INFLUENCE OF CORN AND DEGRADABLE INTAKE PROTEIN SUPPLEMENTS ON DIGESTION, PERFORMANCE, AND RUMINAL ECOLOGY OF BEEF STEERS

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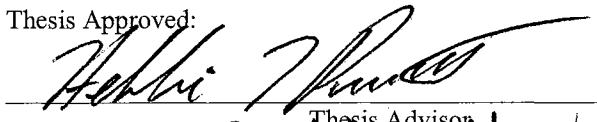

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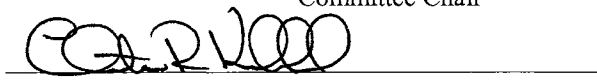

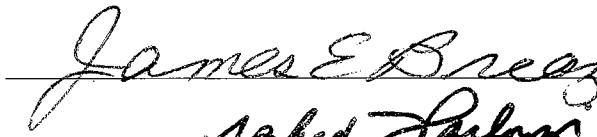

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Format of Dissertation

This Dissertation is presented in the Journal of Animal Science style and format, as outlined by the Oklahoma State University graduate college style manual. The use of this format allows the independent chapters to be suitable for submission to scientific journals. Three papers have been prepared from the data collected for research to partially fulfill the requirements for the Ph. D. degree. Each paper is complete in itself with an abstract, introduction, materials and methods, implications and literature cited section.

Chapter I

Introduction

A wide variety of beef cattle production systems exist in the United States. Many of these systems rely on forages as the primary dietary component to meet animal nutrient requirements. Yet, many forage-based systems will not support necessary levels of animal production. Because forage consumption alone does not always meet animal requirements, many producers provide supplements formulated to provide the necessary nutrients. In many situations, protein and(or) energy are first-limiting. This can result in the need to feed significant quantities of supplement to meet requirements. Yet, providing supplementary energy- and(or) protein-containing feedstuffs may result in a condition where intake and(or) digestion of the supplement may interact with intake and(or) digestion of the basal forge. These interactions between supplementary feedstuffs and the basal forage are called associative effects. Associative effects can be either negative or positive. Negative associative effects have been described as the decrease in digestibility of a component of a mixed diet to a value lesser than would be expected based on the additive digestibility of that component when each feedstuff is fed individually. Positive associative effects are when the opposite situation occurs. These interactions between feedstuffs, as well as the various components in those feedstuffs, decrease our ability to accurately predict animal response. Understanding the effects of various feedstuffs used to formulate supplements helps in assuring that the supplement will aid in reaching the desired production objective.

Even though this area of nutrition has been studied for a considerable length of time, there is still a great deal that is unknown about the causes of associative effects. While

many theories have been postulated about the scientific mechanisms for these effects, the role that ruminally fermentable organic matter and ruminally degradable protein play is still not well understood. Another area where our lack of understanding has limited our ability to adequately model associative effects is the role that the microbial population in the forestomach of cattle plays. Ruminal microorganisms respond to energy with increased growth, along with greater requirements for other nutrients. It has been extensively shown that when the ruminal microbes are protein-deficient, supplying additional energy in the form of rapidly fermented carbohydrates will decrease digestion of fiber (a classic negative associative effect). However, if the protein deficiency is addressed, along with the energy supplement, the ruminal microbes may be able to digest both the grain and the fiber.

This research was designed to determine if negative associative effects due to supplementation of cattle consuming low-quality forages with corn could be alleviated or eliminated by the addition of ruminally degraded protein. Chapter Two provides an overview of molecular methodologies, challenges to applying these technologies to the study of ruminal microbial ecology, and previous research on the effects of nutrition and management on ruminal microbial ecology. Two trials studying the effects of energy and(or) protein supplements fed to beef steers consuming low-quality forages are detailed in chapters Three and Four. Chapter Five is an investigation of the effects of supplementation on ruminal bacterial ecology. Chapter Six is a summary of the research efforts undertaken for the completion of this dissertation. The Appendix contains supporting data relative to Chapter Five that was not included in the journal article format of that chapter.

Chapter II

Review of Literature

Introduction

Nutritional and(or) management alterations of ruminant livestock can determine the substrates available to, and the environment present for, ruminal microbes. These alterations can modify the number and(or) relative proportions of microbial species. When ruminal microbial populations are modified, the production and concentration of intermediates and end-products of microbial fermentation in the rumen are also affected. These alterations may result in a different nutritional profile presented to the host animal, which can cause changes in animal performance. Nutritional factors alter ruminal pH, VFA production, microbial protein production, production of methane (Johnson and Johnson, 1995), and other fermentation related factors. This is indicative of nutrition altering microbial fermentation, which can be traced to changes in ruminal microbial populations. Factors such as geographical location, which may be related to what dietary selections are available to the animal, anti-nutritional factors, such as the effects of *Leucaena leucocephala* on ruminal ecology, stress, osmolality, time spent ruminating, quantity and composition of saliva produced, intake level, feeding frequency, feed processing, diet-dependent diurnal variation, nutrient limitation, water intake, and passage rate can all alter microbial ecology, and can all be related to differences in diet (Yokoyama and Johnson, 1993). Ruminant nutritionists quantify many measurements in the rumen during feeding trials including pH, VFA and ammonia concentrations, passage rate, and ruminal volume and mass. However, changes in the microbial population in terms of total numbers and relative contribution of individual species in the rumen are

rarely measured and may be more significant than pH, and VFA and(or) ammonia concentration. Microbial alterations may provide a clearer explanation of the observed changes in ruminal measures.

Nutritional factors that play a role in ruminal microbial ecology

Ruminant animals have the ability to maintain a population of microbes that have the enzymatic capacity to degrade the β, 1-4 linkages of cellulose in the reticulo-rumen. However, these bacteria have requirements for nutrients that must be met in order for their continued survival and for proper ruminal function. The current Nutrient Requirements of Beef Cattle (NRC, 1996) utilizes the metabolizable protein (MP) system to express animal requirements for absorbed protein in the small intestine. The MP system classifies protein into two major fractions, degradable intake protein (DIP) and undegradable intake protein (UIP). Animals have a requirement for MP, the total protein flow through the small intestine that is absorbed. This protein flow to the small intestine is made up of microbial protein that was synthesized in the rumen from DIP, and UIP, or feed protein, which is passed through the rumen intact. Ruminal microorganisms have a requirement for DIP that must be met in order to ensure adequate ruminal function. This requirement has been based on the energy supplied to the microbes from the feedstuffs ingested by the animal and is quantified as TDN under the NRC (1996) guidelines. Therefore, the MP system accounts for bacterial and animal requirements for protein. This is an important distinction, because all feed proteins do not have equal ruminal availability (NRC, 1996) or intestinal digestibility. As a result, the release of the Nutrient Requirements of Beef Cattle (NRC, 1996) has increased interest in quantifying ruminal degradation of protein in many feeds, because, in order to effectively use the 1996 NRC

and the MP system, estimates of DIP and UIP values of many feedstuffs must be known. Yet, no quick, repeatable, and accurate assay exists for these determinations. Quantification of protein digestion in ruminant nutrition is confounded by the contamination of feed protein in digesta by microbial protein, and thereby complicates estimates of protein degradation and site of digestion. Release rates of ammonia, peptides, and amino acids, as well as the type of carbohydrate, dilution rate, and pH can all influence efficiency of bacterial protein synthesis (NRC, 1996). Russell et al. (1992) suggested that efficiency of microbial protein synthesis could be decreased by imbalances between N and fermentable energy. Many researchers have suggested that pH may be responsible for the negative effects of starch supplementation. However, the lack of consistency in findings involving pH and digestion appear to suggest that factors other than reduced pH are the primary causative agent in decreasing fiber digestion. Horn and McCollum (1987) and Caton and Dhuyvetter (1997) concluded that maintaining ruminal pH would only partially alleviate negative associative effects, that effects would vary with forage type, concentrate level, and forage buffering capacity, that pH could not explain all observed effects, and that additional mechanisms do exist. El-Shazly et al. (1961) found inhibition of cellulose digestion even when pH was maintained by continuous culture. Stern et al. (1978) found decreased in vitro ADF and cellulose digestion with no changes in pH, indicating that depressed fiber digestion was not caused by pH reductions. Hoover (1986) suggested the presence of several effects of feeds on ruminal pH and depressions of forage utilization. This may be due to a "carbohydrate" effect, increased washout, or lag time of attachment. Possibly, pH is an effect that can be measured and correlated to digestion, but is not the primary agent controlling the ruminal

microflora. Ruminal starch fermentation can increase microbial capture of ammonia, amino acids and peptides (Spicer et al., 1986) and consequently increase the requirements for ammonia, amino acids and peptides. Russell et al. (1992) stated that carbohydrate availability determines the fate of peptides between incorporation by amylolytic bacteria or deamination and conversion to ammonia. Ruminal ammonia is a vital nutrient for ruminal cellulolytic microorganisms as fibrolytic bacteria utilize only ammonia as a source of N for microbial CP synthesis, while amylolytic species can use peptides, amino acids, or ammonia (Russell et al., 1992). Horn and McCollum (1987) suggested that ruminal ammonia concentrations are more indicative of the balance between ruminally-degraded protein and energy, which makes interpretation of ruminal ammonia levels difficult. Low ruminal ammonia values may indicate a deficiency of DIP, an excess of fermentable OM, both, or a balance, rather than describing the causative agent. Owens et al. (1991) suggested that ruminal ammonia is a useful indicator of N available for ruminal fermentation while still being a function of supply (DIP) and demand (microbial CP synthesis). Satter and Slyter (1974) also reported that ruminally digested OM controlled ammonia accumulation, pointing to the balance between ruminally degradable nitrogen and energy. Addition of grain to DIP deficient diets will depress ruminal ammonia, and the competition between fibrolytic and amylolytic bacterial species may prevent adequate fiber fermentation. Russell et al. (1992) suggested that non-structural carbohydrate fermenting bacteria reduce ruminal ammonia levels during rapid growth. El-Shazly et al. (1961) reported increasing urea increased in vitro and in vivo cellulose digestibility when the forage to concentrate ratio was greater than 1:1. Hoover (1986) suggested that competition for ruminal ammonia between fibrolytic and amylolytic microorganisms

could also play a part in reductions of fiber degradation when readily fermentable carbohydrates were fed with forage.

Intuitively, increased levels of VFA in the rumen have been suggested as a potential mechanism for decreased ruminal pH levels. In addition, they serve as the primary energy source of ruminant animals. As a result, a great deal of data on the concentrations of VFA in ruminal fluid exists. However, the majority of ruminal VFA data is simply measured and reported as concentrations, rather than actual production of VFA, which is much more difficult to measure. However, concentrations can be impacted by variables such as absorption, particulate and fluid passage rates, ruminal volume, intake of feed and water, and cross-feeding by ruminal microbes. Therefore, based on concentration values, we can only infer the existence of a relationship between experimental treatments and increased VFA production. Various effects of supplementation on ruminal VFA have been reported in the literature. Several studies found that total VFA concentration was not affected by supplementation even though differences were found for individual VFA profiles due to supplements (McCollum and Galyean, 1985; Chase and Hibberd, 1987; Fleck et al., 1988; Krysl et al., 1989; Sanson and Clanton, 1989; Vanzant et al., 1990; Pordomingo et al., 1991; Grigsby et al., 1993; Freeman et al., 1993; Galloway et al., 1993a,b; and Olson et al., 1999). In contrast, other researchers have reported that total VFA levels increased and had various effects on individual VFA profiles (Martin and Hibberd, 1990; Sunvold et al., 1991; Hannah et al., 1991; Barton et al., 1992; Carey et al., 1993; Hess et al., 1996; Köster et al., 1996; Elizalde et al., 1999; and Olson et al., 1999).

Challenges in studying ruminal microbial ecology

Methods for describing the microbial ecology of the rumen have been investigated for many years. However, a vast majority of the ruminal microbes are difficult to study with standard microbiological methods due to their anaerobic nature (Stahl et al., 1988). The fact that many of these microbial species are obligate anaerobes is one of the main difficulties in using culture systems (Atlas, 1983). Estimates are that between 0.1% and 10% of the microbial species present in samples taken from the environment can be isolated using traditional culture-based techniques (Theron and Cloete, 2000). This severely limits the information that can be obtained regarding microbial ecology from traditional studies. There are many limitations on the ability of traditional culture methods. These include our inability to predict proper culture conditions, to choose the correct medium, and the difficulty in selecting individual organisms from a community. One of the greatest challenges to culture methods is the propensity of individual organisms with rapid growth rates under a certain set of conditions to out-grow species that grow more slowly.

Methodologies and techniques used

There are a plethora of molecular techniques and methodologies that biologists routinely use in their research. Those used in the fields of genomics and proteomics are often based on the isolation and downstream manipulation of nucleic acids and proteins and are often focused on the role that genes, gene expression, and protein expression play a role in the control of a specific biological function. The beginnings of this premise can be found in the simple, yet highly complex central dogma of biology: DNA to RNA, RNA to protein, and protein to function.

The first step in many of these procedures is often the isolation of nucleic acids. Extracting DNA from cells involves disruption of the integrity of the cellular membranes. This can be accomplished by mechanical or chemical methods. Most mechanical methods such as blending, homogenizing, grinding in liquid nitrogen, are all considered to be harsh extraction techniques that often result in mechanical shearing of DNA. This can lead to many small DNA fragments. Less harsh mechanical methods include boiling and repeated freeze/thaw cycles. Chemical methods that can be employed include the use of ionic or non-ionic detergents (sodium dodecyl sulfate, cetyltrimethylammonium bromide), organic solvents (guanidine-containing compounds), or lysis in alkaline conditions. Once the cells have been disrupted, the contents of the cell must be separated because many of the molecules inside the cell can inhibit subsequent downstream applications. The presence of proteins, carbohydrates, and nucleases are all of special concern during the purification of nucleic acids. Nucleases are of the greatest concern during the isolation of nucleic acids as intact nucleic acids are extremely important in order to carry out downstream applications successfully. The activity of nucleases can be significantly reduced by the presence of strong chelating agents such as ethylenediamine tetraacetate (EDTA), which bind the magnesium ions that many enzymes require to function properly. Proteins can be removed by a variety of methods. The most common methods include performing a phenol extraction, phenol, chloroform, iso-amyl alcohol extraction, or chloroform extraction. These all utilize the differential precipitation properties of proteins, carbohydrates, and nucleic acids in bi-phasic mixtures of organic and aqueous solutions. The nucleic acids will remain in suspension in the aqueous phase, whereas the proteins will precipitate out in the organic phase. Following this, relatively

pure nucleic acids can be precipitated from the aqueous phase by various techniques. The most common is high-speed centrifugation of nucleic acids in a high concentration of alcohol (usually ethanol or isopropyl), along with a salt (most commonly sodium chloride, sodium acetate, or ammonium acetate). A wide variety of modifications of these procedures are commercially available in kit form, and are quick and easy to carry out. The majority of the commercial kits are based on a chemical cell lysis procedure, followed by either anion-exchange chromatography or selective binding of nucleic acids to resins or membranes, and subsequent elution of relatively pure nucleic acids. In these procedures, the cell lysates are filtered through a column that selectively binds nucleic acids. The contaminants are then washed through the column in a series of steps, followed by a wash with a solution that releases the nucleic acids for recovery and use in subsequent downstream applications.

Gel electrophoresis is the most common method of separating macromolecules such as nucleic acids and proteins. Electrophoresis is a simple, rapid, inexpensive, and highly sensitive (1 to 10 ng of DNA) technique for separation of macromolecules. An electrophoresis apparatus (gel box) consists of two buffer tanks separated by the gel. Samples are loaded in wells formed in the gel and an electrical gradient is applied across the gel from one buffer tank to the other. Nucleic acids and many proteins carry a net negative charge and will migrate towards the positive electrode. The rate of migration of a macromolecule through the gel is controlled by a myriad of factors. These include the concentration of the polymer (agarose or polyacrylamide) used to make the gel, the conformation of the molecule, the voltage applied, the direction of the electric field, the base composition of nucleic acids, the temperature of the gel, the presence of

intercalating agents, the composition of the buffer, and the presence of denaturing agents such as urea, or formamide. The size of an unknown molecule can be determined by running a standard (ladder), which is a series of fragments of that type of molecule (protein or nucleic acids) of known sizes. The two main types of polymers used to make gels used for electrophoretic separation are agarose and polyacrylamide. Agarose is a linear polymer of galactose, which is extracted from seaweed. Depending on the concentration of agarose used, agarose gels can resolve DNA fragments from < 100 bp to 80 kb. Polyacrylamide is composed of a chemical network of acrylamide monomers cross-linked with bis-acrylamide. Gel electrophoresis performed with polyacrylamide has a much greater resolution power than does agarose gel electrophoresis (polyacrylamide can resolve DNA fragments that differ by as little as 0.2%). However, polyacrylamide can separate a much smaller range of molecules (DNA from < 10 bp to 2 kb). Acrylamide is a potent neurotoxin, and gels formed with it are polymerized in the absence of oxygen by free radicals supplied from ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED). Nondenaturing polyacrylamide gels are typically used for the separation of proteins and double-stranded DNA. Denaturing polyacrylamide gels contain chemicals (commonly urea and/or formamide) that suppress base pairing in nucleic acids and result in single stranded DNA. The electrophoretic mobility of DNA (single-stranded) through a denaturing gel is mostly unaffected by base composition and sequence. Denaturing gels are most commonly used for DNA sequencing applications. Electrophoresis can be performed to determine the size of macromolecules, as a preparative technique, or it can be used as both. Electrophoresis can be used to remove contaminants, such as the components of previous assays, that will

interfere with downstream applications. Combined sizing and preparative functions include the use of electrophoresis to purify and select a highly purified molecule of a specific size, or range of sizes, for subsequent manipulations. Detection of nucleic acids is often done by staining with ethidium bromide (0.5 μg/mL), an intercalating dye that fluoresces under UV light (usually at 254 nm). Proteins can be detected by staining with methylelene blue, Coomassie blue, or silver stains, which can be seen by the naked eye. Many newer fluorescent technologies exist for detecting either nucleic acids (SYBR Green or Gold) or proteins SYBR Orange.

