abundance of individual taxa between the extraction methods, the overall microbiome composition as measured by unweighted Unifrac analysis was not significantly different between the Zymo kit and either of the salting out or MO BIO methods (though salting out and MO BIO were different from each other). Across mammalian fecal microbiome studies the hallmark observation is the dominance of *Firmicutes* and *Bacteroidetes*, and it is interesting to note that whereas the samples extracted using the MO BIO kit had approximately a 1:1 ratio of *Firmicutes* : *Bacteroidetes*, samples extracted with the salting out method had a ratio of closer to 5:1. These findings may be relevant to the discussion if the *Firmicutes:Bacteroidetes* ratio predicts obesity (Ley et al., 2006), as use of one extraction method over another may determine whether a correlation is observed (Burbach et al., 2016; Pedersen et al., 2013). Regardless, since the MO BIO kit is a field standard our findings suggests that either MO BIO or Zymo kits in combination with AccuPrime enzyme are preferable to obtain results that will be most compatible with other samples in metaanalyses.

ACKNOWLEDGEMENTS

We acknowledge Kyle Nelson for preparing the samples for sequencing. Funding was provided by BYU startup funds.

TABLES

Table 1-1. Differentially abundant taxa by enzyme, C1 samples
Taxa under each enzyme column exhibit increased abundance with amplification using that enzyme. Differential abundance was determined separately for MO BIO-extracted C1 samples, salting out-extracted C1 samples, and Zymo-extracted C1 samples. FDR-corrected p-values and read count are reported for each taxon. Redundant taxa (taxa significant at multiple phylogenetic levels) are only reported at the lowest taxonomic level they are significant at.
$^{-}$ Taxa are/tend to be gram-negative
$^{+}$ Taxa are/tend to be gram-positive
* Archaea
$^{\#}$ Other

		ACCUPRIME	5 PRIME
MO BIO	Order	*Bacteroidales*$^{-}$ (p = 7.5e-5; 37507) *Clostridiales*$^{+}$ (p = 3.3e-10; 28635)	NA
	Family	*Lachnospiraceae*$^{+}$ (p = 1.5e-6; 5248) *Ruminococcaceae*$^{+}$ (p = 5.3e-11; 5437) *Veillonellaceae*$^{-}$ (p = 6.6e-4; 7529)	NA
	Genus	*Prevotella*$^{-}$ (p = 4.7e-6; 19366) *Ruminococcus*$^{+}$ (p = 4.7e-4; 1547) *Succiniclasticum*$^{-}$ (p =6.7e-3; 3507) *Butyrivibrio*$^{+}$ (p = 3.4e-3; 1931) *Clostridium*$^{+}$ (p = 3.3e-2; 552)	*Treponema*$^{-}$ (p = 3.4e-3; 2078) *Desulfovibrio*$^{-}$ (p = 3.4e-3; 945)
	Species	*Flavefaciens*$^{+}$ (p = 1.4e-3; 635)	NA
SALTING OUT	Order	*Clostridiales*$^{+}$ (p = 3.3e-10; 28635)	NA
	Family	*Christensenellaceae*$^{-}$ (p = 1.5e-3; 799) *Lachnospiraceae*$^{+}$ (p = 1.5e-6; 5248) *Methanobacteriaceae** (p = 2.8e-2; 4926) *Ruminococcaceae*$^{+}$ (p = 5.3e-11; 5437) *Veillonellaceae*$^{-}$ (p = 6.6e-4; 7529)	NA
	Genus	*Methanobrevibacter* (p = 4.6e-2; 4332) *Prevotella*$^{-}$ (p = 4.7e-6; 19366)	*Treponema*$^{-}$ (p = 0.0034; 2078)
	Species	*flavefaciens*$^{+}$ (p = 1.4e-3; 635)	NA
ZYMO	Phylum	NA	*Proteobacteria*$^{-}$ (p = 8.1e-4)
	Class	NA	*Deltaproteobacteria*$^{-}$ (p = 4.9e-4; 1295) *Mollicutes*$^{\#}$ (p = 1.3e-3; 1224)

	Order	*Bacteroidales*$^{-}$ (p = 7.5e-5; 37507) *Clostridiales*$^{+}$ (p = 3.3e-10; 28635)	*RF39*$^{\#}$ (p = 3.3e-3; 1019)
	Family	*Christensenellaceae*$^{-}$ (p = 1.5e-3; 799) *Lachnospiraceae*$^{+}$ (p = 1.5e-6; 5248) *Ruminococcaceae*$^{+}$ (p = 5.3e-11; 5437) *Veillonellaceae*$^{-}$ (p = 6.6e-4; 7529)	NA
	Genus	*Butyrivibrio*$^{+}$ (p = 3.4e-3; 1931) *Prevotella*$^{-}$ (p = 4.7e-6; 19366) *Ruminococcus*$^{+}$ (p = 4.7e-4; 1547) *Succiniclasticum*$^{-}$ (p =6.7e-3; 3507)	NA
	Species	NA	NA

Table 1-2. Taxa differentially abundant by DNA extraction method; C1 samples
Taxa under each extraction method column exhibit increased abundance. Differential abundance was determined separately for AccuPrime-amplified and 5 PRIME-amplified C1 samples.
[−] Gram-negative
[+] Gram-positive
[*] Archaea
[#] Other

		MO BIO	SALTING OUT	ZYMO
ACCUPRIME (C1)	Phylum	NA	*Euryarchaeota*[*] (1.5e-7; 5989)	NA
	Class	NA	NA	NA
	Order	*Bacteroidales*[−] (p = 8.3e-4; 37507)	NA	NA
	Family	*BS11*[−] (p = 1.0e-2; 2022) *Paraprevotellaceae*[−] (p = 6.9e-3; 4792)	*Coriobacteriaceae*[+] (p = 3.1e-8; 551) *Ruminococcaceae*[+] (p = 2.6e-4; 5437)	*Veillonellaceae*[−] (p = 9.7e-2; 7529)
	Genus	*Succiniclasticum*[−] (p = 2.4e-6; 3507) *vadinCA11*[−] (p = 9.2e-4; 1063)	*Methanobrevibacter*[*] (p = 4.5e-8; 4332) *Mogibacterium*[+] (p = 1.4e-7; 901) *SHD*-231[#] (9.2e-4; 1128)	*Treponema*[−] (p = 5.1e-8; 2078)
	Species	*flavefaciens*[+] (p = 4.2e-4; 635)	NA	*flavefaciens*[+] (p = 4.2e-4; 635)
5 PRIME (C1)	Phylum	NA	*Euryarchaeota*[*] (1.5e-7; 5989)	NA
	Class	NA	NA	*Mollicutes*[#] (p = 3.7e-3; 1224)
	Order	NA	NA	*RF39*[#] (p = 2.5e-2; 1019)
	Family	NA	*Coriobacteriaceae*[+] (p = 3.1e-8; 551)	NA
	Genus	*Succiniclasticum*[−] (p = 2.4e-6; 3507) *vadinCA11*[−] (p = 9.2e-4; 1063)	*Methanobrevibacter*[*] (p = 4.5e-8; 4332) *Mogibacterium*[+] (p = 1.4e-7; 901) *SHD*-231[#] (9.2e-4; 1128)	*Desulfovibrio*[−] (p = 9.6e-3; 945) *Ruminococcus*[+] (p = 1.1e-2; 1547) *Treponema*[−] (p = 5.1e-8; 2078)
	Species	*Flavefaciens*[+] (p = 4.2e-4; 635)	NA	*flavefaciens*[+] (p = 4.2e-4; 635)