Restriction enzymes (endonucleases) are a part of a system discovered in bacteria that allows an organism to protect itself from an infection by viruses (eliminates foreign DNA). These enzymes possess the ability to recognize specific sequences of double-stranded DNA, bind to it, and cleave it. Restriction enzymes are named after the organism from which they were isolated. The first letter of the genus is capitalized and followed by the first two letters of the species name in italics. This is followed by a strain designation (if any) and a number in roman numerals that designates the order in which the enzyme was isolated. For example, *Eco*R I was the first restriction endonuclease identified in *Escherichia coli* strain RY13. Restriction enzymes are classed into three groups based on their recognition and cleavage sites, ability to modify (methylate) DNA, and cofactors necessary for enzyme activity. Type I and III enzymes are multi-protein complexes that methylate and cleave DNA, and require ATP and S-adenosyl L-methionine. Type I enzymes bind at their recognition site, and then cleave the DNA at random sites some distance away, whereas Type III bind at a recognition site, but cut at a specific restriction site, which is often 24-26 bp away. In contrast, Type II

endonucleases bind and cleave at specific, consistent, predictable sites. They do not methylate DNA and only require magnesium as a cofactor. These enzymes often recognize a palindromic sequence that is commonly four to eight bases in length. This variation creates differences in the frequency of restriction site occurrence, frequency of cutting, and therefore, difference in the fragment lengths of the cut DNA. The frequency is estimated as the 4^N, where N is number of bases in the recognition sequence and 4 is the possible number of nucleotide bases (A, C, G, T). This results in a frequency of cutting of approximately every 256 bp for an enzyme with a four-base recognition site, referred to as a "four-cutter". Similarly, an enzyme with a six-base recognition site (a "six-cutter") would cleave every 4096 bp on average, whereas an "eight-cutter" would average a recognition site occurring every 65,536 bp. This is significant, as longer DNA fragments will be generated as the frequency of cutting is decreased. This is also important if you are cutting unknown sequences, as using a restriction enzyme with a longer recognition sequence reduces the likelihood of cutting in the middle of a gene of interest. There are many known restriction endonucleases (>1200) that vary greatly in their specificity. New ones are being discovered yearly and a large number of them are commercially available. Restriction enzymes cut double-stranded DNA by cleaving the phosphodiester bond of the deoxyribose sugar backbone of the DNA molecule and leaving a 3' OH group and a 5' phosphate group. Various restriction endonucleases can be selected that will leave different cut ends of the cleaved DNA fragment. Certain restriction enzymes create a blunt end on both DNA fragments by cutting the double-stranded DNA between two pairs of complementary bases. Other restriction endonucleases cut the double-stranded DNA in a staggered manner. This will result in a

portion of one strand of the cut DNA being left single-stranded, and the portion of the DNA that was complementary to it will be single-stranded on the end of the other cut fragment. The presence of these single-stranded ends, or overhangs, is referred to as either a 5' or a 3' overhang, depending on which strand of DNA that the single-stranded fragment overhangs from.

The polymerase chain reaction (PCR) is a technique that amplifies a sequence of nucleic acids by creating copies of them *in vitro*. DNA is synthesized *de novo* using a thermostable DNA polymerase, synthetic oligonucleotide primers that flank or define the sequence of interest, deoxynucleotide tri phosphates (dNTP), MgCl++, and a buffer. DNA polymerase adds nucleotides in a sequence complementary fashion to a growing chain of DNA using 5'-3' polymerase activity. A commonly used thermostable DNA polymerase is *Taq*, an enzyme isolated from and named after *Thermus aquaticus*, a microbe isolated from a Yellowstone Park hot springs in the late 1960's. Both native, as well as newer, cloned versions of *Taq* DNA polymerase are susceptible to errors of incorporation of nucleotides. The error rate of most commercial preparations of *Taq* polymerase is 8 errors per 10^6 base pairs, which can result in up to 16% of the PCR products being mutated after 20 cycles. This makes the choice of DNA polymerase an important factor in the fidelity of the PCR reaction. If the error occurs in an early cycle of the PCR reaction, the error will be greatly amplified. However, if the error occurs in a later cycle, it may have a minimal impact. These mistakes in copying the template can have a range of effects on downstream applications. A single base mis-incorporation may have little effect when copying a short fragment of DNA that will be used for sequencing of a gene that has low homology to any known DNA sequences. However, a

single base mistake in a gene that will be used for protein expression may result in the synthesis of an inactive protein. In order to address this, various other DNA polymerases have been isolated and are commercially available. The majority of these are grouped into a category of DNA polymerases called "high-fidelity" or "proof-reading" polymerases. While both *Taq* and high-fidelity polymerases contain 5' to 3' polymerase activity, the high-fidelity enzymes also have 3' to 5' exonuclease activity, allowing the enzyme to remove and replace (or proof-read) mismatched nucleotides. These polymerases have error rates that range from 1.3 to 2.8 errors per 10^6 bases, resulting in a range of mutated PCR products from 2.6 to 5.6% after 20 cycles. An example of a "high-fidelity" thermostable DNA polymerase is *Pfu*, an enzyme isolated from and named after *Pyrococcus furiosus*, a microbe isolated from deep-sea thermal vents. *Pfu* has the lowest error rate of any thermostable DNA polymerase analyzed currently.

Another important consideration in the PCR reaction is the design of short, synthetic DNA sequences, named oligonucleotides, and commonly referred to as primers. PCR primers must be complementary to a portion of the sequence of the target DNA in order to hybridize, which allows for selection and specificity of the target of interest. One primer is complementary for one strand of the target DNA, whereas, the other primer is complementary for the other strand. When the primers have annealed to the template, their 3'ends are "pointing" towards the site where the other primer has annealed. Because DNA polymerase adds dNTP's using a 5'-3' polymerase activity, the PCR reaction is dependent on both of the primers binding to the template. This must occur in order for the DNA polymerase to synthesize copies of both strands of the template, which is the basis for the exponential amplification of the target DNA that occurs in PCR.

Strand elongation of PCR products starts from the 3' end of each primer and proceeds in a 5' to 3' direction. This orientation results in the primers commonly being called "forward" and "reverse" primers. Once all of the components are mixed in a reaction tube, the tube is placed in a thermocycler and the reaction is then heated (~95°C), which causes the DNA to denature to a single stranded state. The tube is then cooled to allow the primers to anneal to the target DNA (this temperature is dependent on primer design, and varies for each set of primers, an average temperature is ~55°C). Next, the tube is heated to the optimum temperature for DNA polymerase activity (~72°C) to allow strand elongation. After the first cycle, the copy number of the target has been doubled. This is repeated in subsequent cycles, and each cycle doubles the copy number of the previous cycle, resulting in exponential amplification. After 30 cycles, the DNA target of interest has been amplified over one million fold. In a modern PCR machine (thermocycler), this reaction is controlled by a microprocessor, and occurs very rapidly. However, the optimization of the reaction conditions is still an important step in efficient PCR reactions. The amount of time the reaction is held at denaturing, annealing, and elongation temperatures, as well as what temperatures are used, along with the concentrations of primers, dNTP's, magnesium chloride, and polymerase can all have a major impact on the results of a PCR reaction.

There are many methods used to propagate and amplify discrete DNA fragments. One of the most common of these techniques is molecular cloning. The majority of molecular cloning methodologies rely on the assembly of a DNA fragment of interest (often a gene), which is referred to as an insert, and a cloning vehicle, or vector, in a process known as ligation. Ligation involves joining the ends of the double-stranded

insert with the ends of the double-stranded DNA of the vector using ligase, an enzyme that functions to repair DNA strands *in vivo*. One frequently used form of vector is a small, double-stranded, closed-circular, extra-chromosomal, DNA molecule called a plasmid. Plasmids are naturally occurring and are typically found in both bacteria and yeast. They can range in size from 1 to 200 kb, and are replicated and inherited independently of the cellular chromosome. However, even though they are not considered a part of the cell's genomic material, they do rely on the enzymes and proteins of the host cell for replication and transcription. Typically, when found in nature, these plasmids carry genes with an advantageous phenotype, such as resistance to antibiotics, production of a toxin or antibiotic, restriction or modification enzymes, or the ability to degrade complex substrates. This provides a form of evolutionary selection for cells to maintain the plasmid. Often, without this selection pressure, the plasmid will be lost from the cell. Even though plasmids are found in nature, most plasmids frequently used for molecular cloning have been genetically modified to increase their functionality.

Most common cloning plasmids contain an origin of replication, which is a region of the DNA that controls the replication of the plasmid within its cellular host. This region can be modified by the addition of a replicon, a sequence of DNA that controls the copy number of replication, often up to several thousand copies per cell, and may not be dependent on cellular protein expression for replication. This is especially important when antibiotics that inhibit cellular protein synthesis and chromosomal replication (such as chloramphenicol and spectinomycin) are used for selection. Plasmids with this type of replicon use the host's DNA replication mechanisms, which are based on long-lived enzymes. Some plasmids may contain replicons that require cellular protein synthesis

and are expressed in relatively low copy numbers per cell. The majority of commonly used plasmids contain several features making them highly useful in molecular cloning. Most plasmids are relatively small in terms of length in bp. This offers several advantages; first, the insert makes up a greater proportion of the total DNA from plasmid and insert, making it easier to recover and subsequently manipulate the inserted DNA. Second, smaller plasmids are less fragile, making them easier to purify from their hosts. Third, small plasmids replicate better within the cellular host. Lastly, their small size makes the introduction of the plasmid into its cellular host (a process called transformation) more efficient. Another useful feature of plasmids is that they are well characterized, and the knowledge of the complete DNA sequence of the plasmids allows the use of all possible molecular techniques available. Plasmids are also present in the cell in high copy number. This increases the amount of the inserted DNA fragment of interest that can be recovered and makes it easier to separate plasmid DNA from the host's chromosomal DNA. Plasmids also contain a selectable marker that allows easy identification of cells containing plasmids. The primary selection marker found in plasmids is the presence of a gene that confers resistance to a specific antibiotic. When grown on antibiotic-containing medium, cells with the plasmids will proliferate, whereas cells without the plasmid will be susceptible to the antibiotic. The presence of a selectable marker such as antibiotic resistance also aids in ensuring that the host cells will maintain the plasmid. Plasmids frequently contain a second selectable marker at the site of insertion. This second marker is usually a gene with a function that can be easily detected. Examples are genes that confer resistance to a second antibiotic, for enzymes such as β-galactosidase (the *lac* gene) that utilize a chromogenic substrate, or a gene that

expresses a protein that is lethal to the host cell. When the insert is present in the plasmid, it is located in the middle of this gene, disrupting the function of the gene. β-galactosidase will break down X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactoside) into an insoluble dense blue compound causing colonies composed of cells containing the vector, but not having the insert, to turn a blue color, whereas, when the *lac* gene is inactivated because the DNA fragment of interest has been inserted, the host cell will not degrade X-gal, and the colonies will remain white in color. Similarly, when the DNA fragment of interest is inserted into a plasmid and disrupts the expression of a lethal gene, the host cell will grow, whereas a cell with a plasmid without the insert will not grow due to production of the lethal protein. This allows the molecular biologist to select only host cells that contain the inserted DNA fragment of interest. Usually a DNA fragment of interest is obtained by digesting with a restriction enzyme, and if the plasmid is digested with the same enzyme, the DNA fragment can be inserted directly. At the site of insertion, the plasmid contains a region referred to as a multiple cloning site, a polylinker, or a polycloning site. This region contains many unique restriction endonuclease recognition sites in a small area. These specific restriction enzyme recognition sites are not found anywhere else in the plasmid. The circular plasmid is opened up, or linearized, by digesting it with a restriction enzyme. If these sites were in multiple locations, the plasmid would be cut into many fragments that would not reassemble properly during the ligation process. As previously mentioned, this multiple cloning site is often placed in the middle of a gene with an observable function that serves as a second selectable marker. Adjacent to the multiple cloning site, many plasmids also contain powerful promoters that serve as initiation sites for the *in vitro* transcription of large quantities of

mRNA from the inserted DNA fragment through the use of the appropriate RNA polymerase and ribonucleotides.

When a researcher needs to investigate a large amount of unknown genomic DNA, an approach often utilized is the creation of a clone library. While this technique is similar to the creation of cDNA libraries generated from transcribed mRNA sequences, the following description focuses on the creation of genomic DNA libraries. The first step in the creation of a clone library is to generate many smaller DNA fragments. These smaller pieces of DNA are then ligated into a vector and transformed into competent *E. coli* cells. The cells that contain vectors with inserts of interest are streaked at low concentration on plates containing a nutritive medium plus a selection factor (i.e., antibiotic), and allowed to grow in an incubator. The individual cells will establish on the plates, and grow into visible colonies of cells because they contain the vector, which provides them resistance to the antibiotic. Cells without a vector will not survive, as they will be susceptible to the antibiotic. However, not all cells with a vector will contain the desired DNA insert. If the vector has a secondary selection factor (such as the β-galactosidase gene) that is interrupted by a DNA insert, and the plates have been treated with X-gal, the cells with β-galactosidase activity will produce a blue color versus cells that have vectors containing an insert, which will not have β-galactosidase activity, and appear as white colonies. This aids the molecular biologist in screening colonies and increases the likelihood of the DNA insert of interest being included in the library. An advantage of cloning is that each vector will only contain one insert, which is a unique DNA fragment. Upon transformation, each cell will contain only one cloned DNA fragment, and once plated, each colony represents many copies of this unique DNA

fragment. Individual colonies can be selected (picked) and grown in an antibiotic containing medium. Each picked colony represents many copies of a single vector containing a unique DNA fragment of interest. Afterwards, the selected clones can be utilized to create a preliminary library of DNA fragments, with each clone representing a sequence of DNA. Unfortunately, an effective technique does not exist to ensure that there are not redundant sequences or unwanted sequences of DNA in the initial construction of the library of cloned fragments. Because of this redundancy and the presence of unknown inserts, clone libraries must be screened. The library of cloned DNA can be screened on the basis of insert size using PCR with vector specific primers. However, if the inserts were all of similar starting size (such as for PCR products) this may not reduce redundancy, even though it will remove clones without an insert of the correct size, and increase the proportion of the library containing fragments of interest. The inserted DNA can also be screened using PCR, with gene-specific primers. However, this may require a unique set of primers for each clone. If the DNA fragments are of different initial sizes, screening may greatly reduce redundancy, yet, it may occur at the expense of some information because two DNA fragments could be similar in size, yet contain different sequences. Removal of apparently redundant fragments based on size may cause a reduction in the total number of unique DNA sequences recovered in the library. Screening can be accomplished by hybridization techniques to probes of known sequences, or sequences that already exist in another library. However, this technique is limited to situations where there are sufficient differences in DNA sequences that false positives will not occur due to non-specific binding of the probe to the DNA.

Libraries can also be screened by DNA sequencing of the inserts. While this method is definitive, it can become very expensive when performed on large libraries.