Table 1-3. Taxa differentially abundant by DNA extraction method; fecal samples
Taxa under each extraction method column exhibit increased abundance in comparison to the other columns.
[−] Gram-negative
[+] Gram-positive
[*] Archaea
[#] Other

		MO BIO	SALTING OUT	ZYMO
ACCUPRIME (FECES)	Phylum	NA	*Euryarchaeota*[*] (p = 7.7e-4; 5540)	NA
	Class	NA	*Bacilli*[+] (p = 3.4e-2; 732)	NA
	Order	*Bacteroidales*[−] (p = 6.6e-5; 21030)	*Clostridiales*[+] (p = 3.4e-3; 24102)	*Clostridiales*[+] (p = 3.4e-3; 24102)
	Family	*Bacteroidaceae*[−] (p = 9.1e-4; 8269) *Paraprevotellaceae*[−] (p = 4.1e-3; 3639) *Rikenellaceae*[−] (p = 2.4e-3; 980)	*Coriobacteriaceae*[+] (p = 1.9e-4; 1125) *Lachnospiraceae*[+] (p = 4.1e-3; 1885) *Mogibacteriaceae*[+] (p = 1.9e-4; 2768)	*Bacteroidaceae*[−] (p = 9.1e-4; 8269) *Rikenellaceae*[−] (p = 2.4e-3; 980)
	Genus	5-*7N15*[−] (p = 1.2e-3; 2022) *CF231*[−] (p = 1.2e-5; 1810) *Phascolarctobacterium*[#] (p = 4.9e-2; 1041) *Treponema*[−] (p = 1.4e-2; 646)	*Butyrivibrio*[+] (p = 2.5e-3; 443) *Methanobrevibacter*[*] (p = 9.2e-4; 4507) *Mogibacterium*[+] (p = 9.8e-5; 1375)	5-*7N15*[−] (p = 1.2e-3; 2022)
	Species	NA	NA	NA

FIGURES

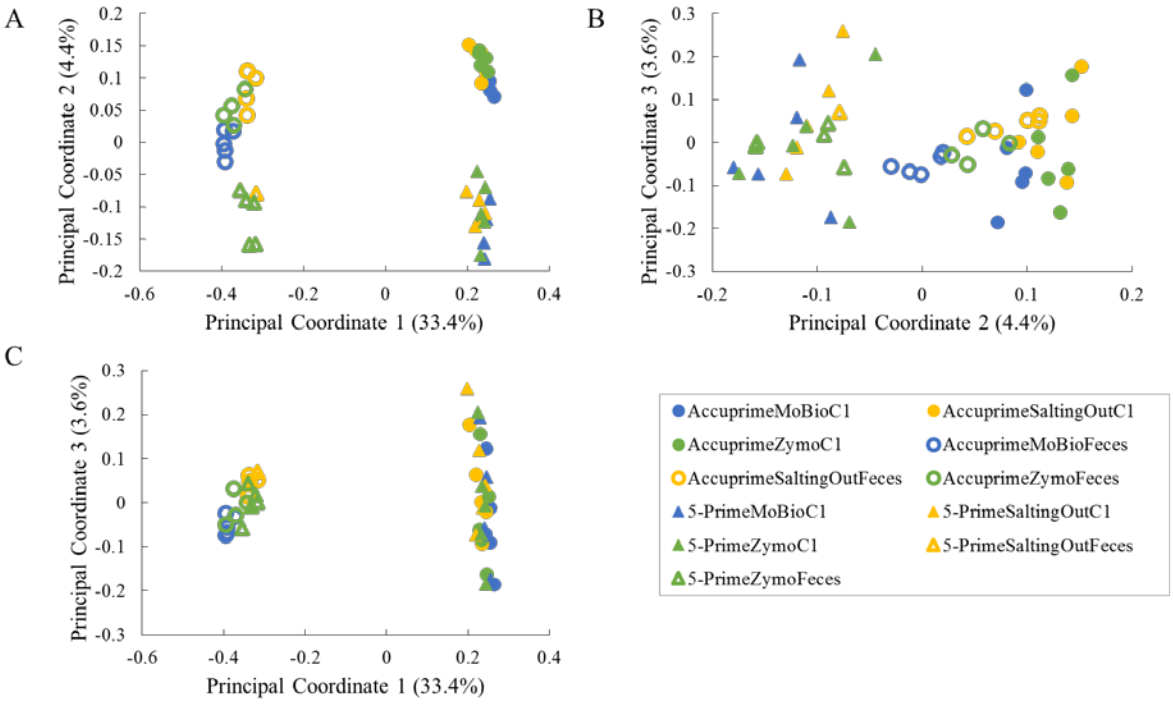

Figure 1-1. Alpaca C1 and fecal microbiomes group by Taq polymerase enzyme
PCoA plots were created using the top three principal coordinates; percentage of variation explained by each principal coordinate is reported next to the axis titles. Principal coordinate analysis of A) all samples using principal coordinates 1 and 2; B) all samples using principal coordinates 2 and 3; and C) all samples using principal coordinates 1 and 3.

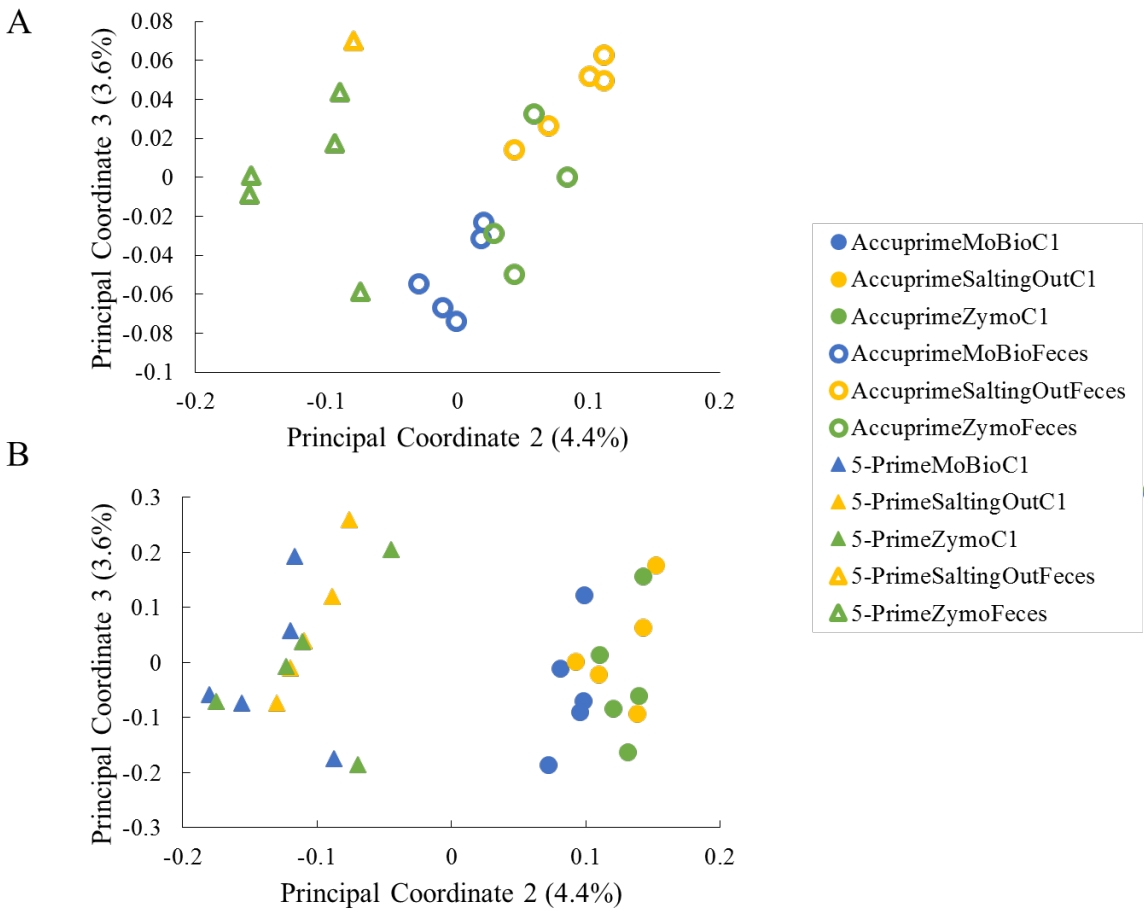

Figure 1-2. Alpaca fecal microbiomes group by extraction method
PCoA plots were created using principal coordinates 2 and 3; percentage of variation explained by each principal coordinate is reported next to the axis titles. Fecal and C1 samples were plotted separately for visual clarity. Principal coordinate analysis of A) fecal samples and B) C1 samples.