Two primary methods of determining the sequence of individual nucleotides in a strand of DNA have been used. They are the chemical degradation method of Maxam and Gilbert, and the enzymatic method of Sanger. While both methods have benefited greatly from modern automation, and both rely on separation using high-resolution polyacrylamide gel electrophoresis, the Sanger method has become the most commonly used technique. The Sanger technique is often referred to as dideoxy-mediated chain termination and involves DNA synthesis from either single- or double-stranded templates. The synthesis reaction is performed using a synthetic oligonucleotide primer (similar to PCR) that anneals to a specific sequence of the template. A DNA polymerase, a mixture of deoxynucleoside triphosphates (dNTPs), and a small amount of a dideoxynucleoside triphosphate (ddNTP) are added to the reaction, and as synthesis occurs, both types of dNTPs will be added to the growing DNA chain. However, ddNTPs lack a 3' OH group on the deoxyribose, preventing the formation of a phosphodiester bond with any subsequent dNTP or ddNTP. This stops further extension of the chain after the incorporation of a ddNTP, hence the name, "dideoxy-mediated chain termination". Incorporation of the ddNTPs will happen at each occurrence of that specific base, terminating the elongation of the DNA at that point, and resulting in a variety of fragment lengths, each ending in a ddNTP. Historically, the ddNTPs were labeled with radioactivity, so that they could be detected by autoradiography. This resulted in each individual ddNTP (A, C, G, T) reaction occurring in one of four separate tubes, and the four reactions were run in four lanes on the gel. However, advancements

in technology have resulted in the further modification of the Sanger dideoxy sequencing method. This modified technique is referred to as "cycle sequencing" because it is performed in a thermal cycler using a thermostable DNA polymerase in a PCR reaction. Cycle sequencing has all but eliminated the use of the Maxam-Gilbert sequencing technique. This modified technique has many advantages including lower amounts of starting template, improved ability to sequence double-stranded template and GC-rich fragments, and it has eliminated the need to clone the fragment before sequencing. Another advantage is the use of four fluorescently labeled ddNTPs, each having a unique fluorescent molecule attached, allowing the reaction to occur in a single tube, and to be run in a single lane. The unincorporated labeled ddNTPs are removed by gel filtration chromatography or ethanol precipitation before electrophoresis through a denaturing polyacrylamide gel, and fluorescently labeled products are detected by automated DNA sequencing systems. During separation by high-resolution polyacrylamide gel electrophoresis, the fluorescent dye is excited by an argon laser that scans back and forth across the bottom of the gel. The emission of the dye is detected and captured by a photomultiplier tube (PMT) and converted to an electronic signal for the base that corresponds to the wavelength detected. These electronic signals are computer processed and converted to a series of emission peaks, identifying the corresponding base, and creating a computer file containing the DNA sequence. This allows for the subsequent analysis of the genetic information with a variety of computer software programs. The majority of current DNA sequencing is done using capillary array electrophoresis systems. Technological improvements in capillary electrophoresis have led to the development of these systems, which use arrays of 96 silica capillaries containing a low-

viscosity polymer matrix that can be reused to separate DNA molecules for sequencing. Detection occurs when a stationary laser at the end of the each capillary excites the fluorescent ddNTP and a CCD camera collects the emission spectrum. The electronic image is converted to an electropherogram, by computer processing, converted to a series of emission peaks, the corresponding base is identified, and a computer file containing the DNA sequence is created. Capillary systems offer several advantages, including greater automation, decreased opportunity for human error (no longer need to mix and pour polyacrylamide gels), eliminates the chance of a sample being confused between lanes, and significantly decreased run times. Sequencing runs can be as rapid as 1.0 to 1.5 hours, while longer reads can be accomplished in no more than 3 hours. These advances have greatly improved the automation, decreased the opportunities for errors, and increased the throughput of DNA sequencing.

Genomic DNA can be used to create "fingerprints" specific to a sequence of DNA, an individual, or an organism. This has been accomplished in the past by using the technique restriction fragment length polymorphism. This technique involves the digestion of large DNA fragments with restriction enzymes into smaller fragments of varying lengths. The differing lengths are a result of the differences in the DNA sequence, which subsequently change where the restriction enzyme recognition sites are. Hence, the use of the name "restriction fragment length polymorphism" or RFLP. Two DNA samples can be digested, and each sample will result in a different pattern of DNA fragments based on length when they are separated via gel electrophoresis. This pattern should be relatively constant for that individual, and can be thought of as a "fingerprint" that has the potential to differentiate two DNA samples. However, given the large size of

most genomes (> 1 Mb), even an enzyme that cuts rarely (eight-cutter; $4^8 = 65,536$), can give upwards of 50 bands for a relatively modest genome like *E. coli*. A six-cutter can give hundreds, if not thousands of bands when total genomic DNA is used, resulting in a smear that makes interpretation difficult. A related technique that has been used extensively for DNA fragments smaller than total genomic DNA, but larger than ~ 10 kb is restriction mapping. Restriction mapping typically involves sequential digestion of a DNA fragment with two enzymes, or simultaneous digestion with two or more enzymes. A related procedure for smaller DNA fragments is amplified fragment length polymorphism. PCR is performed on a sequence of interest, the PCR product is digested, and the different fragment lengths are separated via electrophoresis. This has the advantage of only detecting restriction sites in the PCR product, and not in the original template, due to the high copy number of the PCR product. This form of fragment analysis fingerprinting has been applied to the gene encoding the ribosomal RNA, it has been used extensively for taxonomic studies of many bacteria, and is commonly referred to as ribotyping. It is capable of differentiating between unique species as well as between strains of the same organism, and is often applied in epidemiology due to the great intraspecies diversity present in the ribopatterns. An offshoot that has combined these two modified procedures is terminal restriction fragment length polymorphism (T-RFLP). In this technique, a specific gene or sequence of DNA that is common to all samples is targeted. This gene must have a portion of sequence that is conserved across species for primer binding sites; yet also have a variable sequence region. The presence of the variable region of sequence allows for changes in the occurrence of the restriction enzyme recognition site, which will result in different fragment lengths. The samples of

interest are then amplified in a PCR reaction where one of the primers is labeled, either with radioactivity or with a fluorescent molecule. Then the PCR product is digested with the restriction enzyme, and the terminal fragments are detected by autoradiography or fluorescence detection. This technique utilizes a restriction enzyme with a more commonly occurring recognition site (i.e., a four-cutter $4^4 = 256$ bp) without increasing the difficulty in interpreting the output from the gel. This is due to the fact that only the fragment that is end-labeled (terminal fragment) is detected. The changes (polymorphisms) in the location of the first occurrence of the recognition site will result in different fragment lengths. Because they are not labeled, any fragments resulting for a restriction cleavage at a further downstream site will not be detected. This greatly decreases the complexity of interpreting the results of the electrophoresis. This technique also lends itself to greater precision and automation. Because most PCR products are relatively small (< 4 kb), restriction fragments can be resolved using polyacrylamide rather than agarose gel electrophoresis. In addition, the use of a fluorescent molecule as a label, allows the detection of the terminal fragments by an automated DNA sequencing apparatus. When combined with an internal size standard on a high-resolution sequencing gel, single base pair differences in the occurrence of the first recognition site can be detected. This makes the technique extremely sensitive, and has allowed its adaptation to the study of complex microbial ecosystems. While this sequence-dependent technique was not designed to analyze microbial community ecology, many researchers have begun to incorporate its use into their research (Marsh, 1999; Moeseneder et al., 1999; Blackwood et al., 2003; Nagashima et al., 2003). The application of this technique to microbial ecology is based on the presence of highly conserved genes in microbial

species. The primary gene that meets these criteria in nature is the gene that codes for the RNA that makes up a portion of the small subunit of the ribosome and is unique between kingdoms.

In cells, the ribosome is made up of two subunits, commonly called the large subunit and the small subunit, based on their sizes. Ribosomes make up a large portion of cellular mass, and their associated RNA (rRNA) comprise a majority (~97%) of the total RNA found in a cell. The ribosomal RNA associated with the small subunit of the ribosome of prokaryotes are 5S and 16S rRNA, based on size. 16S rRNA molecules are approximately 1500 bp in length. The genes that code for the 16S rRNA not only have highly conserved regions, but they also have hypervariable regions. This allows for selection of PCR primers that will amplify a targeted group of microbial species, while still allowing differentiation of these species on the basis of the internal sequence of that gene. The ability to characterize communities in their natural habitat is paramount to our understanding of the function of complex systems of interactions that occur in these diverse species. Additionally, our inability to reproduce these systems in vitro prevents us from gaining any great understanding of the mechanisms that play a role in the function of these systems. Current estimates are that less than 1% of microbial species are culturable in the lab. These estimates have been inferred from microbial communities studied in soils, where DNA-DNA re-annealing curves suggest the presence of up to 10,000 unique genomes; yet only a fraction of these have been isolated by traditional culture techniques. An additional advantage of using the 16S rRNA gene for molecular microbial species ecology research is the presence of a large database of genetic information. This allows comparisons to be made between known and unknown

organisms and for the phylogeny of organisms to be determined. The information that the previous section is based on came from Sambrook et al. (1989), Ausubel et al. (1995), Alcamo (2001), Sambrook and Russell (2001), and from various class notes.

Ruminal microbial ecology techniques

Traditional ruminal microbiology methods have included growing microbial populations in the laboratory from inoculations of ruminal contents, including culturing and counting colony forming units (CFU) on selective medium using plates, or tubes (Hungate, 1950), or with the most-probable number technique (Dehority et al., 1989), counting and identifying microbes by morphology using microscopy (Dehority and Orpin, 1997), flow cytometry (Avgustin and Lipoglavsek, 2000), and measuring metabolites produced from fermentation of various substrates. Several newer procedures include sorting by cell size (Baker and Munyard, 2000) and quantifying cellular fatty acid profiles (Marinsek Logar et al., 2000). However, molecular methodologies have been gaining popularity (Pace et al., 1986; Stahl et al., 1988; Macheboeuf et al., 2000; Tepsic and Avgustin, 2000). In the last 15 years, the advent of new and improved technology and techniques, such as PCR and rapid sequence determination systems, have resulted in extensive DNA, RNA, and protein sequences being determined, which has spurred research in the field of ruminal microbial ecology (Olsen et al., 1994; Wood et al., 1998; White et al., 1999; Tajima et al., 2001). A method to describe microbial populations using sequences of RNA that combine with proteins to make up the ribosome of microorganisms has been developed (Pace et al., 1984). Pace et al. (1986) described the use of oligonucleotide probes for ribosomal RNA (16S rRNA) of microbial origin to hybridize with specific nucleotide sequences that allow detection of various taxonomic

levels of microorganisms. Because the rRNA molecule is single-stranded and made up of specific sequences of nucleotides (A, U, G, C), a short oligonucleotide with ^{32}P-labeled nucleotides can be hybridized to the extracted microbial rRNA and the counts of radioactivity can be measured to quantify specific microbial species. These "tags" can be designed so that the oligonucleotide will bind to all microbes, or to specific major groups of related microbes, or to a particular species of microorganism. This technique has greatly expanded the amount of research done in the area of microbial ecology. Use of 16S rRNA profiling has been utilized effectively in ruminant systems to describe changes due to ionophore feeding (Stahl et al., 1988; Krause and Russell, 1996), various nutritional effects (Briesacher et al., 1992), and protein supplements (May et al., 1993). While technically demanding, molecular techniques utilizing 16S rRNA can provide an effective method to describe ruminal microbial ecosystems. However, the level of technical difficulty, absolute precision required, and equipment and facilities that most ruminant nutritionists do not have access to, has made broad adoption of this molecular technique difficult.

The 16S rRNA methods, when used to describe microbial populations using sequences of microbial ribosomal RNA (16S rRNA), has recently been gaining popularity since it became available (Pace et al., 1986; Stahl et al., 1988). Many researchers have utilized the 16S rRNA technique for profiling microbial ecology (Stahl et al., 1988, Briesacher et al., 1992; May et al., 1993; Odenyo et al., 1994a,b; Krause and Russell, 1996; Kalmokoff and Teather, 1997; Franks et al., 1998; Zoetendal et al., 1998; Simpson et al., 1999; Davies et al., 2000; Forster et al., 2000; Hold et al., 2000; Rupf and Eschrich, 2000, Leser et al., 2000; Macheboeuf et al., 2000; Mikkelsen and Jensen, 2000;

Ozkose et al., 2000; Rieu-Lesme et al., 2000; Tepsic and Avgustin, 2000; and Thyer et al., 2000).

More recently, the 16S rRNA procedure has been modified to probe the genes (16S rDNA) for 16S rRNA, and has been further modified to use a real-time PCR system (ABI Prism, Light-Cycler, etc.) with fluorescent primers to provide an effective method to describe ruminal microbial ecosystems that can be easily, quickly, and accurately used on a wide-scale (Higgins et al., 2001; Tajima et al., 2001). This also removes the reliance on radioactive labeled compounds, and the associated disposal, containment, and health risks for researchers, technicians, students, and institutes. This approach is being used to research the evolution of microbial populations in the specific areas where microbial ecology is altered the most, and is greatly adding to improving the understanding of microbial communities in a wide range of areas of livestock animal agriculture. These areas include increasing the efficiency of animal production, reducing the potential for negative environmental impact, assessing the possibilities to decrease the use of feed anti-microbial agents, and gaining a better understanding of new methods to prevent, diagnose, and treat metabolic disorders and disease states.

The incorporation of these types of technology have been undertaken in human nutrition, health, and disease research, dentistry, soil microbiology, environmental hazard and cleanup research, ruminant nutrition, animal livestock production, entomology, phylogeny, taxonomic classification, the study of evolution, and a great many other areas.

Ruminal microbial ecology responses to nutritional factors

Even though ruminal microbes has been extensively studied (Hungate, 1966; Bryant, 1977; Hobson and Wallace, 1982a,b), there is still a great deal that remains unknown

about the impact that nutritional changes play in altering ruminal microbial ecology. Ruminal microbial populations can be altered by many nutritional factors. For example, it has been documented that changes in nutrition (Bryant and Burkey, 1953; Bryant and Robinson, 1968; Briesacher et al., 1992; May et al., 1993), the use of ionophores (Chen and Wolin, 1979; Dennis et al., 1981; Whetstone et al., 1981; Bergen and Bates, 1984), and ruminal acidosis during the transition from forage to high-grain diets (Hungate, 1966; Slyter, 1976; Coleman, 1979; Mackie and Gilchrist, 1979) all result in vastly altered ruminal microbial ecology. However, the detailed alterations in microbial species due to these perturbations are still unknown.

General nutritional factors. Many early researchers found alterations in ruminal ecology as a result of dietary shifts (Bryant and Burkey, 1953; Bryant and Robinson, 1968; Van Gylswyk, 1970). Dehority and Orpin (1997) reviewed a great deal of literature and found that time, diet, level and frequency of feeding, seasons, domestic vs wild ruminants, and antibiotics all played a role in altering total microbial populations as well as species or specific groups.

Effects of monensin on microbial composition. Monensin is an inhibitor of gram-positive ruminococci bacteria, and can result in the increase of gram-negatives such as *Fibrobacter succinogenes* and *Selenomonas ruminantium* (Owens et al., 1991). Ionophores select against the gram-positive bacteria and allow rapid selection of ionophore resistant gram-negative species while also selecting against *streptococci* and *lactobacilli* that may aid in reducing acidosis (Yokoyama and Johnson, 1993). Monensin has little effect on methanogenic microbes; however, it does inhibit the bacteria that produce hydrogen and formate, both precursors of methane production, and is also

inhibitory to several obligate amino acid fermenters (Russell, 1996). Yang and Russell (1993) and Krause and Russell (1996) both found significant decreases in these species when monensin was included in chopped cool season grass hay diets, as did Stahl et al. (1988), when alfalfa and grain diets were fed. Ionophores will typically reduce coccidia loads to near zero within 30 d (Owens et al., 1991). Monensin is thought to be fungistatic, it decreases protozoal and gram-positive bacteria populations, it results in increased populations of microbes that produce succinate or propionate, and will decrease methane loss (Owens et al., 1991). Because profiles of fermentation products change within minutes of the addition or removal of monensin (Stahl et al., 1988; Owens et al., 1991) the mode of action is relatively rapid. While monensin decreases the deamination of feed protein by reducing the numbers of obligate amino acid fermenters, it may not result in an overall increase in duodenal CP flow, due to decreased efficiency of microbial growth (Owens et al., 1991).

Roughage, concentrate, and protein. Protein supplementation has been shown to increase microbial populations while having no effect on *Fibrobacter succinogenes* proportions (May et al., 1993). When cellulose was the only substrate, gram-negative micrococci and small rods were the predominate microbes, and the addition of starch replaced them with gram-negative crescent and oval rods, cocciods, and gram-positive diplococci (el-Shazly et al., 1961). Leedle et al. (1982) found greater cellulolytic bacteria on forage than grain diets. Ha et al. (1995) found increased cellulolytic microbes with various treatments of cellulose to increase digestibility. Varel et al. (1995) introduced large quantities of *Clostridium longisporum* into emptied rumens, and found that it was undetectable after 48 h, even though this species is of ruminal origin. Cummings et al.

(1995) found alterations in the sulfate-reducing bacteria populations when polioencephalomalacia was induced as a result of high dietary sulfur intakes. Varel and Dehority (1989) found no differences for cattle, bison, and cattle-bison crosses fed alfalfa with three levels of corn. Cellulolytic bacteria do not grow on cellulose when pH is below 6.0 (Hoover, 1986; Russell and Wilson, 1996; Weimer, 1996, 1998). When animals are adapted to high-grain diets, acid-tolerant lactate utilizers replace the acid-sensitive lactate utilizers while amylolytic bacteria are replaced by lactate producers (Slyter, 1976; Yokoyama and Johnson, 1993). Mackie et al. (1978) found no differences in total bacteria, but did note that lactate-utilizing bacteria always increased following an increase in amylolytic microbes during adaptation to grain diets by sheep. Grain overload has caused increased numbers of amylolytic and sugar-using bacteria, which have rapid generation intervals, often resulting in increases of *Lactobacillus* species (Slyter, 1976; Huntington, 1993). Under normal conditions, lactate-producing microbes mainly produce acetate, propionate, small amounts of butyrate, formate, and ethanol (Hishinuma et al., 1968; Russell and Hino, 1985). However, when glucose is in excess (i.e., when ruminants are fed large amounts of grains that are high in starch, a storage form of glucose), lactate is produced, pH drops, and the lactate-utilizing bacteria are inhibited along with the cellulolytic microbes (Slyter, 1976; Russell and Dombrowski, 1980; Newbold and Wallace, 1988). Eventually, the reduction in pH will inhibit *Streptococcus bovis* along with the sugar-utilizers, and further inhibit the lactate-utilizers, resulting in a proliferation of *Lactobacillus* species (Slyter, 1976; Newbold and Wallace, 1988). Overall, during acidosis, there is a shift towards increased numbers of rapidly growing gram-positive bacteria, specifically rods and cocci (*Lactobacillus* species and

Streptococcus bovis), and greatly decreased numbers of gram-negative bacteria (Slyter, 1976). Yet, during sub-clinical acidosis, total bacterial numbers and gram-positive bacteria numbers may increase (Mullen, 1976). As a result of these changes in the relative contributions of specific species to the ecology of the rumen in addition to the changes in the activities of specific groups, the addition of grain to the ruminant diet can result in several negative effects. Even though the most efficient animal gains can be realized by feeding grain, changes that occur in the rumen during the transition from forage to grain diets can have large negative effects on animal production, health, and well-being. The negative effects of grain on forage utilization could be circumvented if the fibrolytic bacterial species could maintain their cellulolytic activity even at high levels of grain inclusion in the diet.

Protozoa. Addition of 10% forage to concentrate diets did not change total protozoal concentrations (which can contribute 50% of ruminal microbial mass), yet forage addition has been shown to increase the percentages of *Isotricha* and *Epidinium* (Franzolin and Dehority, 1996). Eadie et al. (1970) suggested that *Isotricha* are more numerous during grain overload. However, others have suggested that protozoa increase with decreasing pH up to about 5.5 and then rapidly decrease after that with the *Holotrichs* being more susceptible to this decrease in pH than the *Entodiniomorphs* (Yokoyama and Johnson, 1993). Slyter et al. (1965) suggested that feeding high-grain diets causes reductions in protozoal numbers. Stern et al. (1978) found no effect of level of starch or urea inclusion on protozoal numbers in continuous culture. Protozoal populations are greater when high-molasses diets are fed (Bird and Leng, 1978) and

changes in bacterial populations can result in changes in protozoal composition (Yokoyama and Johnson, 1993).

Ruminal microbial ecology response to substrate competition. As grain is added to the diet of ruminants consuming low-quality forages, pH declines, and fibrolytic ruminal microorganisms are selected against by a variety of mechanisms, including microbially produced anti-microbial peptides (lantibiotics) commonly known as bacteriocins. The production of bacteriocins in the rumen may occur in response to the decreasing pH that occurs as a result of large additions of grain to the diet. As these mechanisms become elucidated, further research will allow increases in the viability, survivability, and ability of fibrolytic microorganisms to ferment roughage under conditions of starch addition and lowered pH. Discovering the mechanisms that induce bacteriocin production could allow changes in management to specifically alter microbial diversity, improve the efficiency of fermentation by altering the ratios of fermentation metabolites, allow greater fiber digestion, improve animal health, performance, and efficiency.

Anti-microbial peptides are found throughout nature in microorganisms, as well as in mammals. Lactic acid bacteria produce a broad group of these bactericidal substances known as bacteriocins. Bacteriocins are typically selective against related strains of organisms, with some of the anti-microbial peptides having broader ranges of activity. These compounds have been isolated from a large number of microbes occurring in a variety of environments and have even been used as a safe and natural food preservative in the human food industry. Bacteriocins have bactericidal action as a result of forming pores in the cytoplasmic membrane of sensitive bacterial species (targets) and cause potassium ion efflux resulting in dissipation of the membrane potential of the bacteria

(Herranz et al., 2001). Bacteriocins have been classed into three groups. Group I bacteriocins are the lantibiotics, or peptides that are post-translationally modified and contain lanthionine residues. Group II bacteriocins are not post-translationally modified, are small (<10 kDa), heat-stable peptides, and are subdivided into four sub-groups: sub-group IIa contains bacteriocins with similar N-terminal sequences (YGNGVXC); sub-group IIb consists of bacteriocins with the two-peptide systems; sub-group IIc bacteriocins are *sec*-dependent; and sub-group IId is made up of the remainder of bacteriocins in class II that don't fall into one of the other three sub-groups. Finally, Class III bacteriocins consist of large (>30 kDa), heat-labile proteins.

There is limited knowledge of the mechanisms that ruminal microorganisms employ during degradation of feedstuffs to compete for substrate availability. Even though it has been suggested that bacteriocins may be important in bacterial ecology (Riley, 1998), and may play a role in competition for substrates via competitive advantage (Pagie and Hogeweg, 1999), bacteriocins have been studied very little in the context of ruminant animal production, nutrition, and management.

While bacteriocins are known to exist in the rumen (Iverson and Mills, 1976; Laukova and Marekova, 1993; Odenyo et al., 1994a,b; Kalmokoff et al., 1996; Kalmokoff and Teather, 1997; Wells et al., 1997; Kalmokoff et al., 1999; Whitford et al., 2001; Rychlik and Russell, 2002), they have been much more widely studied in other areas of research, such as food science, microbiology, biochemistry, and protein research (Bradley, 1967; Tagg et al., 1973, 1976; Barefoot and Klaenhammer, 1983; Geis et al., 1983; Klaenhammer, 1988, 1993; Delves-Broughton, 1990; Delves-Broughton et al., 1992; Holck et al., 1992; Barefoot and Nettles, 1993; Jack et al., 1995; Saucier et al.,

1995). Recently, this area of research has gathered more attention in the field of ruminant nutrition (Kalmokoff and Teather, 1996), resulting in the isolation of several bacteriocins from a variety of ruminal microbial species. These species include: *Butyrivibrio fibrisolvens* (Kalmokoff and Teather, 1997; Kalmokoff et al., 1999; Rychlik and Russell, 2002); *Lactobacillus fermentum* (Wells et al., 1997); *Pseudomonas aeruginosa* (Duncan et al., 1999); *Ruminococcus* spp. (Odenyo et al., 1994a,b; Chan and Dehority, 1999; Chen and Weimer, 2001); and *Streptococcus* spp. (Whitford et al., 2001). While a large variety of microbial strains have been screened for bacteriocin production, it has been suggested that the lack of production of bacteriocins in laboratory culture may not necessarily indicate that the strain is not a producer in the environment. The signals and mechanisms that result in induction of bacteriocin production are as of yet unclear, making duplication of these environmental conditions difficult in laboratory culture systems. Add these difficulties to the complexities of culturing environmental anaerobes emphasizes the absolute necessity for research to be conducted on bacteriocin production under the normal environmental conditions that induce the production of the bactericidal peptide compounds. For example, Fondevila and Dehority (1996) only found inhibition in forage cellulose digestion when *F. succinogenes* and *R. flavefaciens* were co-cultured. As a result of these challenges, other researchers have suggested that the presence of genes encoding the enzymes for the production of lanthionine can be used to determine if a strain is a producer (Nes and Tagg, 1996). Bacteriocin transcription is growth-phase dependent, stimulated by extra-cellular bacteriocin, and regulated by two-component systems (Kuipers et al., 1995; Dean et al., 1996; Ra et al., 1996; Gomez et al., 2002). It appears that when pH is decreased as a result of feeding large amounts of grain

(providing abundant glucose via fermentation of the starch in the grain), there is a negative effect on the major fibrolytic ruminal bacteria (*Butyrivibrio fibrisolvens, Fibrobacter succinogenes, Ruminococcus albus*, *Ruminococcus flavefaciens*; Dehority and Tirabasso, 1998). However, the decreases in the major cellulose fermenting bacteria cannot be explained by pH alone (Hoover, 1986; Horn and McCollum, 1987; Caton and Dhuyvetter, 1997). Researchers have found a decrease in cellulose digestion even when pH was maintained artificially in vitro (el-Shazly et al., 1961; Stern et al., 1978). This has been termed the "carbohydrate" effect (Hoover, 1986) and has been suggested to be caused by many factors including preferential use of substrate, substrate limitation (ammonia), increased washout of bacteria, increased passage rates, increased lag time required for microbial attachment to feed particles (which has been shown to be increased as a result of bacteriocin production; Rychlik and Russell, 2002), decreased pH, or buffering capacity, or some other factor (such as bacteriocin production). It is possible that the addition of starch to the ruminant diet results in an increase in bacteriocin production, and that the primary fibrolytic bacteria are more susceptible to these anti-microbial peptides than the amylolytic bacteria. In vitro studies have shown that the addition of higher levels of glucose to pure bacterial cultures will result in the increased production of these compounds. Also, other studies have shown that bacteriocins are not produced until the late exponential growth phase, when glucose and ammonia are limiting for growth, the pH of these pure cultures has dropped significantly, and bacteriocin production has had time to occur. Shi and Weimer (1997) showed that when available glucose dimers became limited, di-cultures of ruminal cellulolytic microbes were converted into monocultures, in comparison with complex substrate (cellulose),

where niche specialization occurred, and di-cultures were maintained (Shi et al., 1997). Addition of the homolactic fermenter *Selenomonas ruminantium* (amylolytic, one of the major ruminal starch fermenters) to a tri-culture of cellulolytic microbes resulted in *Selenomonas ruminantium* predominating (90%), with similar results occurring when *Streptococcus bovis* (amylolytic, also one of the major ruminal starch fermenters and a known bacteriocin producer, Whitford et al., 2001) was added to the three cellulolytic strains (Chen and Weimer, 2001). The addition of either amylolytic strain also increased the relative prevalence of *Ruminococcus albus*, a fibrolytic microorganism, in comparison with *Fibrobacter succinogenes* or *Ruminococcus flavefaciens* (two of the predominant fibrolytic bacteria in the rumen). *Ruminococcus albus* is a known bacteriocin producer (Odenyo et al., 1994a,b; Chan and Dehority, 1999). However, to date, there is no in vivo data to support this theory (Chen and Weimer, 2001).

Conclusions. There are many mechanisms that interact to influence the ecosystem of the microbial species in the bovine rumen, many of which are poorly understood. Much of the research that is currently being undertaken is focusing on the effects of manipulations on a handful of known bacterial species in the rumen. However, because we lack a thorough understanding of what species exist and how changes in species composition alters ruminal function, this direction of research may provide us with limited conclusions. We have a great deal to learn about the microbial species present in the rumen, what role they play in ruminal function, at what levels they exist, how they change over time, and if we can manipulate them.

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Chapter III

Effects of supplemental energy and(or) degradable intake protein on performance, grazing behavior, intake, digestibility, and fecal and blood indices by beef steers grazed on dormant native tallgrass prairie

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Abstract: To evaluate the effects of balancing total diet degradable intake protein with dietary TDN, we conducted 2 studies during two years with 100 (302 ± 8 kg initial BW) mixed-breed yearling steers and 12 ruminally cannulated steers (526 ± 28 kg). Steers individually received one of four supplements 5d/wk while grazing dormant native tallgrass prairie. Supplements included: 1) corn and soybean meal, balanced for total diet degradable intake protein in relation to total diet TDN (CRSBM), 2) corn and soybean hulls, equal in supplemental TDN to CRSBM (CORN), 3) soybean meal, equal in supplemental degradable intake protein to CRSBM (SBM), or 4) a cottonseed hull-based control supplement (CONT). At each feeding (5 d/wk), steers consumed 13.6, 13.6, or 4.2 g of dry matter/kg of body weight, or 178 g of DM, respectively, of supplement. Steers fed CRSBM had greater ($P < 0.01$) average daily gain than cattle fed CORN or SBM. Feeding soybean meal (CRSBM, SBM) resulted in improved ($P < 0.01$) efficiency of supplement. Grazing time, intensity, and harvesting efficiency were reduced ($P < 0.05$) by corn supplementation (CRSBM and CORN), whereas the number of grazing bouts per day was increased ($P < 0.08$). Intake and digestibility of forage OM were reduced ($P < 0.01$) for steers supplemented with corn (CORN and CRSBM) vs cattle not fed corn (SBM and CONT). Total diet digestibility ($P < 0.12$) and digestible organic matter intake ($P < 0.01$) were greater for CRSBM-fed steers than for cattle fed

either CORN or SBM. Steers fed CRSBM had greater ($P < 0.01$) fecal nitrogen and serum insulin than cattle fed CORN or SBM. Corn-fed cattle had lesser ($P < 0.01$) fecal pH and ADF concentration than steers not consuming grain. Cattle fed supplements with soybean meal (CRSBM and SBM) had greater ($P < 0.01$) serum urea nitrogen than steers fed supplements without soybean meal (CORN, CONT). Supplemented steers grazing dormant tallgrass prairie had greater rate of gain with the greatest response in animal performance occurring when grain supplements were balanced for total diet degradable intake protein in relation to total diet TDN. These results lead us to suggest that grain-supplemented cattle grazing dormant tallgrass prairie require a balance of total diet degradable intake protein in relation to total diet TDN to optimize animal performance.

Key Words: Beef Cattle Performance, Blood Analysis, Corn, Foraging, Soyabean Oilmeal, Supplement Feeding Programs

Introduction

Cattle grazing native tallgrass prairie during the dormant season may encounter many nutrient deficiencies. Supplementation is necessary to allow stocker cattle to gain weight during this period. Protein is typically considered the primary limiting nutrient; however, increasing forage intake with protein supplements might not result in adequate increases in energy intake for animal performance to achieve a desired rate of gain (Bowman and Sanson, 1996). Summaries of previous research have indicated that forage intake and digestibility will decrease when high-starch, low-protein grains are used as a supplement for cattle consuming low-quality (protein deficient) forages (Horn and McCollum, 1987; Bowman and Sanson, 1996; Caton and Dhuyvetter, 1997). These decreases in forage intake and digestibility have resulted in lower than expected energy

intake when corn was supplemented to cattle consuming low-quality forages (Chase and Hibberd, 1987). However, more recent research (Hibberd et al., 1987; Olson et al., 1999; Bodine et al., 2000b, 2001) has indicated that the addition of degradable intake protein **(DIP)** to grain supplements will increase the utilization of low-quality forages. This increased utilization will result in greater energy intake for grain plus DIP- supplemented cattle grazing low-quality forage. Therefore, our objective was to determine the effects of energy and/or protein supplementation using the current NRC (1996) model to balance DIP in relation to TDN for steers grazing low-quality forage.

Materials and Methods

Study Site, Vegetation, and Stocking Rate. We conducted two experiments during 2 yr in which 100 crossbred stocker steers grazed 130 ha of native tallgrass prairie located at the Oklahoma State University Bluestem Range, 11 km southwest of Stillwater, OK (long 36°03'N; lat 97°12'W; elevation 331 m; Payne County). Climate at the experimental location is continental with an average frost-free growing period of 204 d, extending from April to October. Annual precipitation averages 831 mm with 65% falling as rain from May to October. The mean annual temperature is 15°C, and ranges from an average daily minimum of -4.3°C in January to an average maximum of 34°C in August (Myers, 1982; NOAA, 1999). Range sites at the study location include shallow savannah, sandy savannah, eroded sandy savannah, loamy prairie, shallow prairie, eroded prairie, shallow claypan, and eroded clay sites. The majority of slopes range from 0 to 8%, with stream drainages having steep slopes (8 to 45%). Livestock had ad libitum access to drinking water from free-flowing streams, ponds, and improved water sources.

Bulldozers mechanically cleared the pasture to reduce cover of dominant woody plant species approximately 10 yr prior to the trial, and the OSU Research Range Fire Crew burned the pasture to control woody plant species 18 mo prior to the initiation of the trial. Woody plant species currently present at the experimental site include eastern red cedar (*Juniperus virginiana* L.), post oak (*Quercus stellata* Wangenh.), and blackjack oak (*Quercus marilandica* Muenchh.). Herbaceous vegetation is typical of tallgrass prairie in a late seral state. Dominant forage grass species are big bluestem (*Andropogon gerardii* Vitman), little bluestem (*Schizachyrium scoparium* [Michx.] Nash), and indiangrass (*Sorghastrum nutans* [L.] Nash). Subdominant grass species include switchgrass (*Panicum virgatum* L.), tall dropseed (*Sporobolus asper* [Michx.] Kunth), sideoats grama (*Bouteloua curtipendula* [Michx.] Nash), and Scribner's dicanthelium (*Dicanthelium oligosanthes* [J. A. Schultes] Gould). Forbs include lespedeza species (*Lespedeza* spp.) and western ragweed (*Ambrosia psilostachya* DC).