CHAPTER TWO

Survey of the alpaca digestive tract microbiota under different dietary regimes and its relationship to body condition

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ABSTRACT

Animal-associated microbes ('microbiota') have key impacts on the nutrition of their hosts, especially in ruminants and pseudoruminants that consume high-cellulose diets. Examples include the pseudoruminant alpaca, which is economically important in Peru and especially to impoverished communities in the high Andes. To better understand how body site and diet influence the alpaca microbiota we performed two 16S rRNA gene surveys. In the first, we surveyed six sites along the digestive tract of alpacas fed a grass hay (GH; tall fescue) or alfalfa hay (AH) diet for 30 days. In the second we performed a compartment 1 (C1) survey of alpacas fed a series of two-week mixed grass hay (MGH) diets supplemented with barley, quinoa, amaranth, or soybean meal. Samples from GH- and AH-fed alpacas grouped by diet and body site but none of the four supplements significantly altered C1 microbiome composition, relative each other. To explore the relationship between alpaca energy extraction and the microbiota we calculated correlations between operational taxonomic units (OTUs) and volatile fatty acid (VFA) abundances in matched alpaca C1 samples. We found no significant correlations between VFA and OTU abundance, and further could not identify any OTUs that were differentially abundant between alpacas with normal versus poor energy extraction. Taken together, our findings of diet- and body-site specific alpaca microbiota are consistent with previous findings in

ruminants and other mammals but failed to provide evidence that links changes in alpaca body condition with variation in microbiota abundance or identity.

IMPORTANCE

Alpacas have long been economically important in the South American countries of Peru and Bolivia, which together contain 99% of the world's alpaca population, where alpacas are raised mainly for their luxurious fibers and meat. Pseudoruminant camelids (camels, llamas and alpaca) depend on cellulolytic microbes in their gastrointestinal tracts, mainly the forestomach, to access nutrients from otherwise indigestible plant material they consume. The camelid foregut, but not other gastrointestinal tract sites, has been the subject of previous microbiome surveys. In this study, we identified distinct microbiomes at different sites in the alpaca digestive tract. We also identified microbial taxa that are more abundant with specific diets. However, we failed to identify any bacterial OTUs that are significantly correlated with energy extraction or that were differentially abundant between animals that naturally maintain healthy versus unhealthy body weights. Together, these findings provide no evidence for a microbial role in poor body condition of alpacas.

INTRODUCTION

The alpaca (*Vicugna pacos*) is a South American camelid of industrial and household importance, especially in Peru and Bolivia. For example, 90% of the world's alpaca fiber (hair) production for use in the textile industry comes from Peru (Paredes et al., 2013). However, it is the communities of Peru's high Andes that are arguably most dependent on these animals. Many people within these highly impoverished communities are alpaca herders and depend on alpaca

fiber for clothing, income, and meat. Alpaca meat is highly nutritious, low in cholesterol, and an important source of protein for rural families in these areas, and family livelihood depends critically on herd health, size, and productivity (Cristofanelli et al., 2005).

Ruminants and camelids rely on the microbes in their gastrointestinal (GI) tracts to access energy and nutrients from the plant material they consume. In ruminants, the forestomach is especially important because its first chamber, the rumen, acts as a fermenting chamber for microbial degradation of otherwise indigestible vegetation. Unlike true ruminants which make use of a four-chambered forestomach, the camelid family is classified as a pseudo-ruminant and possess a three-compartment forestomach. The first two compartments, of which the first (C1) comprises most of the volume, function similarly to the rumen/reticulum and omasum of true ruminants (Vallenas et al., 1971). Due to their greater feed retention time (San Martin and Bryant, 1989), increased microbial yield, and presence of glandular saccules in the forestomach, camelids possess a higher efficiency of fiber degradation when compared with ruminants, particularly when fed low-quality, low-protein forages (Genin and Tichit, 1997; Rübsamen and von Engelhardt, 1979; Van Soest, 1994). Volatile fatty acids (VFAs) - primarily acetic, propionic, and butyric acid - are released as a by-product of the microbial fermentation in the C1 or rumen (Stevens et al., 1980) and are used by camelids and ruminants as a major energy source (Bergman, 1990).

It has been shown that adequate energy balance, a health factor linked to body condition (Pryce et al., 2001), is crucial to reproductive success and survival in both ruminants (Pryce et al., 2001) and alpacas (Van Saun, 2008). Alternatively, low body condition (LBC) or negative energy balance can be associated with low productivity of the herd (Kristjanson et al., 2007; Reyna, 2005). Even within a well-conditioned alpaca herd, a number of animals tend to exhibit

chronic LBC despite efforts to treat all animals equally in regards to factors such as deworming, vaccination, and access to feed and water. Because the microbiota is postulated to influence energy balance (Bäckhed et al., 2005; Wang et al., 2012), we sought to define the alpaca microbiome as a way to better understand its contributions to alpaca nutrition.

Little is known about the composition of the alpaca microbiota outside of C1 (Henderson et al., 2015; Pei et al., 2010; Pei et al., 2013) or how it responds to dietary perturbation. To test the prediction that the alpaca microbiota varies with both body site and diet, we surveyed five sites along the digestive tract of ten time-, age- and herd-matched alpacas fed one of two forage diets (5 animals per diet). We tested for influence of minor dietary variation on the microbiome by surveying the C1 of a second alpaca cohort (5 alpacas total) all fed the same staple diet supplemented sequentially with different natural grains. We also tested if any microbial operational taxonomic units (OTUs) were significantly correlated with differences in energy extraction between the different animals. Finally, based on evidence for microbial OTU abundance correlations with VFAs, we tested the prediction that the C1 microbiota of LBC and GBC alpacas differs. Overall, there was variation in the alpaca gastrointestinal microbiome with both body site and with some, but not all administered diets. However, despite finding OTUs that were correlated with changes in C1 energy extraction the C1 microbiomes of GBC and LBC alpacas were not different, suggesting that variation in identity and abundance of the microbiota may not be a key determinant of alpaca body condition.

MATERIALS AND METHODS

Sample Collection

Two experiments were designed in accordance with animal care and use guidelines (McGlone et al., 2010) and with approval of The Camelid Center and BYU Animal Use Committee (#16-1104). Within each experiment, alpacas were treated equally in regards to environment and feed administration. Digesta samples were taken upon completion of each experiment. All samples within an experiment were taken at the same time and from the same location within each organ unless otherwise noted.

Forage diet experiment

Ten adult male alpacas (3+ years old; 65 kg BW) were divided into two groups. Each group was fed a different diet (grass hay [GH; tall fescue, *Festuca arundinacea*] or alfalfa hay [AH; *Medicago sativa*]) for thirty days. The alpacas were housed in drylot paddocks and fed once daily *ad libitum.* They were also provided with water and a commercial free-choice salt and mineral supplement *ad libitum.* The animals were sacrificed at a commercial slaughtering facility two hours post-feeding on the last day of the trial. The digestive tract was removed as quickly as possible and divided into C1-3, duodenum, jejunum, ileum, cecum, and large intestine. Digesta samples were taken from C1 and from each of the intestinal subsections and stored at -20ºC.

Grain supplement experiment

Four C1-fistulated male (7± 1.5 years old; 61kg BW) alpacas were fed a series of 5 diet treatments in a random order: mixed grass hay (MGH; orchard, *Dactylis glomerata*; meadow bromegrass, *Bromopsis biebersteinii*; smooth bromegrass, *Bromus inermis*), and MGH

supplemented with barley, amaranth, quinoa, or soybean meal (SBM) (Table S1) (Nilsen et al., 2015). Prior to the start of the experiment, the animals were acclimated to a MGH diet which was fed *ad libitum* at 0700 h for thirty days. During the acclimation period, water was also provided *ad libitum*. During the trial phase each alpaca was fed each of the 5 diet treatments, and no alpaca was fed the same diet twice. Three hours post-feeding on day 14 of each treatment period, fluid from the C1 caudal region was collected through the fistula via a rumen sampler tube and stored at -20ºC for microbiome analysis.

Body condition experiment

Eighteen adult (~ 8 years old) female alpacas were randomly selected for C1 sampling and body condition scoring. All individuals within the herd were fed on a mixed grass pasture for 90 days prior to being switched to a MGH diet for 30 days, provided with water ad libitum, examined by a veterinarian, and current on vaccinations and free of internal parasites. C1 samples were collected using an orogastric tube and stored at -20ºC for microbiome analysis. Each alpaca was assigned a body condition score (BCS) of 1-5 with a BCS of 1 indicating very low body condition and a BCS of 5 representing very high body condition. Scores were assigned by palpating the hip bones and lumbar and thoracic vertebrae of each animal (Fowler, 1998). Two separate DNA extractions and PCR amplifications were performed on these samples.

DNA Extraction

Microbial DNA was isolated from each sample in the forage diet and supplement experiments using the PowerFecal® DNA Isolation Kit (MO BIO, Carlsbad, CA). Body condition experiment samples were extracted using the ZR-96 Fecal DNA Kit™ (Zymo

Research). Analysis of five paired C1 samples from which DNA was extracted by each of the Zymo ZR Fecal DNA MiniPrep and MO BIO DNA extraction kits revealed no extraction-method-dependent differences in microbiome composition (Olsen, Carroll, and Chaston, unpublished data).

PCR

DNA was prepared for 16S rRNA gene V4 region sequencing exactly as described previously (Kozich et al., 2013). Briefly, the V4 region of the 16S rRNA gene was amplified individually from each sample with AccuPrime *Pfx* SuperMix (Invitrogen; Carlsbad, CA) using a dual-indexing strategy as described previously (Kozich et al., 2013) (Table S2). Samples were normalized using the SequalPrep Normalization kit (Applied Biosystems; Waltham, MA). Sample QC and preparation for sequencing was performed in the BYU DNA Sequencing center, and samples plus 10% PhiX control DNA were sequenced with the Illumina MiSeq 2x250 v2 kit at the U. of Wisconsin-Madison or on a HiSeq 2500 at the BYU DNA Sequencing center, following manufacturer's recommendations. Sequences are in the process of being deposited to the SRA.

Illumina Sequencing and Analysis

Sample reads were demultiplexed on the Illumina platform and quality filtered using default parameters in QIIME (Caporaso et al., 2010). Open-reference OTU picking was performed using uclust (Edgar, 2010) with OTUs grouped at 97% similarity. The reads were aligned to the GreenGenes Core reference alignment (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010). Taxonomy was assigned according to the GreenGenes taxonomy using

RDP Classifier 2.2 (Wang et al., 2007) and a GreenGenes reference base (McDonald et al., 2012; Werner et al., 2012) and the phylogenetic tree was built with FastTree 2.1.3 (Price et al., 2010). OTUs in each experiment were subsampled to allow for comparisons between samples. For the forage experiment, diet-dependent changes in the microbiome were calculated from an OTU table that was subsampled to 4,890 reads per sample, leading to the discarding of all duodenum samples and one jejunum sample. In the same experiment, correlations between VFAs and OTU abundances in the C1 samples only were calculated from an OTU table subsampled to 6,700 reads. The OTU table in the supplement experiment was subsampled to 11,010 reads per sample to include all samples. In the body condition experiment, OTU read counts in a sample were discarded if the reagent-only controls had higher read counts. In the first and second sets, reads were subsampled to 7,400 and 74,000 reads per sample, respectively.

Statistical Analysis

Statistical analyses were performed in QIIME. Unless otherwise specified, beta diversity was calculated using unweighted Unifrac distance (Lozupone and Knight, 2005) and differences between samples were confirmed by adonis. If there was a significant group effect ($p < 0.05$), linear models of the distance matrices were calculated to discover which groups had different ($p<0.05$) microbiomes using the R multcomp package (Hothorn et al., 2008). Custom R scripts were used to test for differences in OTU abundance in the alpacas fed an AH or GH diet. Because different experiments had varying read totals, we analyzed each experiment using a different set of cutoff parameters (see figure legends for details). Total microbial reads attributed to different taxonomic levels (phylum through OTU ID) were compared between groups using linear models to test if the abundance of each taxon differed between groups. P-values were

FDR-corrected within each taxonomic level and, for diet x body site effects with FDR $p < 0.05$, post-hoc Tukey tests were performed to define differences within the group (Hothorn et al., 2008). Differences in OTU abundance were determined by FDR-corrected Welch's two-sample t-tests (FDR $p < 0.05$). The Spearman rank correlations between BCS and taxonomic abundances, clustered at the phylum through OTU levels, were calculated and FDR-corrected (FDR $p < 0.05$) in R.

VFA Correlations

A subset of each sample from the forage diet experiment, which were from a mixture of solid and liquid material, was fractionated via centrifugation and the supernatant was stored at -20°C for microbiome and VFA analyses. The fractionated supernatant samples were sequenced as described previously. VFA composition from these samples were previously defined by gas chromatography (GC) and values presented are exactly from our previous report (Oldham et al., 2014). The Spearman rank correlation between microbial abundances in C1 samples from the forage experiment, clustered at the phylum through OTU levels, and absolute VFA levels (mM acetate, butyrate, or propionate) were calculated and false-discovery rate (FDR) corrected in R for all taxonomic groups that bore at least 100 reads across all the samples.

RESULTS

Diet- and Body-Site Specific Microbiomes in the Alpaca

To better understand how body site and diet contribute to differences in the alpaca microbiome, we performed a 16S rRNA gene survey of 6 sites along the digestive tract of alpacas fed grass hay (GH) or alfalfa hay (AH) diets. Digesta samples were taken from each alpaca's C1 compartment, duodenum, jejunum, ileum, cecum, and large intestine. A total of

1,222,207 reads were obtained on a partial Illumina MiSeq lane, with an average of 17,974 reads per sample and 13,244 total OTUs.