Stocking rates were 33.5 and 22.5 animal-unit-days (**AUD**)/ha, for yr 1 and 2, respectively. This corresponds to traditional stocking rates of 2.5 and 2.7 ha/steer (0.4 and 0.37 steers/ha) for the winter grazing season (96 and 70 d), for yr 1 (52 steers) and 2 (48 steers), respectively. We calculated animal units as BW, $kg^{0.75}$ divided by $454^{0.75}$ (Vallentine, 1990), using the initial shrunk BW of the steers in each experiment. In the second year, four fewer steers grazed the pasture, resulting in a decreased stocking density. In addition to this reduction in stocking density, the length of the experimental grazing period was shortened by 26 d because of earlier than expected spring growth of forage grasses in yr 2. The combination of fewer steers and a reduced number of days on study resulted in the reduced stocking rate in the second year.

Animals. All experimental protocols were approved by the Oklahoma State University Animal Care and Use Committee. In both years, fall-born calves from the same two herds grazed 65 ha of native tallgrass prairie during the late summer, and we trained the steers to enter individual feeding stalls by offering each steer 0.9 kg of a 20% CP cubed supplement two times/week for a period of 4 wk prior to the initiation of the trials. Steers were not implanted prior to weaning (verified by palpation of ears), nor were they implanted while on trial during either year. We weighed steers approximately 3 wk prior to the initiation of each study (d –21), at trial initiation (d 1), at the completion of each trial (~d 83), and approximately 1 mo (~d 116) after the completion of the studies. These weights were taken following an overnight (14 h) removal of access to feed and water and used to calculate weight gain and rate of gain to determine animal performance responses to treatments. We weighed cattle without removal of access to feed and water 1 d prior to the initiation of the trial (d -1), adjusted the weights for a 4% mathematical shrink, and used these shrunk weights to calculate preliminarily supplement intake (g/kg of BW) and to randomly allot cattle to treatments. On d 1, steers were ear tagged, randomly allotted to treatment, treated for parasites according to label directions (Ivomec, Merial Limited, London, UK), and allowed to graze for 4 h prior to the feeding (start of adaptation) of supplements. We also weighed steers without removal of access to feed and water at approximately 28 and 56 d of each study to recalculate supplement intake (g/kg of BW; using a 4% mathematical shrink) to adjust for increased body weight as a result of gain. In both years, we weighed cattle approximately 3 wk (~d -21) prior to the initiation of the study to determine a pretrial rate of gain. Steers grazed together as a group prior to trial initiation and no differences were noted between the subsequent

treatment groups prior to trial initiation. In an attempt to determine the effects of our supplementation treatments on subsequent animal performance, we weighed steers approximately 1 mo (~33 d) after the completion of each trial to determine post-trial animal performance. During this period, steers grazed 25 ha of standing dormant and early spring growth of Old World bluestem (*Bothriochola ischaemum*) and were fed 1.2 kg of DM/(steer·d) of a 34% CP (as-is) range cube prorated for feeding four times weekly. Twelve ruminally cannulated (10 cm i.d.) steers (526 ± 28 kg; Angus and Angus x Hereford; 3 to 5 yr old) were used for masticate sample collection, and to validate Cr recovery from total fecal collection using fecal collection bags. Cannulates were randomly allotted to supplement treatments, grazed the experimental site along with the intact steers, and were included in the calculations of stocking rate expressed as AUD/ha.

Experimental Diets and Feeding. Treatments (Table 1) consisted of: 1) 7.5 g dry-rolled corn DM/(kg of BW·d) and added an adequate amount of soybean meal to balance total diet DIP requirements (**CRSBM**), 2) 7.5 g dry-rolled corn DM/(kg of BW·d) and added soybean hulls to achieve an equal amount of supplemental TDN, g/(kg of BW·d) as CRSBM (**CORN**), 3) soybean meal to supply an equal amount of supplemental DIP g/(kg of BW·d)] to CRSBM (**SBM**), or 4) a cottonseed hull-based control supplement (**CONT**). This level of supplemental corn feeding is based on our previous metabolism studies (Bodine et al., 2000b; 2001), and is similar to quantities of corn fed by Chase and Hibberd (1987) and Sanson and Clanton (1989), and provides 41 g of supplemental DM/kg of $BW^{0.75}$, which is above the level of 30 g of DM/kg of $BW^{0.75}$ suggested by Horn and McCollum (1987) to depress forage intake. Requirements for DIP were determined using the NRC (1996) level 1 model software with estimated forage intake

[(18 g DM)/(kg of BW·d)] and estimated forage chemical composition: 93% OM; 74% CP; DIP 70% of CP; 75% NDF, and 60% TDN (estimated from in vitro organic matter disappearance) from historical masticate samples (1993 to 1998) previously collected from the experimental pasture (Basurto et al., 2000). Other model inputs included measured steer BW supplemental corn intake, and assumed an average value of 10.25% microbial protein yield from TDN. This value of microbial protein yield from TDN agrees with data we have collected previously (our unpublished results) and is at the upper end of the range described in the text of the NRC (1996) for low-quality forage diets with 50 to 60% digestibility, and is similar to the value suggested by Cochran et al. (1998). The decision to use a value at the upper end was made in order to ensure that DIP requirements were met. We added soybean meal to the CRSBM diet until DIP requirements were met and added pelleted soybean hulls (**SBH**) to the CORN supplement to achieve equal TDN intake g/(kg of BW·d) to CRSBM. Steers consumed supplements in quantities based on the mean BW measured during the previous experimental period of all non-cannulated steers on CRSBM, CORN, and SBM; cannulated steers received those supplements based on the mean initial BW of all cannulates on each treatment. The CONT supplement was fed to all steers on that treatment at 128 g of DM/d. Steers individually received their supplements 5 d/wk in individual stalls at 0800. We calculated daily supplement intake (g of DM/kg of BW), multiplied it by seven to determine weekly intake, and divided weekly intake by five to determine the quantity offered at each feeding. This resulted in quantities of supplement (CRSBM, CORN, SBM, and CONT) fed of 4.4 kg, 4.4 kg, 1.4 kg, and 178 g of DM per feeding, respectively. Cattle were adapted to supplements during the first 6 d of the trial.

Adaptation was accomplished by feeding the total quantity of CONT and the total quantities of soybean meal and soybean hulls, along with 50% of the corn, until most steers consumed all supplement fed. Steers received increasing quantities (454 g/d) of corn until the target quantity of corn intake was achieved. Beginning on approximately d 50, we top-dressed supplements with 100 g of a 7.5% chromic oxide, 92.5% dried molasses supplement [7.5 g of chromic oxide/(steer·d)], and continued top-dressing supplements for the 5 d prior to (~ d 50 to 54) and during the 5-d fecal collection period (~d 55 to 59). During this period, steers consumed all supplement and feeders were clean at the end of the 1-h feeding period. Steers had ad libitum access to covered mineral feeders containing a trace mineralized salt mix while on trial in both years.

Sample Collection and Preparation. We estimated forage mass from 30 clipped 0.1-m^2 quadrats taken at the initiation and completion of the trial, and at approximately d 28 and 56 of each trial. Clipped samples were dried in a forced air oven at 55°C for 48 h and weighed. We collected masticate samples as described by Lesperance et al. (1960) from two unsupplemented, cannulated steers at the initiation and completion of the trial, and from the supplemented, cannulated steers on approximately d 28 and 60 of each trial. During each trial, feed ingredients were sampled weekly, and because all feeds in each year were from a single source, they were composited within each year at the completion of the trial. Ingredients were ground to pass a 2-mm screen in a Wiley mill (Thomas Scientific, Philadelphia, PA) for determination of DM, OM, NDF, ADF, acid detergent insoluble ash (**ADIA**), starch, CP, and DIP.

We estimated grazing time during the 5-d fecal collection periods that occurred starting at about d 55 of each year by using 12 grazing collars with vibracorders. Three

steers per treatment wore grazing collars on 1 d prior to (~d 54) the initiation of grazing time measurements and for five consecutive days (~d 55 to 59), during which time supplements were fed.

We collected feces at 0800 via rectal grab samples 1 day prior to the initiation of each study to provide a pretreatment baseline for fecal indices, and again 1 mo after the completion of the studies, to provide a posttreatment measurement of fecal indices. Fecal samples were also collected at about d 28, and for five consecutive days starting at about d 55 of each trial from each steer to evaluate the responses of fecal indices to our treatments. We placed fecal samples on ice, transported them to the lab, and directly determined fecal pH using a portable combination electrode pH meter (Corning 314i pH/mV/temperature portable pH meter with an ion-selective field effect transistor electrode, Corning, NY). Fecal samples were dried (55°C, 72 h), ground to pass a 2-mm screen in a Wiley mill (Thomas Scientific), and stored for later analyses. Because fecal output was estimated from Cr concentration in the feces, we performed total fecal collection on the cannulated steers in an attempt to measure Cr recovery to validate fecal output estimates obtained from Cr concentration. Recovery of Cr was calculated as grams of Cr recovered per day in feces divided by grams of Cr fed per day multiplied by 100. Cannulated steers wore fecal collection bags, that were changed twice daily (0800/1700), starting at about d 55 of each trial for 5 d (d 55 to 59), during which time supplements were fed. Contents of the fecal bags were weighed, mixed thoroughly, and subsampled. The subsamples were weighed, dried (55°C, 72 h), re-weighed, ground (2-mm screen), and reserved for later analyses.

We collected blood samples at 0800 1 d prior to the initiation and 1 month after the completion of the studies to provide pre- and post-trial measures of serum urea nitrogen and serum insulin. Blood samples were also collected within 1 h of feeding on the fifth day of five consecutive days of feeding supplements at approximately d 28 and 59 of both years to measure responses to treatment. All blood samples were collected via tail venipuncture, placed ice, and transported to the lab where they were stored at 4°C overnight (~20 h) prior to centrifugation, at which time we harvested serum and stored it frozen (-20°C) for further analyses.

Laboratory Analyses. Dry matter and ash content were determined by oven drying at 105°C for 24 h, followed by ashing at 500°C for 6 h in a muffle furnace. A combustion method (Leco NS2000, St. Joseph, MI) was utilized in accordance with AOAC (1996) to determine N content. Degradable intake protein concentrations of masticate and supplement samples were estimated from an enzymatic in vitro degradation technique (Roe et al., 1991). Supplement and masticate sample NDF (procedure A, without sodium sulfite) and supplement, masticate, and fecal sample ADF and ADIA concentrations were determined as described by Van Soest et al. (1991). We used ADIA as an internal marker to estimate forage and total diet OM digestibility (Van Soest et al., 1991; Van Soest, 1994). Starch content of feeds was estimated enzymatically from α-linked glucose by a colorimetric procedure (Galyean, 1997). We ashed and digested composite fecal samples in a solution of phosphoric acid, manganese sulfate, and potassium bromate with heat, according to the procedure outlined by Williams et al. (1962), and quantified Cr concentration in fecal composites using inductively coupled argon plasma optical emission spectroscopy (SpectroFlame, Spectro Analytical Instruments Inc., Fitchburg,

MA). Serum urea N was determined using an enzymatic (urease/Berthelot) technique (Sigma Procedure No. 640, Sigma Diagnostics, St. Louis, MO) that uses phenol-hypochlorite as the colorimeteric agent. Serum insulin was determined using RIA kits (Coat-A-Count Insulin, DPC, Los Angeles, CA) using bovine insulin for standards. A 48-h in vitro procedure similar to the method of Goering and Van Soest (1970) was used to determine in vitro OM disappearance. Masticate samples (0.5 g) were incubated in buffered (casein added as a N source) ruminal fluid (4:1) for 48 h. Samples were frozen immediately following the 48-h incubation to stop microbial activity. Samples were thawed and an NDF extraction was performed on the residue, which was then dried and ashed.

Calculations. To improve brevity and clarity, the calculations performed in this study are listed in Table 2. Details regarding these calculations and the basis for their use can be found in Owens and Goetsch (1993), Van Soest (1994), and Galyean (1997). Many of the values reported in this study are based on markers and the associated assumptions that accompany marker-derived data. We attempted to validate Cr recovery under experimental conditions and observed 73% recovery. In addition, we used ADIA as an indigestible internal marker for forage digestibility. The use of 100 minus supplemental TDN value to calculate indigestible supplement is supported by previous work that has shown little or no effect of supplemental treatment on starch digestibility (Vanzant et al., 1990; Chan, 1992; Bodine et al., 2000a,b). However, changes in the digestibility of the supplement would alter estimates of forage digestibility, especially given the quantities of supplement fed in this study. Estimates of forage intake are dependent on estimates of fecal output from Cr and forage digestibility from ADIA.

Because steers were fed for 10 consecutive days (5 d of adaptation to Cr plus 5 d of Cr feeding and fecal sampling), the observed intake and digestibility values are reflective of the quantity of supplement fed per feeding. Therefore, fecal output and ADIA values were adjusted based on this quantity of supplement. However, it might be expected that given 2 d without supplementation, forage intake, digestibility, fecal measures, and grazing behavior would be slightly different than the values we observed. Based on the assumptions made, some caution should be taken when interpreting absolute values, however, relative differences between treatments are still valid.

Evaluation of the NRC (1996) Model Predictions vs Observed Values. Because this trial was based on the NRC (1996) level 1 model using the metabolizable protein (**MP**), we evaluated how well the model predicted animal performance, intake, digestibility, ruminal pH, and DIP and MP balances. The model was evaluated using observed environmental measures with the grazing option turned off, steer age, type, condition, and breed, mean steer BW, no feed additives or implants, observed forage chemical composition, intake, and digestibility, and supplement amount and chemical composition.

Experimental Design and Statistical Analyses. Experimental design for both years was a completely randomized design. We included year, supplemental dietary treatment, and their interaction in the model as fixed effects (Littell et al., 1996). Because steers individually received supplements and they grazed a common pasture, individual steer was considered the experimental unit (Adams et al., 2000). We analyzed all response variables using PROC MIXED (SAS Inst. Inc., Cary, NC), calculated means using least squares means (LSMEANS option), and separated the means using least significant differences methods only when the overall F-value< 0.05. Observed significance levels

were adjusted with the Tukey procedure to account for the number of comparisons made. Interactions among years and treatments did not occur ($P > 0.24$) for any of the response variables. The order of ranking of treatments was similar for all variables among years, and as a result, data were pooled across years and mean responses for all variables are reported.

Results and Discussion

Forage Mass, Forage Allowance, and Diet Quality. Forage mass and allowance (Table 3) were similar among years and were relatively similar among times, and never seemed to be a limiting factor for animal performance. However, diet quality decreased as time progressed. Based on chemical composition of masticate samples, forage quality was such that response to supplementation would be expected by steers (McCollum and Horn, 1990). Chemical composition of masticate samples collected from cannulated steers on different supplementation treatments did not differ ($P > 0.62$). Because of these similarities, diet quality was pooled within time period across treatments and years, and means are reported by time periods.

Animal Performance. Cattle had similar ($P > 0.88$) ADG (Table 4) for the 3 wk before the initiation of the trials [0.11 kg/(steer·d)], as well as similar ($P > 0.49$) initial BW at the start of the experiments. Steers fed CRSBM had greater ($P < 0.01$) ADG and final BW ($P < 0.10$) than either CORN- or SBM-fed cattle. All treatments gained more ($P < 0.01$) weight per day and had heavier ($P < 0.01$) BW at trial completion than the CONT cattle. Cattle responded to both energy and protein, as demonstrated by increased animal performance resulting from the addition of either corn or soybean meal to the diets. However, the greatest response in animal performance occurred when soybean

meal was fed with corn to adequately balance DIP for total diet TDN. Steers fed three times the supplemental TDN with half the protein (CORN vs SBM) and one-fifth the DIP did not have different ADG ($P < 0.16$). Cattle fed equal supplemental TDN with twice the protein (CRSBM vs CORN) had three times the ADG. When steers were fed similar supplemental protein (CRSBM vs SBM) with half the DIP but three times the TDN, they had twice the ADG. Grain supplements fed with DIP have previously improved animal performance of forage-fed beef cattle than those not fed added protein (DelCurto et al., 1990; Garcés-Yépez et al., 1997; Bodine et al., 2001). Feeding either energy (Horn and McCollum, 1987; Bowman and Sanson, 1996; Caton and Dhuyvetter, 1997) or protein (McCollum and Horn, 1990; Owens et al., 1991; Moore et al., 1999) supplements to cattle consuming low-quality forages in our study resulted in similar observations of improved animal performance, as many other researchers have noted. However, the combination of energy and protein in a single supplement resulted in the greatest response in animal performance, which also agrees with previous findings (Sanson et al., 1990; Beaty et al., 1994; Heldt et al. 1998).

For the month after the completion of the trial, steers previously fed CORN had greater ($P < 0.08$) ADG than steers that had been fed SBM, possibly suggesting a form of compensatory gain, increased rumen fill, or both. However, at the end of the post-trial periods, steer BW was similar in rank to steer BW at trial completion, and overall ADG (trial + post-trial) was similar in rank to trial ADG. Even though greater post-trial gain occurred for cattle with lesser rates of winter gain, treatment effects were of a magnitude that differences could be detected approximately one month after the cessation of treatments.