We performed principal coordinate analysis (PCoA) and adonis tests using unweighted Unifrac distances to compare the microbiome composition of different alpaca body sites in alpacas fed different diets. At a 4,890-read subsampling depth, the samples grouped by body site in a PCoA (Fig. 1A). Principal coordinates 1 (30.19%) and 2 (18.06%) separated the samples into three general locations within the digestive tract- the C1 compartment; the small intestine (jejunum and ileum); and the distal intestine (cecum and large intestine). Duodenum samples were excluded because they uniformly bore few reads, but a PCoA with shallower subsampling to include the duodenum samples showed similar trends and that the duodenum samples clustered with ileum and jejunum samples (Fig. S1). Only one negative control yielded enough reads to meet the subsampling depths and was visually separated from the digestive tract samples in the PCoA plot (data not shown). Unweighted Unifrac distance analysis confirmed the visual differences in the microbiota composition between but not within each general location (Fig. S2 and S3). Differences between communities could be attributed in part to the phyla dominating different sites, including *Bacteroidetes* in the C1 and large intestine; and *Actinobacteria* and *Euryarchaeota* in the small intestine (Fig. 1B and 1C, Fig. S1). *Firmicutes* were abundant in all body sites. At the family level, *Ruminococcaceae* were dominant in the C1 and distal intestine while *Lachnospiraceae* was most abundant in the C1 and small intestine. *Prevotellaceae* was abundant in the C1 and *Methanobacteriaceae* was dominant in the small intestine (Table 1). These findings are generally consistent with previous C1 and rumen microbiome surveys of alpacas and other ruminants, demonstrating concordance between this and previous work, and extending previous analyses by adding previously unstudied body sites (Espinosa et al., 2015;

Gharechahi et al., 2015; Henderson et al., 2015; Pei et al., 2010; Pei et al., 2013; Samsudin et al., 2011).

Principal coordinate 3 revealed sample separation by diet (Fig. 1A, 4.50% of variance). Unweighted Unifrac distance analysis confirmed that at each of the six body sites, diet significantly influenced the sampled microbial communities (Fig. S2 and S3). Some of the differences were uniform across all body sites: grass hay-fed animals bore higher numbers of *Bacteroides* and *BF311* but fewer *Butyrivibrio*, *Pseudobutyrivibrio* and *Methanosphaera* (Table 2; Table S4). In some cases, such as for *BF311* and the *Methanosphaera,* most genus-level reads were attributable to a single OTU (Table S4). We also detected body-site specific effects of the diet treatments. For example, a GH diet increased *Coriobacteriaceae* and *Prevotella* in the ileum and the C1, respectively, but not in other body sites (Fig. S4; Table S5). Together, these results demonstrate significant differences in read abundances with both body site and diet.

Grain Supplement Effects on the C1 Microbiome

To test if dietary supplements in MGH diet can alter the C1 microbiota, a 16S rRNA gene survey was conducted on C1 material from C1-fistulated male alpacas fed MGH or MGH supplemented with one of amaranth, quinoa, barley, or soybean meal. At the end of each two-week period, liquid from the C1 was sampled and surveyed by 16S rRNA gene sequencing. 478,392 filtered reads were obtained by Illumina MiSeq sequencing, and reads were subsampled to 11,010 reads per sample; the reagent-only control was not analyzed because it produced only 230 reads. None of the four supplements significantly altered microbiome composition relative to MGH alone or to each other (Fig. 2, Fig. S5). Overall, these results suggest that, unlike a 6-

week regime using tall fescue GH and AH, feeding alpacas a 2-week dietary supplement together with MGH was insufficient to alter their C1 microbiome composition.

C1 Microbes and VFA Abundances

We were interested to test if, consistent with microbial effects on health and weight in other animals, the alpaca microbiome might contribute to alpaca body condition (Henning et al., 2010; Nkrumah et al., 2008; Turnbaugh et al., 2006; Wang et al., 2012). VFAs are a key source of energy in the alpaca C1, and previous work has shown increased VFA extraction in animals fed alfalfa hay (Oldham et al., 2014). We reasoned that the increased VFA extraction might be due to changes in microbial identity or abundance in the C1 of alpacas fed an alfalfa diet. To test the hypothesis that diet-dependent microbiome variation might be correlated with VFA extraction we measured OTU abundances in fractionated rumen fluid of the same AH- and GH-fed alpacas, from which acetate, butyrate, and propionate had been previously measured, and calculated Spearman rank correlations between OTU counts and each VFA's abundance (Table S6). VFA abundances were previously measured from the same samples used in the microbiome analysis (Oldham et al., 2014). Under these conditions no taxa showed any correlation with absolute abundance of acetate, butyrate, or propionate after correcting for multiple tests.

One concern with the approach above is that very few correlations would be expected to yield significant p-values with our small sample size (N=3 AH, 5 GH samples) and thus a lack of significant p-values might not reflect underlying biological variation. To test if increasing the sample size led to detection of significant correlations we recalculated correlations after adding a second set of microbiome measures. The microbiomes in the second set of samples were from the samples in the forage diet experiment, which had been measured from the unfractionated

portion of the same rumen samples tested in the VFA analysis above. Both the first set and second set of microbiome samples were compared to VFA measures from the fractionated C1 material (i.e. N=8-10 microbiome samples (3-5 from fractionated, 5 from unfractionated) versus 5 VFA measures (all from fractionated)). Using this approach there were few correlations with significant effects, and the largest number were for butyrate abundance (Table 3; Table S6). Three genera were associated with decreased butyrate and more abundant in GH-fed alpacas- *Paraprevotellaceae* genera *YRC22* and *CF231;* and *BF311* ($p = 0.04$), an uncultured genus in *Bacteroidetes* whose growth in rumens is stimulated by nitrate addition (Zhao et al., 2015) (Fig. S6A-C). *CF231* and *BF311* were also correlated with decreased propionate abundances (Fig. S7). *Shuttleworthia*, a genus belonging to the family *Lachnospiraceae*, was the only genus that correlated with increased butyrate abundance (Fig. S6D) and, of the four taxonomic groups that were significantly different between samples, it was the only genus more abundant in AH-fed alpacas. Together, these findings reveal candidate microbial taxa whose abundance is correlated with VFA levels and are candidate strains with possible influence on C1 energy extraction; but come with the caveat of pseudoreplication of the VFA measures in the rank correlation calculations.

Body Condition and the C1 Microbiome

Our previous analyses revealed diet-dependent variation in the alpaca C1 microbiome and identified *Shuttleworthia* abundance is associated with increased energy extraction in alpacas fed a VFA-extraction-promoting diet. We reasoned that if the abundance of *Shuttleworthia* or any other taxonomic group is causally associated with VFA extraction, its abundances would differ between GBC and LBC alpacas. To compare the microbiomes of LBC and GBC alpacas

we performed a 16S rRNA gene survey on C1 samples from 12 LBC alpacas and 6 GBC alpacas. 3,779,411 total reads were sequenced, filtered, and subsampled to 74,000 reads per sample for beta diversity analysis. PCoA of weighted Unifrac distances between samples revealed no visual clustering of the samples by body condition, which was statistically confirmed by adonis ($p = 0.329$) (Fig. 3). Additionally, no taxonomic groupings of reads were differentially abundant between LBC and GBC alpacas, including *Shuttleworthia*, *BF311*, *CF231*, and *YRC22* (Fig S8). Thus, we found no evidence to support the idea that the presence or abundance of any of these four genera or any other bacterial species is associated with variation in alpaca body condition.

DISCUSSION

Our goals were to survey the alpaca digestive tract microbiome, test how diet modifies the detected communities, and identify candidate strains with possible influence on alpaca energy extraction. A survey of six body sites in GH-fed alpacas – C1, duodenum, jejunum, ileum, cecum, and large intestine – revealed that the different body sites clustered into three unique microbiome groups – the C1, small intestine, and distal intestine. Comparisons of age-matched animals from the same herd fed an AH diet revealed significant impacts of diet on gut microbiota composition in each body site. In contrast, supplementing MGH with each of four different grains was insufficient to significantly alter the C1 microbiota, although this interpretation comes with the caveat that our randomized design might have led to confounding effects of diet order and historical contingency. Finally, the C1 genera that correlated with decreased C1 butyrate abundance were more abundant in a diet that promoted lower VFA extraction, and those that correlated with increased butyrate abundance were more abundant in a diet promoting greater VFA extraction (Oldham et al., 2014), initially suggesting a possible

relationship between these microbes and VFA production. This hypothesis was not supported by subsequent finding that no OTUs were differentially abundant between GBC and LBC alpacas. Thus, our work reveals diet- and body site-specific alpaca microbiomes but provides no evidence that microbial identity or abundance alone are significantly associated with alpaca health.

The results of the alpaca microbiome C1 surveys are generally consistent with existing literature and add novel findings about the microbiota of the alpaca small and large intestine. Rumen microbial analyses are common in studies of camelids and various ruminants (Gharechahi et al., 2015; Henderson et al., 2015; Jami and Mizrahi, 2012; Jewell et al., 2015; Jones, 1972; Kong et al., 2010; Li et al., 2012; Pei et al., 2010; Samsudin et al., 2011), but GI data beyond the rumen is sparse with no published studies of the microbial communities in pseudoruminant intestines. The most obvious difference in our study, relative to previous work (de Oliveira et al., 2013; Gharechahi et al., 2015; Henderson et al., 2015; Jami and Mizrahi, 2012; Jewell et al., 2015; Kong et al., 2010; Li et al., 2012; Mao et al., 2013; Mao et al., 2015; Myer et al., 2015; Samsudin et al., 2011), was the greater abundance of archaea within the alpaca gut, particularly the small intestine. This may be attributable to the different primer sets used between studies, as most studies either did not report the presence of archaea in their samples (de Oliveira et al., 2013; Jami and Mizrahi, 2012; Jewell et al., 2015; Kong et al., 2010; Li et al., 2012; Mao et al., 2013; Mao et al., 2015; Myer et al., 2015; Samsudin et al., 2011) or reported on archaeal communities and bacterial communities separately (Henderson et al., 2015). Regardless, the dominant OTUs detected in our analyses -*Firmicutes* and *Bacteroidetes*- are generally consistent with those found in previous studies of ruminants and pseudoruminants (de Oliveira et al., 2013; Gharechahi et al., 2015; Henderson et al., 2015; Jami and Mizrahi, 2012; Jewell et al., 2015; Kong et al., 2010; Li et al., 2012; Mao et al., 2013; Mao et al., 2015; Myer et al., 2015;

Samsudin et al., 2011), and support previous conclusions of broad similarities in the set of microbes shared between the 2 suborders.

Our microbial survey demonstrates the presence of at least three distinct microbial communities within an individual alpaca; the C1, the small intestine (duodenum, jejunum, and ileum), and the distal intestine (cecum and large intestine). The presence of at least three different microbial populations is consistent with previous studies in goats and Holstein dairy cattle that showed differences in the foregut, small intestine, and large intestine or hindgut microbiomes (Mao et al., 2013; Mao et al., 2015). Different nutritional functions are known for these three subsections of the digestive tract. The rumen and C1 use microbes to ferment plant material resulting in the production of the VFAs acetate, propionate and butyrate and are also the site of VFA absorption, whereas the small intestine is responsible for further digestion and absorption of nutrients. The cecum and large intestine are sites of fermentation, VFA production, and water absorption. The presence of different microbial communities in each subsection may contribute to their physiological functions (Mao et al., 2015). For example, *Ruminococcaceae*, which contain numerous species capable of producing short-chain fatty acids by fermenting fiber (Dehority, 1993), were abundant in the alpaca cecum and large intestine in this study, whereas *Prevotella*, a diverse genus of bacteria that vary in their abilities to degrade polysaccharides and proteins (Avguštin et al., 1997), were more abundant in the C1 (see Fig. S4). Focusing studies on these bacteria may reveal more about the physiological processes linked to the digestive tract.

We detected diet-dependent variation in the alpaca microbiome, consistent with the current model that diet strongly shapes mammalian GI-tract microbiota composition (Carmody et al., 2015; Henderson et al., 2015; Muegge et al., 2011). Alpacas fed either AH or GH for 30 days displayed significant differences in microbiome composition at each of the six tested body sites,

most obviously attributable to site-specific shifts in *Bacteroidaceae*, *Prevotella*, and *Actinobacteria*, with significant differences in the less abundant taxa *Tenericutes* and *SR1*. In contrast, supplementing a mixed bromegrass and orchard diet with each of four different grains did not influence the microbiome. However, the non-replicated randomized design of supplement administration makes it difficult to distinguish noise from real biological effects of the supplements. For example, the experiment did not control for the order of diet administration. Therefore, we are unable to completely rule out that addition of minor supplements to the diet influences the microbiome; however, our data suggest that if there is an effect it is smaller in magnitude that the AH vs. GH diet effect.

Because increased fat deposition and body condition may stem from greater VFA production, we sought to identify C1 OTUs that were correlated with VFA abundance. We detected relatively few taxa whose abundance correlated with VFA abundance, and only *Shuttleworthia* was more abundant in AH-fed animals. Its association with an increase in butyrate, a precursor to de novo fatty acid synthesis, is in line with reports of greater rumen butyrate concentrations and increased *Shuttleworthia* abundance in more efficient cattle (Nkrumah et al., 2008). If AH increases *Shuttleworthia* abundance then AH, which is usually considered unhealthy for the alpacas due to its high protein content and concerns that alpacas are overfed protein (Van Saun, 2006), could possibly function as a short-term prebiotic to stimulate fat deposition in low body condition alpacas. This speculation comes with two caveats: first, that our findings do not establish a causal relationship between AH and *Shuttleworthia* abundance; and second, that *Shuttleworthia* was only detected using pseudoreplicated VFA measures. Thus, our findings suggest that intervention with a diet that substantially alters the alpaca microbiota

and VFA extraction (Oldham et al., 2014) relative to a standard GH diet does not lead to commensurate changes in body condition in chronic LBC alpacas.

Regardless of the potential health impacts of *Shuttleworthia* or any other bacterial taxa, we observed no differences between the C1 microbial communities of LBC and GBC alpacas. We found this surprising since the microbiome has been associated with weight or body condition scores in numerous other animals (Nkrumah et al., 2008; Turnbaugh et al., 2006; Wang et al., 2012), and propose at least three, non-exclusive explanations. First, it is possible that while taxonomic identity and abundance do not vary between GBC and LBC animals, the genes do, e.g. by different gene content or gene expression of bacterial strains that have similar V4 sequences (Zhu et al., 2015). Second, there could be genotype x microbiome interactions, where alpacas with LBC respond differently to the same microbial communities in the gut (Dobson et al., 2015; Shen et al., 2016). Third, the microbiome may make minimal contributions to LBC, which may have stronger contributions from host genotype independent of the influence of associated microorganisms. We do not provide any data to favor one possibility over any of the others, and future survey experiments (e.g. metagenomic, metatranscriptomic, GWA studies) could help to address some of these gaps. The current work establishes the need for more in-depth analyses.

In summary, this study used Illumina sequencing technology and volatile fatty acid profiling to survey the gut microbiome of alpacas fed different forages and link specific microbes with VFA production. The presence of unique microbial populations at different parts of the digestive tract with different diets suggested the alpaca microbial communities are sufficiently flexible to be modified by dietary interventions. However, the absence of any microbiome or taxonomic differences between LBC and GBC alpacas suggests that body condition scores may

not have a strong link to the gut microbiota. We recommend that further studies on the microbial gene activities (expression) and host genetics underlying alpaca low body condition could prove fruitful in efforts to improve the health and wellness of unproductive herd members.