Supplement Efficiency. Added gain (gain greater than that observed for the CONT-fed steers) per unit of added supplement (supplement fed greater than the CONT steers were fed) was greatest ($P < 0.01$) for SBM, least ($P < 0.01$) for CORN, and intermediate ($P < 0.01$) for CRSBM (Table 4). If these efficiencies are transformed into supplement conversions, SBM, CRSBM, and CORN treatments converted 1.5, 3.5, and 7.2 kg of added supplement into a kilogram of added gain. These values are similar to those suggested in a review paper by McCollum and Horn (1990) for either protein- or energy supplemented grazing livestock. Supplement conversions of less than 3:1 are symptomatic of a N deficiency (McCollum and Horn, 1990), and indicate that a response greater than could be attributed to the energy supplied by the supplement alone (positive associative effect) was occurring, very similar to our results for the SBM-fed cattle. They also suggest that conversions of 8:1 or greater were typical of energy supplementation and might be a result of substitution or inefficient utilization of the supplemental nutrients (negative associative effect), which agrees with our observations of the CORN-supplemented steers. However, when we fed grazing steers CRSBM, we observed an improvement in supplement conversion compared with CORN-fed cattle. The steers converted the CRSBM supplement into added gain at a rate that was at the high end of typical protein supplements and at the low end of typical energy supplements, indicating that N was not deficient in relation to the supplemental energy (no associative effects, only an additive effect). The responses in ADG and supplement conversions are indicative of a situation where animals are deficient in both protein and energy, and the greatest response occurs when both deficiencies are addressed in the supplement.

Grazing Behavior. Steers fed supplements with corn (CRSBM and CORN) had reduced ($P < 0.01$) grazing time and intensity (Table 4) and an increased ($P < 0.08$) number of grazing bouts vs cattle not receiving supplemental grain (SBM and CONT). Cattle fed corn (CRSBM and CORN) were similar ($P > 0.35$) in grazing time, bouts, and intensity, and steers that did not receive grain (SBM and CONT) were similar ($P < 0.26$). This agrees with Krysl and Hess (1993), who reported that supplementation seemed to decrease grazing time and intensity while increasing grazing bouts. It is possible, as suggested by Adams (1985),that feeding of large quantities of an energy supplement in the middle of the morning grazing period also contributed to the reduced grazing time of corn-fed (CRSBM and CORN) steers. Time spent foraging was reduced by the quantity of grain supplementation fed in our study, which might result in decreased energy expenditure from grazing, as suggested by Caton and Dhuyvetter (1997). However, decreases in grazing time did not always result in improved ADG, since CORN-fed cattle grazed less time (possibly reducing energy cost) and had lower gains than SBM-fed steers. Grain supplementation decreased ($P < 0.05$) harvesting efficiency vs steers fed SBM, which does not agree with the general conclusion drawn by Krysl and Hess (1993), who suggested supplementation had little effect on harvesting efficiency. We believe that the decrease in harvesting efficiency in our study is because the large quantities of corn supplementation fed in our study decreased forage OM intake. Harvesting efficiency of steers fed supplements with grain (CRSM and CORN) were similar ($P > 0.71$), CRSBM and CONT treatments were similar ($P > 0.21$), and supplements without grain (SBM and CONT) were similar ($P > 0.76$). The addition of soybean meal (CRSBM vs CORN, SBM vs CONT) numerically increased harvesting efficiency in our study,

which agrees with previous reports of increased harvesting efficiency from protein supplementation (Barton et al., 1992; Krysl and Hess, 1993).

Forage Intake. Cattle fed corn (CRSBM and CORN) had reduced ($P < 0.01$) forage intake (Table 4) vs those not supplemented with corn (SBM and CONT). The decrease in forage OM intake is supported by the decreases in grazing time, intensity, and harvest efficiency that were observed due to feeding these relatively high levels of corn-based supplements. The decreased forage OM intake as a result of corn supplementation is in agreement with the findings of Sanson and Clanton (1989), Sanson et al. (1990), and Garcés-Yépez et al. (1997), whose corn supplements decreased intake of low-quality forages. Forage OM intake for CRSBM-fed cattle was not different ($P > 0.22$) vs CORN supplemented steers, which does not agree with our previous findings that adding soybean meal to grain supplements will increase forage intake of low-quality prairie hay (Bodine, et al., 2000b, 2001). However, CRSBM supplemented steers did have numerically greater forage intake and the lack of differences may be a result of the variation in the data due to the use of markers. We believe that the lack of a protein response by CRSBM- vs CORN-fed cattle in our study might be a result of the relatively high quantity of supplemental TDN fed (Moore et al., 1999), supplementation-caused alterations in grazing behavior (Krysl and Hess, 1993), the opportunity for diet selection by grazing cattle (Vavra and Ganskopp, 1998), the high variation associated with marker-derived data, or some combination of these factors. Protein supplementation (CRSBM and SBM) did not increase ($P > 0.50$) forage OM intake vs CORN- and CONT-fed cattle. Previous work (McCollum and Horn, 1990; Owens et al., 1991; Bodine et al., 2000b) has suggested that a response would be expected from a forage with this ratio of digestible

OM to crude protein (Hogan, 1981; McCollum and Horn, 1990; Moore and Kunkle, 1995) or DIP as a percentage of TDN (Cochran et al., 1998). However, numerical increases were observed in forage intake for SBM vs CONT treatments, which is in agreement with previous work. The fact that added soybean meal did not increase forage intake (CRSBM vs CORN, or SBM vs CONT) might be as a result of diet selection (Vavra and Ganskopp, 1998), grazing behavior (Krysl and Hess, 1993), energy expenditure of foraging (Krysl and Hess, 1993; Caton and Dhuyvetter, 1997), environmental exposure (Fox et al., 1988), the added variation caused by markers, or some combination of these factors. An additional explanation for the lack of differences may be a result of the method that was used to collect the data. Forage intake may have been further depressed by the feeding of this amount of corn for the 10 consecutive days required to obtain the necessary fecal samples. Because of the use of markers, their associated assumptions, and the methods used to collect the data, some caution should be used in the interpretation of these intake values as absolute.

Forage Digestibility. Forage OM digestibility (Table 4) was decreased ($P < 0.01$) for steers fed corn-based supplements (CRSBM and CORN) vs steers not fed supplemental grain (SBM and CONT), with the least value for steers fed CORN. This agrees with our findings of reduced grazing time, intensity, harvesting efficiency, and forage OM intake as a result of supplementing with large quantities of corn. Similar decreases in digestibility of low-quality forages have been reported when corn has been used as a supplement (Chase and Hibberd, 1987; Sanson et al., 1990). However, steers that were supplemented with corn and adequate DIP (CRSBM) had greater ($P < 0.01$) forage OM digestibility than cattle fed supplemental grain that were deficient in DIP

(CORN). Other researchers (Hibberd et al., 1987; Olson et al., 1999; Bodine et al., 2000b) have noted a similar increase in forage digestibility when DIP has been added to grain supplements. Yet the increased forage OM digestibility for CRSBM- vs CORN-fed steers in our current study did not result in a statistically significant increase in forage OM intake for cattle supplemented with CRSBM, which is what we would have expected based on our previous research (Bodine et al., 2000b, 2001). The increased forage OM digestibility among CRSBM- vs CORN-fed steers might help to partially explain the greater ADG of CRSBM-steers. Similarities in forage OM digestibility for CONT- and SBM-fed steers agree with previous research that has reported no effect of protein supplementation on extent of forage digestibility (Owens et al., 1991; Bodine et al., 2000b; 2001). However, although similarities in digestibility are typically accompanied by increases in passage rate and intake, no statistically significant increase in intake occurred in our study. This may be reflective of the differences in grazing vs pen-fed animals, or as a result of variation associated with marker-derived data. The observed value of 14% forage OM digestibility by steers on the CORN treatment is lower than would be expected. However, it is similar to the value of 18% reported by Chase and Hibberd (1987), and a forage OM digestibility of 21% is required for the NRC (1996) model to predict an ADG similar to our observations of ADG by CORN-fed cattle. An additional explanation for the extremely low values observed by CORN- and CRSBM-fed steers may be a result of the method that was used to collect the data. Forage digestibility may have been further depressed by feeding this relatively large amount of corn for 10 consecutive days. The usage of markers in this study also requires caution in the interpretation of digestibility values as absolute.

Total Diet OM Intake and Digestibility, and Digestible OM Intake. Feeding corn with added protein (CRSBM) tended to increase ($P > 0.17$) total-diet OM intake on a per-day basis (Table 4). Feeding CORN or SBM supplements resulted in similar ($P > 0.99$) total-diet OM intakes, whereas steers fed greater supplemental TDN but similar DIP (CRSBM) tended to have greater ($P < 0.09$) total OM intake than SBM-fed cattle. Steers fed CORN and SBM had similar ($P > 0.33$) total OM digestibility and digestible OM intake. This similarity can be explained by the differences in supplemental feeding amount, forage intake and digestibility. Cattle fed CRSBM tended ($P < 0.12$) to have greater total-diet OM digestibility and had greater ($P < 0.01$) intake of digestible OM than SBM-fed treatments. This was a result of the greater quantity of highly digestible supplement fed, even though forage intake and digestibility were greater for cattle fed SBM. Steers fed CRSBM had greater ($P < 0.01$) total-diet OM digestibility and digestible OM intake ($P < 0.01$) than CORN-fed cattle. These increases were a result of improved forage digestibility by CRSBM-fed steers vs CORN-supplemented cattle as a result of the addition of DIP to a diet deficient in ruminally degraded protein and numerically greater forage intake, because supplement intakes (DM and TDN) were similar. The increased forage OM digestibility as a result of greater protein intake for CRSBM- vs CORN-fed cattle aids in partially explaining the greater ADG of CRSBM-supplemented steers. Forage and total OM intake and grazing time were similar between these two treatments; however, forage and total-diet OM digestibility, and digestible OM intake were greater for cattle fed CRSBM supplements. When steers fed adequate quantities of DIP (SBM) were given additional energy in the form of corn (CRSBM), total-diet OM intake and digestible OM intake were increased. These increases in intake

and digestibility agree with the observed increases in ADG by steers fed CRSBM vs SBM-supplemented cattle in our study. These results support the assertion of Chase and Hibberd (1987), who observed that supplemental corn resulted in decreased total-diet OM intake, digestibility, and digestible OM intake when compared with protein-supplemented cattle, that their findings were the result of a deficiency of DIP. No differences were detected ($P > 0.21$) in ADF digestibility.

The addition of supplemental feeds improved the total-diet digestible OM:CP and DIP as a percentage of TDN ratios. The greatest improvement was when soybean meal was added without grain. Adding soybean meal to grain supplements resulted in the total-diet digestible OM:CP ratio being lesser than the value that Moore and Kunkle (1995) suggested as a threshold for protein adequacy in relation to energy, which agrees with our findings. Even though DIP as a percentage of TDN was not adequate according to Cochran et al. (1998), it was improved vs CORN-supplemented steers, which also aids in explaining our findings. These results emphasize the importance of adequate DIP when grain supplements are fed with low-quality forages. When DIP is not balanced for total-diet TDN of grain supplemented steers, animal performance will be reduced because the basal forage does not supply as much energy as would be expected as a result of decreased forage digestibility.

Evaluation of Experimental Data Using the NRC (1996) Model. Using the previously listed model inputs, we evaluated the NRC (1996) model 1 predictions of ADG, intake, digestibility, ruminal pH, and DIP and MP balances (Table 5). Observed daily gains (kg/d) for CRSBM, CORN, SBM, and CONT treatments were 0.73, 0.24, 0.39, and -0.17. Predicted ME allowable daily gains were 1.15, 0, 1.34, and 0.58, and

MP allowable daily gains were 1.37, not estimable (ERR), 0.95, and 0.18, respectively. Total intakes (kg/d) observed for CRSBM, CORN, SBM, and CONT treatments were 7.96, 6.99, 7.29, and 5.55, and predicted total intakes were 7.49, 5.39, 7.45, and 6.99. Because supplement intake was fixed, observed forage intakes were calculated as a percentage of total intakes, and predicted forage intakes were calculated by multiplying that percentage by predicted total intakes. Observed forage intakes (kg/d) for CRSBM, CORN, SBM, and CONT treatments were 4.76, 3.94, 6.33, and 5.42, and predicted forage intakes were 4.48, 3.04, 6.47, and 6.83. It appears that when supplements contain added DIP, predictions of total and forage intake are similar to the observed values. However, when CORN and CONT supplements were evaluated, predictions of intake did not agree with observed values.

Forage OM digestibility estimated with Cr and ADIA were 37.4, 13.8, 56.7, and 56.1% for CRSBM, CORN, SBM, and CONT, respectively. Using forage OM digestibility as a value for forage TDN, and changing it until predicted and observed ADG was equal, resulted in digestibilities of 30.3, 21.3, 43.1, and 42.8%. The values for the supplements that contained corn are low, but are somewhat similar to observations, whereas observed forage digestibility was considerably greater than predicted TDN for SBM and CONT supplements.

Observed ruminal pH values for CRSBM, CORN, SBM, and CONT were 6.11, 6.12, 6.35, and 6.44; and predicted values were 6.25, 6.21, 6.46, and 6.46, respectively, indicating good agreement between observed and predicted values.

Using the observed data, the NRC (1996) evaluated the DIP balances for CRSBM, CORN, SBM, and CONT at -113 -114, 56, and -155 g/d, respectively. Metabolizable

protein balances were 65, 172, 116, and -122 g/d for CRSBM, CORN, SBM, and CONT, respectively. Degradable intake and MP balances were highly subject to the low DIP concentrations measured using the enzymatic in vitro procedure for CRSBM (36% of CP), CORN (30% of CP), and masticate samples (51% of CP), and the extremely high value for SBM (86% of CP). Using more realistic estimates of DIP concentration in corn (50% of CP), soybean hull pellets (67% of CP), and soybean meal (50 to 65% of CP), the CRSBM supplement would have been expected to supply 50 to 60% of the CP as DIP, and the CORN supplement 54%. Using these values, the NRC (1996) predicts DIP balances of -11, -41, and 19 g/d for CRSBM, CORN, and SBM, respectively. These balances would change based on the forage DIP and digestibility values used, but appear to be much more in line with what was observed in the experiment than the values derived using the enzymatic method of DIP determination.

Using the predicted forage TDN values, the forage consumed by steers on CRSBM, CORN, and SBM, and CONT supplied 22, 0, 95, and 96.5%, respectively, of the NE_m requirement of these cattle, and 13.2, 0, 69, and 96.5% of the total NE_m intake.

Fecal Output and Indices. Fecal OM output (Table 6) was greater ($P < 0.02$) for diets with corn (CRSBM and CORN) than for diets without corn (SBM and CONT). Feeding corn (CRSBM and CORN) reduced ($P < 0.01$) fecal ADF output and concentration compared with supplements without corn (SBM and CONT). Increased fecal OM output, reduced ADF output, and reduced ADF concentration by corn-supplemented steers (CRSBM and CORN) might be related to decreased digestibility of forage, lesser forage intake, increased total OM intake, greater digestibility of the supplements than the basal forage, or some combination of these factors.

Fecal pH (Table 6) was unchanged ($P > 0.23$) for CONT- and SBM-fed steers when compared during the 1 d prior to, during, or 33 d after the trial, indicating that these supplements did not affect fecal pH. Neither of these supplements was fed at a quantity, or are of a type that would be expected to decrease ruminal digestion or alter site of digestion and consequently increase large intestinal fermentation (Hannah et al., 1991). However, fecal pH values observed during the experiments from CRSBM- and CORN-fed cattle were lesser ($P < 0.01$) than 1 d pre-trial and 33 d post-trial fecal pH values. Feeding corn (CRSBM and CORN) decreased ($P < 0.01$) fecal pH vs supplements without grain (SBM and CONT). This reduction in pH seems to indicate an increase in large intestine fermentation, as suggested by Galyean et al. (1979) and Russell et al. (1980). The fecal pH values observed in our study from cattle fed diets with supplemental corn are similar to values observed by other researchers for high-concentrate diets, whereas the fecal pH values from steers fed supplements without corn are similar to values reported for forage-fed cattle (Hovde et al., 1999; Russell et al., 2000; Scott et al., 2000). It is interesting to note that similar fecal pH values occurred for our corn-supplemented low-quality forage diets (approximately 60:40 forage:concentrate) as was reported from steers on high-concentrate finishing diets, indicating the possibility that significant quantities of fermentable OM reaching the large intestine when corn is fed. In our study, reduced fecal pH was more likely as a result of changes in site of digestion of the basal forage. Previous research conducted in our laboratory has found increased forage digestibility, decreased fecal ADF concentration, and indicated little change in total-tract starch digestibility or fecal concentrations of starch when low-quality forage-fed steers were supplemented with corn and DIP (Bodine et al, 2000a). In

addition, quantities of starch fed in the current studies would not be expected to overwhelm the capacity for ruminal fermentation, or digestion and absorption in the small intestine (Huntington, 1997). The increased fecal pH values for diets with added protein (CRSBM vs CORN and SBM vs CONT) agree with previous work in which added protein increased fecal pH (Haaland et al., 1982). This suggests that additional protein will increase ruminal fermentation and result in less fermentable substrate being presented to the lower tract for colonic fermentation.

Fecal N (Table 6) was similar ($P > 0.64$) among steers on all dietary treatments 1 d prior to and 1 mo after the completion of the study. Steers fed supplements had increased ($P < 0.01$) fecal N concentration after treatment initiation than were observed prior to the initiation of the studies because of their greater N intake during the studies. This agrees with the observations of Wofford et al. (1985), who reported a strong relationship between fecal N and dietary N intake. However, the majority of previous research conducted has correlated fecal N with forage quality (Raymond, 1948; Ward et al., 1982; Lyons and Stuth, 1992), or animal performance (Erasmus et al., 1978; Holechek et al., 1982; McCollum, 1990), and not with supplemental feeding. Little research exists on the effect that supplementation of grazing animals would have on correlations among plane of nutrition and fecal indices (McCollum, 1990; Lyons et al., 1993). The use of fecal N to predict dietary N intake and animal performance might be more complicated in supplemented animals, than in livestock not offered supplemental feed. Steers fed CRSBM had the greatest increase ($P < 0.01$) in fecal N after the initiation of the experiments, which is also related to increased N intake, similar to the relation between dietary N intake and fecal N reported by Holechek et al. (1982). While on study, steers

supplemented with corn and DIP (CRSBM) had greater ($P < 0.01$) fecal N than steers supplemented with similar quantities of either TDN (CORN) or protein (SBM) alone. Feeding SBM resulted in similar ($P > 0.90$) fecal N than steers supplemented with CORN, even though N intake was considerably different. Along with the decreased fecal pH and digestibility, the increased fecal N in steers supplemented with corn (CRSBM, CORN) appears to be related to increased large intestinal fermentation. The range of fecal N values in our study agrees with ranges reported by Holechek et al. (1982), Wofford et al. (1985), and McCollum (1990). Fecal N did not accurately describe treatment ranking of N intake, animal performance, or serum urea N. Leite and Stuth (1990) also reported that no single fecal measure could be highly correlated with observed animal dietary variables.

Blood Indices. Concentrations of serum urea N (Table 6) were similar ($P > 0.49$) among all treatments 1 d before and 33 d after studies ended, indicating similar N and plane of nutrition status of experimental animals when dietary treatments were similar, which would be expected according to Hammond (1996). In comparison with pretreatment values, serum urea N increased ($P < 0.01$) after the initiation of the study for all treatments, with SBM-fed steers having the greatest increase ($P < 0.01$), followed by steers fed CRSBM supplements, and steers receiving CORN tending ($P < 0.12$) to have a greater increase in serum urea N than CONT cattle. During the trials, steers fed SBM supplements had the greatest ($P < 0.01$) serum urea N, CRSBM-fed steers had the second greatest, and CORN supplemented cattle were greater ($P < 0.05$) than CONT steers. Feeding soybean meal has previously been found to increase serum urea N (Barton et al., 1992; Marston et al., 1995). Feeding similar amounts of DIP with different quantities of

energy (CRSBM vs CORN) resulted in lower serum urea N, similar to the results observed by Chase et al. (1993). Serum urea N concentrations of cattle fed low DIP supplements (CORN and CONT) were below quantities (7 mg/dL) of blood urea N suggested to respond positively to protein supplementation by Hammond (1996). The serum urea N concentrations of steers with the greatest rates of gain (CRSBM, SBM) were similar to serum urea N values previously reported for optimal gain by growing steers (Byers and Moxon, 1980). After trial completion, serum urea N concentrations of CONT steers did not change ($P > 0.56$), whereas serum urea N of CORN-fed steers increased ($P < 0.01$), and steers that had consumed soybean meal (CRSBM and SBM) showed a decrease ($P < 0.01$). The reduction in serum urea N of protein-supplemented steers was a result of a decreased N intake when experimental supplements were no longer fed. A possible explanation of the increase in serum urea N of CORN-fed steers after the cessation of energy supplementation is a decreased demand for N necessary for ruminal fermentation, which is supported by the findings of Chase et al. (1993), or the feeding of a protein supplement, or the combination of the two.

Even though fecal indices are extremely easy to collect in a production setting, the current supplementation strategy in use will impact the fecal N concentrations of cattle and might result in erroneous conclusions if used to predict N status of grazing animals. This has been suggested previously (Lyons et al., 1993) and is supported by the lack of a relationship between fecal N and ADG in our study. Cattle that were fed CORN supplements had greater fecal N but lesser serum urea N and N intake. Particular caution should be taken when using fecal N concentrations as an indicator of N status for cattle grazing low-quality forages and receiving energy-based supplements without added DIP.

Even though blood urea N is more difficult to collect, it seems to be a better measure of N status across a variety of supplement types for grazing cattle.

Serum insulin concentrations (Table 6) were similar ($P > 0.55$) among all treatments 1 d prior to the initiation, and one month after the completion of trials. After the initiation of the studies, CORN- and CONT-fed steers had similar ($P > 0.84$) serum insulin concentrations, which were similar ($P > 0.21$) to pre-trial values. However, serum insulin increased ($P < 0.01$) for both CRSBM- and SBM-supplemented steers compared with pretrial concentrations. This agrees with previous work reporting insulin responses to increased energy or protein intake (Barton et al., 1992; Marston et al., 1995). While on study, steers fed corn plus soybean meal (CRSBM) had greater ($P < 0.01$) serum insulin than was observed in steers fed either similar supplemental TDN (CORN) or DIP (SBM). Supplemental energy (CORN) or protein (SBM) had similar ($P > 0.18$) serum insulin values while on trial. Steers fed supplements (CRSBM, CORN, SBM) had increased ($P < 0.01$) serum insulin vs animals not offered supplement (CONT). These results are in agreement with Wettemann et al. (1987) and Yelich et al. (1995, 1996), who reported that nutrient intake influenced insulin concentrations. The increased insulin concentrations for CRSBM- vs CORN-supplemented cattle might have been a result of greater digestible OM and N intake, greater total-diet digestibility (as a result of the added DIP) resulting in greater VFA production, greater protein flow to the small intestine, or a combination of these factors (Trenkle, 1978; Harmon, 1992). The increased energy and protein intake by CRSBM-fed steers would provide a greater supply of gluconeogenic precursors and insulinotropic factors (Trenkle, 1978; Harmon, 1992; Marston et al., 1995). This

suggestion is supported by the observation that steers on the CRSBM treatment had the greatest measured ADG.

Implications

Grain-based supplements fed in large quantities can be efficaciously utilized on low-quality forages when adequate degradable intake protein is included in the supplements. Corn fed alone with no added ruminally degradable protein will exacerbate the preexisting deficiency of degradable intake protein, resulting in the potential for reduced intake and digestibility of low-quality forages. When fed with adequate degradable intake protein, corn-based supplements can have acceptable efficiencies and conversions and will increase performance of cattle grazing low-quality forages because of greater forage digestibility than when corn is fed alone. Feeding large amounts of a supplement that provides both energy and adequate degradable intake protein to digest not only the supplement but also the basal forage diet, allows cattle to achieve greater rates of gain while grazing low-quality forages than either similar levels of grain alone or low-level protein supplementation.

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Table 1. Ingredient, chemical composition, and intake of supplements fed 5 d/wk to steers grazing dormant tallgrass prairie

Supplement	**Supplement[a]**			
Ingredient, (% of DM)	**CRSBM**	**CORN**	**SBM**	**CONT**
Corn (dry-rolled)	78.5	78.1	---	20
Soybean hulls (pelleted)	---	21.9	---	20
Soybean meal (49% CP)	21.5	---	100	---
Cottonseed hulls	---	---	---	55
Molasses	---	---	---	3
Salt	---	---	---	2
Chemical Composition, (% of DM)	**CRSBM**	**CORN**	**SBM**	**CONT**
Dry Matter	87.9	88.0	89.9	90.0
Organic Matter	97.8	98.1	94.3	95.4
Crude Protein	18.3	9.8	53.2	7.6
Degradable Intake Protein, % of CP[b]	36.6	29.3	84.9	38.2
Neutral Detergent Fiber	10.3	21.2	10.8	64.2
Acid Detergent Fiber	5.5	14.1	6.4	46.2
Acid Detergent Insoluble Ash	0.7	0.7	0.7	0.7
TDN[c]	89.3	89.2	87	62
DOM:CP[d]	4.9	9.1	1.6	8.2
DIP:TDN[d]	7.5	3.2	51.9	4.7
Supplement Intake, (DM/steer)	**CRSBM**	**CORN**	**SBM**	**CONT**
kg/feeding, (5 d/wk)	4.44	4.27	1.34	0.18
kg/d, (7 d/wk)	3.19	3.05	0.96	0.13
g/(kg of BW·d), (7 d/wk)	9.78	9.58	2.98	0.43
g/(kg of $BW^{0.75}$·d), (7 d/wk)	41.6	40.5	12.6	1.8

[a]**CRSBM** = 4.4 kg of DM/feeding of dry-rolled corn plus soybean meal to meet NRC (1996) degradable intake protein requirements; **CORN** = 4.3 kg of DM/feeding of dry-rolled corn plus soybean hull pellets, equal in TDN to CRSBM; **SBM** = 1.3 kg of DM/feeding of soybean meal, equal in degradable intake protein to CRSBM; **CONT** = 178 g of DM/feeding of a cottonseed hulls based control supplement.

[b]Estimated using the enzymatic procedure of Roe et al. (1991).

[c]Estimated using tabular TDN values (NRC, 1996).

[d]Estimated using tabular TDN to approximate DOM, and measured CP values, similar to Moore and Kunkle (1995), and estimated by multiplying measured CP and degradable intake protein values, and using tabular TDN values, similar to Cochran et al. (1998).

Table 2. Calculations used to determine forage allowance, fecal output, forage and total diet intake and digestibility, and harvesting efficiency

Item	Abbrev.	Units	Calculation
Forage Allowance	FA	kg of DM/kg of BW	(forage mass*ha)/(mean BW*steer #'s)
Fecal Output[a]	FO	g of OM/d	(g Cr dose/[fecal Cr])*(Cr recovery)[b]
Fecal Output, Forage	FOF	g of OM/d	FO – g of indigestible supplement
Fecal ADIA	FO_{ADIA}	g of ADIA/d	(FO*[Fecal ADIA])
FO_{ADIA}, Forage	FOF_{ADIA}	g of ADIA/d	FO_{ADIA} – g of supplemental ADIA
Forage OM Intake[a]	FOMI	g of OM/d	FOF_{ADIA}/[ADIA]masticate
Fecal [ADIA], Forage	$FOF_{[ADIA]}$	%	(FOF_{ADIA}/FOF)*100
Forage OM Digestion	FOMD	%	100-([ADIA]masticate/FOF_{ADIA})*100
Total OM Intake[a]	TOMI	g of OM/d	FOMI + g of supplement OM Intake
Total OM Digestion	TOMD	%	((TOMI – FO)/TOMI)*100
Digestible OM Intake[a]	DOMI	g of OM/d	TOMI*(TOMD/100)
Harvesting Efficiency	HE	g OM/(kg BW·min)	(FOMI/BW)/grazing time, min

[a]Also adjusted to a g/kg of BW basis.

[b]Measured from g of Cr recovered in total fecal collection of ruminally cannulated steers fitted with fecal bags and harnesses.

Table 3. Forage mass, forage allowance, and masticate sample chemical composition by period for steers grazing dormant tallgrass prairie during both years

	Portion of the winter grazing period[a]		
Item	**d 1 - 28**	**d 29 - 56**	**d 57 - end**
Forage Mass, kg of DM/ha	3797 ± 1976	4461 ± 1495	4012 ± 1603
Forage Allowance, kg of DM/kg of BW[b]	31.6 ± 16	36.9 ± 12	31.3 ± 12
Chemical Analysis (% of DM)			
Organic Matter	87.7	87.3	87.1
Crude Protein	9.1	5.8	5.7
Degradable Intake Protein, % of CP[c]	55.0	52.1	50.3
In Vitro OM Disappearance	66.1	60.6	58.9
Neutral Detergent Fiber	67.9	69.5	71.6
Acid Detergent Fiber	43.1	46.0	46.9
Acid Detergent Insoluble Ash	4.9	5.6	5.9
DOM:CP[d]	7.3	10.5	10.3
DIP:TDN[d]	7.6	5.0	4.9

[a]Average length of winter grazing period is 83 days.

[b]Calculated for each period from the mean forage mass/ha multiplied by the total ha grazed and divided by the quantity of mean steer BW multiplied by the number of steers.

[c]Estimated using the enzymatic procedure of Roe et al. (1991).

[d]Estimated using in vitro OM disappearance to approximate DOM, and measured CP values, similar to Moore and Kunkle (1995), and estimated by multiplying measured CP and degradable intake protein values, and using in vitro OM disappearance to approximate TDN values, similar to Cochran et al. (1998).

Table 4. Performance, grazing behavior, intake, and digestibility of steers grazing dormant tallgrass prairie and fed one of four supplements 5 d/wk

	Supplement[a]				
Item	**CRSBM**	**CORN**	**SBM**	**CONT**	**SEM[b]**
Steers, #	25	25	25	25	---
Initial BW, kg	296	308	304	304	5.6
Final BW, kg	357^{rx}	328^{s}	338^{ry}	293^{t}	5.4
Post-Trial BW, kg[c]	365^{rux}	343^{rv}	346^{ruvy}	307^{s}	5.4
Trial ADG, kg/d[c]	0.73^{r}	0.24^{s}	0.39^{s}	-0.17^{t}	0.05
Post-Trial ADG, kg/d[c]	0.11^{xy}	0.33^{x}	0.08^{y}	0.26^{xy}	0.07
Trial+Post ADG, kg/d[c]	0.60^{r}	0.31^{s}	0.36^{s}	0.03^{t}	0.04
Grazing Time, min	445^{r}	422^{r}	516^{s}	544^{s}	10.6
Grazing Bouts	12.3^{r}	11.7^{rsx}	8.7^{t}	9.8^{sty}	0.6
Grazing Intensity, min/bout	39.6^{r}	38.2^{r}	63.5^{s}	59.7^{s}	3.1
Harvest Efficiency, g/(kg BW·min grazing)	0.024^{rsuv}	0.022^{ru}	0.031^{sw}	0.029^{rsvw}	0.002
Forage OM intake, g/kg of BW	12.7^{r}	10.8^{r}	17.2^{s}	15.8^{s}	0.7
Forage OM digestibility, %	37.4^{s}	13.8^{r}	56.7^{t}	56.1^{t}	2.6
Total OM Intake, g/kg of BW[d]	26.2^{r}	24.1^{r}	21.1^{s}	16.4^{t}	0.7
Total OM Intake, g/kg of BW[e]	22.3^{rx}	20.3^{rxy}	20.0^{ry}	16.2^{s}	0.7
Total OM Digestibility, %[d]	63.1^{r}	56.6^{s}	59.4^{rs}	56.2^{s}	1.2
Digestible OMI, g/kg of BW[f]	16.5^{r}	13.7^{s}	12.6^{s}	8.6^{t}	0.6
Total Diet DOM:CP[g]	5.7	7.4	4.8	9.7	---
Total Diet DIP:TDN[g]	7.2	5.2	15.0	5.2	---
Supplement Efficiency[h]	0.29^{r}	0.14^{s}	0.67^{t}	---	0.03

[a]**CRSBM** = 4.4 kg of DM/feeding of dry-rolled corn plus soybean meal to meet NRC (1996) degradable intake protein requirements; **CORN** = 4.3 kg of DM/feeding of dry-rolled corn plus soybean hull pellets, equal in TDN to CRSBM; **SBM** = 1.3 kg of DM/feeding of soybean meal, equal in degradable intake protein to CRSBM; **CONT** = 178 g of DM/feeding of a cottonseed hulls based control supplement.

[b]SEM = Standard error of the means, n = 25.

[c]Average trial period was 83 d, average post-trial period was 33 d, and average Trial+Post period was 116 d.

[d]Expressed with supplement OM intake on a per feeding (5 d/wk) basis.

[e]Expressed with supplement OM intake adjusted for a per d (7 d/wk) basis.

[f]Calculated using estimated fecal OM output and forage OM intake, and with supplement OM intake expressed on a per feeding (5 d/wk) basis.

[g]Estimated using total diet OM digestibility to approximate DOM, and measured CP values, similar to Moore and Kunkle (1995), and estimated by multiplying measured CP and degradable intake protein values, and using total diet OM digestibility to approximate TDN values, similar to Cochran et al. (1998).