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TABLES

Table 2-1. Most abundant families at each body site of GH- and AH-fed alpacas
The most abundant microbial families based on read count in a 4,890-read rarified OTU table. Both read count and OTU count are displayed.
* significant diet effect on read count
# significant diet x body site effect on read count

	# OTUs (# reads)	% Bacteria (% reads / % OTUs)	**Top 3 Orders (reads / OTUs)**
Grass hay			
C1	2,528 (24,450)	96.2% / 95.8%	*Prevotellaceae* (5709 / 369)# *Ruminococcaceae* (2144 / 322) *Lachnospiraceae* (1853 / 271)*
Jejunum	1,001 (19,560)	77.5% / 97.1%	*Methanobacteriaceae* (4376 / 17) *Coriobacteriaceae* (2420 / 110)# *Lachnospiraceae* (1867 / 150)*
Ileum	1,225 (24,450)	77.7% / 97.5%	*Methanobacteriaceae* (5436 / 22) *Coriobacteriaceae* (2987 / 119)# *Lachnospiraceae* (2735 / 194)*
Cecum	2,247 (24,450)	95.2% / 98.0%	*Ruminococcaceae* (6361 / 801) *Bacteroidaeae* (2990 / 68)# *Paraprevotellaceae* (1396 / 39)
LI	2,200 (24,450)	95.6% / 97.4%	*Ruminococcaceae* (6752 / 816) *Bacteroidaceae* (3098 / 69)# *Paraprevotellaceae* (1513 / 39)
C1	2,558 (24,450)	93.1% / 95.3%	*Prevotellaceae* (4244 / 331)# *Lachnospiraceae* (3203 / 352)* *Ruminococcaceae* (2084 / 287)
Jejunum	1,067 (24,450)	71.7% / 96.4%	*Methanobacteriaceae* (6866 / 19) *Lachnospiraceae* (4140 / 201)* *Mogibacteriaceae* (2541 / 63)
Ileum	1,261 (24,450)	71.7% / 96.0%	*Methanobacteriaceae* (6984 / 27) *Lachnospiraceae* (3699 / 224)* *Mogibacteriaceae* (2493 / 81)
Cecum	2,344 (24,450)	97.4% / 98.0%	*Ruminococcaceae* (5347 / 721) *Methanobacteriaceae* (3054 / 23) *Lachnospiraceae* (1626 / 253)*
LI	2,328 (24,450)	91.4% / 97.3%	*Ruminococcaceae* (5771 / 780) *Methanobacteriaceae* (1924 / 19) *Bacteroidaceae* (1549 / 45)#

Table 2-2. Diet-dependent variation in bacterial genus abundance
OTUs were rarified to 4,890 reads/sample and any OTUs with less than 10 reads in each sample were discarded. Genus-level significance was determined using linear models (read count ~ diet x body site) on all families with at least 500 reads in the entire dataset, and t-tests were performed on all genera with significant diet effects.
* Genera with significant FDR-corrected t-test p-values (FDR $p < 0.05$).

	Grass: Alfalfa Ratio	P-value	FDR	T-test p-value	T-test FDR
*Methanosphaera**	0.11 : 1	2.34E-14	5.61E-13	1.21E-06	1.57E-05
*BF311**	43.61 : 1	1.01E-06	1.21E-05	2.46E-04	1.60E-03
*Bacteroides**	8.84 : 1	1.58E-06	1.26E-05	5.43E-03	0.014
*Butyrivibrio**	0.34 : 1	2.28E-06	1.37E-05	1.43E-03	6.20E-03
*Pseudobutyrivibrio**	0.25 : 1	1.40E-04	6.74E-04	3.63E-03	0.012
Mogibacterium	0.68 : 1	1.95E-04	7.80E-04	0.2	0.26
Clostridium	1.41 : 1	2.74E-04	9.38E-04	0.29	0.31
rc4-4	0.29 : 1	6.52E-03	1.50E-03	0.049	0.091
SHD-231	0.52 : 1	1.45E-03	4.34E-03	0.029	0.062
Prevotella	1.31 : 1	1.79E-03	4.77E-03	0.62	0.62
YRC22	1.58 : 1	6.89E-03	0.015	0.1	0.15
5-7N15	1.58 : 1	0.012	0.024	0.26	0.31
Treponema	0.40 : 1	0.017	0.032	0.065	0.106

Table 2-3. Genera correlated with absolute butyrate extraction

Welch's two-sample t-tests were performed on all taxa at each phylogenetic level to determine whether taxon abundance differed with diet. Correlations were determined at each phylogenetic level on all differentially abundant taxa (FDR $p < 0.05$) using Spearman rank correlation tests of C1 taxon read counts (solid and liquid samples from AH-fed and GH-fed alpacas; OTU table subsampled to 6,720 reads) and total butyrate extraction (mM) per sample. All OTUs with 10 or more reads in at least one sample were retained for this analysis, and at each taxonomic level, only taxa with at least 3,000 total reads were considered.

Taxon	Average Reads/Sample		Spearman Rank	Rho	T-test p-value	Spearman p-value
	Alfalfa	Grass				
BF311	0.25	119	1030.5	-0.515	0.008	0.041
Shuttleworthia	15	4.375	248.770	0.634	0.009	0.017
YRC22	96	161.375	1166.150	-0.715	0.013	0.007
CF231	64.5	134.25	1047.9	-0.541	0.025	0.041

FIGURES

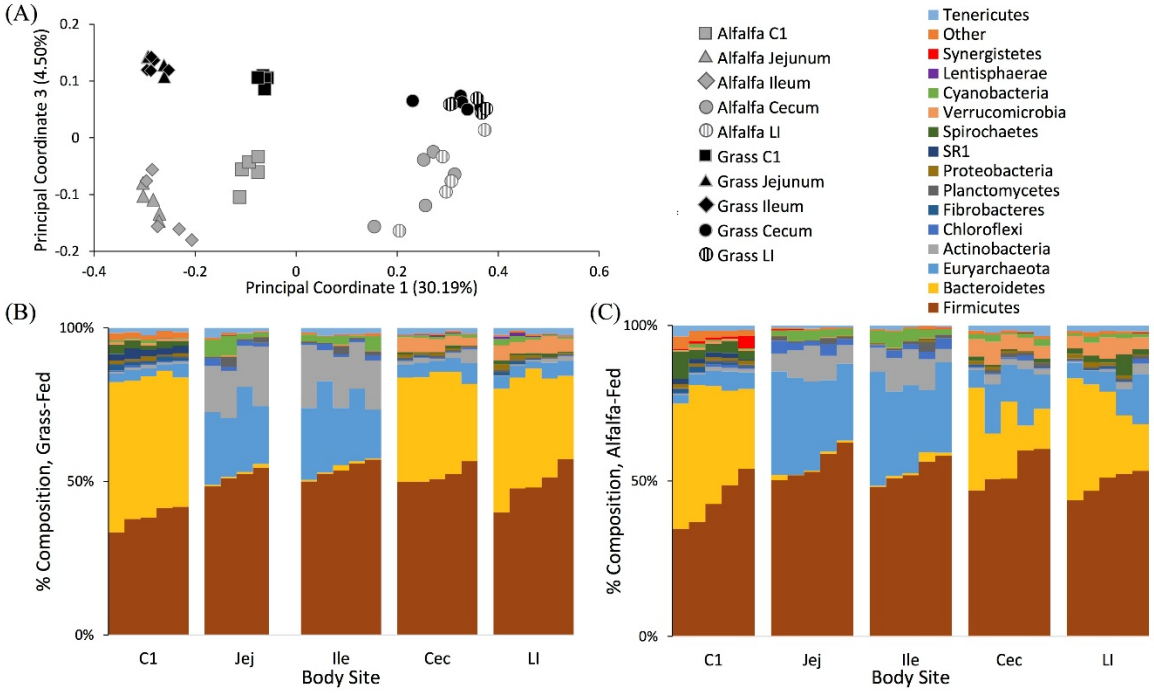

Figure 2-1. Microbial communities of the GH-fed and AH-fed alpaca digestive tracts
(A) Principal coordinate analysis of samples from GH-fed and AH-fed alpacas. 16S rRNA gene sequences from alpaca C1, small intestine (jejunum, and ileum), and distal intestine (cecum and large intestine) samples were subsampled to 4,890 reads and clustered by unweighted PCoA using QIIME. Principal coordinates 1 and 3 visually separate the samples by body site and diet, respectively. Percent composition of microbes at the phylum level in C1, jejunum (Jej), ileum (Ile), cecum (Cec), and large intestine (LI) from GH-fed (B) and AH-fed (C) alpacas.

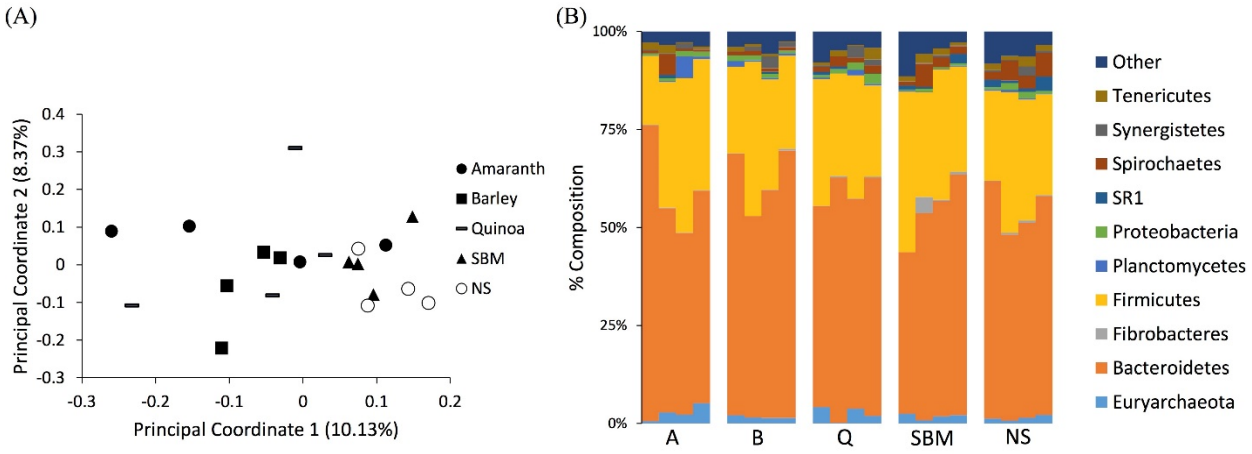

Figure 2-2. The C1 microbiota of alpacas fed mixed grass hay or mixed grass hay plus one of four trial supplements

Each alpaca was fed mixed grass hay supplemented with amaranth (A), barley (B), quinoa (Q), soybean meal (SBM), or no supplement (NS). Each treatment was fed to each animal for two-week periods in a randomized order, and no alpaca was fed the same treatment twice. (A) Principal coordinate analysis of the C1 microbiota. Principal coordinate analysis was performed on unweighted Unifrac data from 11,010 reads subsampling depth. (B) Microbial composition of each sample at the phylum level, rarified to 11,010 reads per sample.

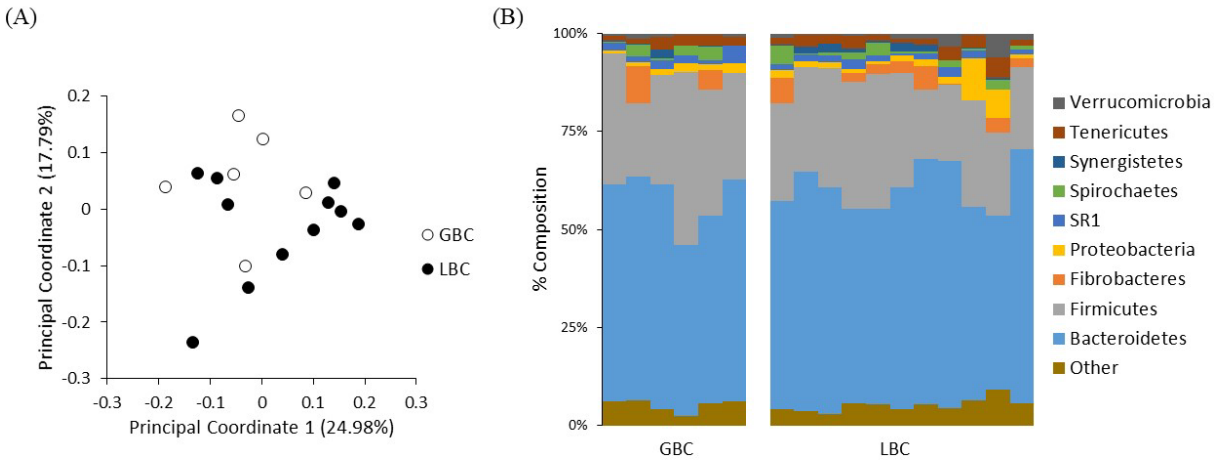

Figure 2-3. C1 microbiota of LBC and GBC alpacas
(A) Weighted principal coordinate analysis and (B) phylum percent composition of C1 samples from body condition-scored alpacas, subsampled to 74,000 reads/sample. Alpacas with body condition scores of ≥ 3 were considered to have good body condition (GBC); those with body condition scores of < 3 were considered to have low body condition (LBC).

DISCUSSION

The goals of these studies were to 1) find a combination of DNA extraction method and polymerase enzyme that will optimize alpaca gut microbiome sequencing; 2) survey the alpaca gut microbiome, including digestive sites that have rarely or never been surveyed in camelids; identify the effects of different diets on the alpaca gut microbiota and volatile fatty acid production; and 4) explore the idea that the C1 microbial community may influence host body condition. Overall, we hoped to contribute to the study of an animal that is important to the economy and livelihood of many people, and yet poorly studied. Scientific literature about alpacas and camelids in general lacks the breadth of study given to cattle and other ruminants.

Our research lays groundwork for additional studies of the alpaca and other camelids. Metagenomic study lacks a standardization of library preparation methods. Although the scientific community may never commit to a single extraction method or polymerase for metagenomics studies, our trial of three extraction methods and two polymerases can help in the quest toward a more narrow set of parameters which would ultimately reduce the amount of bias seen across studies. Our discovery of the similarity between the MO BIO and Zymo DNA extraction kits may also make studies of the alpaca gut microbiota more feasible. The Zymo kit is less expensive than the MO BIO kit, a standard in the field.

A true understanding of alpacas and other camelids will only come from scientific study specifically focused on these animals. Our survey of DNA extraction methods and polymerase enzymes sheds some light on which methods are optimal for microbiome studies of these animals. Our 16S rRNA gene amplicon survey of sites along the alpaca digestive tract may serve as a springboard for related studies about alpaca digestion and how it compares to that of other SACs and ruminants; for instance, studies of species along the digestive tract and how they relate

to the unique digestive vasculature of camelids may be interesting. In addition, our inability to find a relationship between BCS and the C1 microbiota can focus further studies on the unexplained LBC status of alpaca individuals in a herd. Our studies add valuable information to an area lacking in adequate study and enable further studies of the alpaca, which can ultimately make a difference in the economic, environmental, veterinary, and animal welfare spheres.

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