[h]Supplement efficiency calculated as kg of added daily gain (greater than CONT-fed steers) divided by added kg of supplement DM fed (greater than CONT-fed steers).

[r,s,t]Within a row, means without a common superscript letter differ ($P < 0.01$).

[u,v,w]Within a row, means without a common superscript letter differ ($P < 0.05$).

[x,y,z]Within a row, means without a common superscript letter differ ($P < 0.10$).

Table 5. Observed (experimental) versus predicted (NRC, 1996) values for animal performance, intake, forage energy value, ruminal pH, and degradable intake and metabolizable protein balances

	Supplement[a]			
Item[b]	**CRSBM**	**CORN**	**SBM**	**CONT**
Mean BW, kg	327	318	321	299
Obs. ADG, kg	0.73	0.24	0.39	-0.17
Pred. ME allow ADG	1.15	0.00	1.34	0.58
Pred. MP allow ADG	1.37	ERR	0.95	0.18
Obs. Forage DMI	4.76	3.94	6.33	5.42
Pred. Forage DMI	4.48	3.04	6.47	6.83
Obs. Total DMI	7.96	6.99	7.29	5.55
Pred. Total DMI	7.49	5.39	7.45	6.99
Obs. Forage OMD	37.4	13.8	56.7	56.1
Pred. Forage TDN[c]	30.3	21.3	43.1	45.8
Obs. Rumen pH	6.11	6.12	6.35	6.44
Pred. Rumen pH	6.25	6.21	6.46	6.46
DIP balance, g	-113	-114	56	-155
MP balance, g	65	72	116	-122
Pred. Forage NE_m Mcal/kg	0.23	-0.14	0.72	0.82
Pred. Forage NE_m Supply Mcal/d	1.07	0	4.62	4.42
Forage NE_m, Mcal, % of Requirement	22	0	95	96.5
Forage NE_m, Mcal, % of Total Diet	13.2	0	69	96.5

[a]**CRSBM** = 4.4 kg of DM/feeding of dry-rolled corn plus soybean meal to meet NRC (1996) degradable intake protein requirements; **CORN** = 4.3 kg of DM/feeding of dry-rolled corn plus soybean hull pellets, equal in TDN to CRSBM; **SBM** = 1.3 kg of DM/feeding of soybean meal, equal in degradable intake protein to CRSBM; **CONT** = 178 g of DM/feeding of a cottonseed hulls based control supplement.

[b]Obs. = observed value estimated from this experiment; Pred. = predicted value using the NRC (1996) level 1 model.

[c]Forage OM digestibility value used as forage TDN value in order for predicted (NRC, 1996) ADG to equal observed ADG.

Table 6. Fecal measures and blood indices of steers grazing dormant tallgrass prairie and fed one of four supplements 5 d/wk

	Supplement[a]				
Item	**CRSBM**	**CORN**	**SBM**	**CONT**	**SEM[b]**
Steers, #	25	25	25	25	---
Fecal OM Output, g/kg of BW	9.6^{r}	10.3^{r}	8.5^{s}	7.8^{s}	0.3
Fecal ADF Output, g/kg of BW	2.9^{r}	3.2^{r}	3.9^{s}	3.7^{s}	0.1
Organic Matter, % DM	85.8	87.6	83.1	84.3	0.6
Acid Detergent Fiber, % DM	26.1^{r}	27.1^{r}	37.6^{s}	39.9^{t}	0.5
Pre-Trial Fecal pH	6.86^{xy}	6.73^{x}	6.80^{xy}	6.87^{y}	0.05
Trial Fecal pH	5.97^{r}	5.74^{s}	6.88^{t}	6.81^{t}	0.05
Post-Trial Fecal pH	6.84	6.87	6.93	6.85	0.07
Pre-Trial Fecal N, % DM	1.8	1.7	1.8	1.7	0.07
Trial Fecal N, % DM	2.5^{r}	2.1^{s}	2.1^{s}	1.7^{t}	0.07
Post-Trial Fecal N, % DM	2.4	2.3	2.4	2.3	0.08
Pre-Trial Serum Urea N, mg/dL	2.9	2.9	2.8	2.8	0.2
Trial Serum Urea N, mg/dL	10.4^{s}	4.2^{tv}	15.8^{r}	5.6^{tu}	0.4
Post-Trial Serum Urea N, mg/dL	5.8	5.9	6.5	5.9	0.4
Pre-Trial Serum Insulin, ng/mL	1.6	1.7	1.6	1.7	0.08
Trial Serum Insulin, ng/mL	2.2^{r}	1.7st	1.9^{s}	1.6^{t}	0.06
Post-Trial Serum Insulin, ng/mL	1.2	1.1	1.2	1.1	0.1

[a]**CRSBM** = 4.4 kg of DM/feeding of dry-rolled corn plus soybean meal to meet NRC (1996) degradable intake protein requirements; **CORN** = 4.3 kg of DM/feeding of dry-rolled corn plus soybean hull pellets, equal in TDN to CRSBM; **SBM** = 1.3 kg of DM/feeding of soybean meal, equal in degradable intake protein to CRSBM; **CONT** = 178 g of DM/feeding of a cottonseed hulls based control supplement.

[b]SEM = Standard error of the means, n = 25.

[r,s,t]Within a row, means without a common superscript letter differ ($P < 0.01$).

[u,v,w]Within a row, means without a common superscript letter differ ($P < 0.05$).

[x,y,z]Within a row, means without a common superscript letter differ ($P < 0.10$).

Chapter IV

Interactions between starch and degradable intake protein on intake, digestibility, and ruminal kinetics of prairie hay diets fed to beef steers

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ABSTRACT: Eight ruminally cannulated beef steers were fed prairie hay plus one of eight supplements and the interrelationships between supplemental energy and protein were evaluated. Supplement treatments were arranged as a 2 × 4 factorial and consisted of dry-rolled corn at either 0 or 0.75% of BW along with one of four graded levels of degradable intake protein (**DIP**) coming from soybean meal. Intake of fiber, neutral detergent solubles (**NDS**), N, and S quadratically increased ($P < 0.05$) with increasing DIP. Digestibility of fiber, N, and S linearly increased ($P < 0.01$) as DIP increased for steers fed corn-based supplements. Added protein tended to linearly increase ($P < 0.06$) NDF digestibility, tended to quadratically increase ($P < 0.09$) hemicellulose and NDS digestion, and quadratically increased ($P < 0.01$) N and S digestibility by steers fed supplements without corn. Fecal ADF and NDF output responded quadratically ($P < 0.01$) as protein increased for steers fed supplements without corn, whereas NDF and hemicellulose tended to respond quadratically ($P < 0.07$) and ADF decreased linearly ($P < 0.01$) when corn was fed. Output of NDS, N, metabolic fecal N, and S quadratically increased ($P < 0.03$) as DIP increased. At similar ruminal ammonia levels, supplements with corn had decreased ($P < 0.01$) rate of ruminal ADF digestion as supplements without corn. Corn-fed steers had a linear increase ($P < 0.01$) in fecal N, whereas supplements without corn resulted in a linear increase ($P < 0.01$) as DIP increased.

Increasing supplemental DIP intake increased ($P < 0.01$) rates of in situ forage DM disappearance and proportion of DM in the B-fractions, tended to increase ($P < 0.07$) effective ruminal degradation of forage DM, and decreased ($P < 0.02$) lag time and C-fractions. Supplements without corn had more rapid ($P < 0.04$) disappearance rates of in situ forage DM and protein, greater ($P < 0.05$) B-fractions, decreased ($P < 0.01$) lag times, and tended to have lesser ($P < 0.10$) C-fractions than supplements with corn. Increasing dietary DIP intake tended to quadratically increase ($P < 0.07$) rate of forage protein digestion. Feeding supplements with corn tended to increase ($P < 0.08$) the UIP concentration of the basal prairie hay. When supplemental corn was fed to beef steers consuming prairie hay, providing additional supplemental DIP increased intake and digestibility, appeared to increase ruminal fiber digestion, and ameliorated the negative associative effects of starch.

Keywords: Associative Effects, Beef Cattle, Degradable Intake Protein, Digestibility, Fecal Indices, Supplementation

Introduction

Negative associative effects have been described as the reduction in digestibility of a component of a mixed diet to a value lesser than would be expected based on the additive digestibility of that component when each feedstuff is fed individually (Ferrell, 1993; Van Soest, 1994). Reviews of supplementation have concluded that grain supplements cause negative associative effects whn fed with low-quality forages (Bowman and Sanson, 1996; Caton and Dhuyvetter, 1997; Horn and McCollum, 1987). However, many studies that report negative associative effects have been grossly deficient in degradable intake protein. Chase and Hibberd (1987) attributed the

decreased forage and fiber digestibility that occurred in corn-supplemented prairie-hay fed steers to a deficiency of ruminal ammonia caused by adding ruminally fermentable organic matter to a diet already deficient in degradable intake protein. In a follow up study (Hibberd et al., 1987) and in more recent research (Bodine et al., 2000; Heldt et al., 1999a; Olson et al., 1999) the inclusion of ruminally degradable protein has been found to reduce or eliminate the negative associative effects of starch on fiber digestion. Yet, supplying adequate protein along with readily available carbohydrates is not a novel concept, as the effects have been previously detailed (Burroughs et al., 1949; Mitchell et al., 1940; Williams et al., 1953) and have resulted in a variety of proposed feeding models (Burroughs et al., 1975; Chalupa, 1984; Russell et al., 1992), including the basis of the current beef (NRC, 1996) and dairy (NRC, 2001) cattle systems.

The objectives of the our studyl were to elucidate how degradable intake protein alters the negative associative effects typically expected when starch-based supplements are fed with low-quality forages. These effects include intake, digestibility, fecal excretion, in situ degradation of forage dry matter and protein, and to evaluate how closely one of the previously mentioned feeding models (NRC, 1996) predicts these measures.

Materials and Methods

Two studies were conducted in order to evaluate the effects of degradable intake protein (**DIP**) on negative associative effects. We have described these in detail elsewhere (Bodine et al., 2000). Briefly, steers were given ad libitum access to prairie hay (Table 1) and individually hand-fed supplements daily. Supplements were cracked corn fed at 0 or 0.75 % of BW, with one of four levels of DIP coming from soybean meal

to result in between 0 and 1.3 g of DIP/kg of BW (Table 2). Steers were housed individually and fed during14-d periods, during which sampling occurred during the last four days. Sampling procedures were described by Bodine et al. (2000). The Oklahoma State University Animal Care and Use Committee approved all experimental protocols.

In Situ Procedures. Dacron bags (10 × 20 cm, 53 ± 15 μm pore size, Ankom, Fairport, NY) with heat-sealed edges were used to determine in situ DM disappearance of forage. Five grams (as-fed) of ground (2-mm screen) prairie hay were incubated in dacron bags in the rumen for 0, 2, 4, 8, 12, 16, 24, 36, 48, or 96 h. Two bags containing hay for each incubation time were placed in the rumen at 0800 on d 11 after soaking for 20 min in 39°C water except for 0-h bags, which were soaked for 20 min and not incubated in the rumen. Bags were placed under the ruminal mat in nylon mesh bags (36 × 42 cm). After removal, bags were rinsed with 39°C water to remove particles adhering to the outside of bags and stored frozen (Bodine et al., 2003). In situ forage protein fraction degradation (neutral detergent insoluble nitrogen [**NDIN**] Bodine et al., 1999; Mass et al., 1999) was determined from hay-containing bags incubated for 2, 12, or 96 h, using similar dacron bag techniques as described for in situ DM disappearance. At the completion of the trial, all bags were thawed and washed in a washing machine on delicate setting 10 times for 1-minute rinse and 2-minute spin cycles with a maximum load of 100 bags. Bags were oven-dried (50°C, 72 h) and weighed. We have shown that this technique has minimal effect on in situ DM disappearance measures (Bodine et al., 2003).

Laboratory Analyses. We determined dry matter and ash content by placing samples in a forced-air oven at 105°C for 24 h and then in a muffle furnace at 500°C for 6 h.

Nitrogen and S concentrations were determined by a combustion method (Leco NS2000, St. Joseph, MI; [AOAC, 1996]). Fiber constituents (neutral detergent fiber, **NDF**, procedure A, without amylase or sodium sulfite; acid detergent fiber, **ADF**; and acid detergent insoluble ash, **ADIA**) were estimated as described by Van Soest et al. (1991). Starch content was determined enzymatically (Megazyme, Wicklow, Ireland) using α-amylase, amyloglucosidase, and a colorimetric glucose determination reagent (glucose oxidase-peroxidase, high purity) in accordance with AOAC (1996). Crude protein was classified as undegradable intake protein (**UIP**) or DIP by the enzymatic procedure of Roe et al. (1991). Fiber-bound protein (NDIN) of the residue from dacron bags was determined by NDIN analysis (Bodine et al., 1999; Mass et al., 1999).

Calculations. Values for intake and fecal output of individual chemical components were calculated by multiplying their corresponding percentages (DM basis) by intake and output of total DM. Hemicellulose was calculated as the difference between NDF and ADF. Neutral detergent solubles were calculated as the difference between DM and NDF. Neutral detergent insoluble N (fiber-bound N) in the feces was considered to be undigested feed N and used to determine true N digestibility. Digestibility of each component was calculated as the quantity of intake minus output subsequently divided by intake and then multiplied by 100 to convert to a percentage. Metabolic fecal N (**MFN**) output was estimated from neutral detergent soluble N in the feces, which was calculated as total fecal N minus fecal NDIN (fiber-bound N). Metabolic fecal N output was also estimated from the intercept obtained when apparent total tract N digestibility was regressed on dietary N concentration. Output of MFN was also estimated using the following equations: MFN = (kg of fecal DM output * 68) / 6.25, which is based on fecal

DM output (NRC, 1978); MFN = (kg of DMI * 33.44) / 6.25, which is based on DM intake (NRC, 1984); MFN = (kg of indigestible DM output * 14.4) which is based on indigestible DM output (NRC, 1985) where apparent OM digestibility was used to estimate TDN and adjusted for an intake greater than maintenance levels of a diet of greater than 40% roughage by multiplying estimated TDN by 0.92. Indigestible OM concentration was then calculated as one minus adjusted TDN, and this value was multiplied by DMI to obtain indigestible DM output. Estimates of MFN were also obtained from the software provided with the current beef cattle NRC (1996). Fractional rate constants for ruminal digestibility of ADF (K_d) were estimated by subtracting fractional particulate passage rate constants (K_{pp}; estimated from ADIA) from fractional ruminal disappearance rate constants (K_t; Owens and Goetsch, 1993; Owens, personal communication). Based on ruminal degradability, in situ forage DM was classified into one of three fractions as follows: A = the immediately soluble fraction; B = the fraction that is degraded at a measurable rate; and C = the ruminally undegradable fraction. Extent of in situ forage DM disappearance (C-fraction) was considered to be DM residue remaining after washing bags that had been ruminally incubated for 96 h. The soluble portion of the DM (A-fraction) was calculated as 100 minus the sum of the B and C fractions. Effective ruminal degradability of DM was calculated as the A-fraction plus the quantity of the B-fraction multiplied by the quantity of the in situ K_d divided by the sum of in situ K_d and K_{pp} (Orskov and McDonald, 1979). Fractional rate constants (K_d) and extent of in situ NDIN disappearance were calculated by regressing the natural logarithm of the percentage of potentially digestible NDIN remaining (corrected for 96 h

NDIN) on time of incubation (2 to 12 h) as outlined by Bodine et al. (1999) and Mass et al. (1999).

Statistical Analyses. Effects of corn and DIP combinations (2 x 4 factorial) were analyzed using analysis of variance techniques appropriate for a crossover design (Kuehl, 1994) for intake, digestibility, fecal output, ruminal ADF digestibility fractional rate constants (K_d), effective ruminal DM degradability, forage DIP and UIP concentrations, and fractional rate constants (K_d) of in situ disappearance of forage NDIN. Analyses of variance were performed using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC) with Satterthwaite degrees of freedom approximation techniques. Fixed effects included in the model were the main effects of corn and DIP and their interaction and random effects included in the model were steer and period. The covariance structure was tested and modeled using variance components (VC) structure. Means were calculated using least squares means (LSMEANS) and were subjected to further analyses when a significant ($P < 0.05$) F-test was observed for the main effects, or if the interaction F-test was significant ($P < 0.10$). Main effects were examined using linear and quadratic orthogonal polynomial contrasts for level of DIP across levels of corn, and the interaction was examined using linear and quadratic orthogonal polynomial contrasts for level of DIP within each level of corn. When linear and quadratic contrasts were significant ($P < 0.10$), means separation between levels of corn within each level of DIP were performed using tests of simple effects (SLICE). Treatment effects on extent of in situ forage DM disappearance were analyzed using a model similar to the one previously described that was modified for a repeated measures in time analysis. Time was included in the model, along with all interactions of time with corn and DIP. Steer × period

combinations defined the subjects on which the repeated measures were taken. Heterogeneity of variance among periods and a first-order autoregressive [AR(1)] correlation structure among repeated measures were adopted. Results were supported by the model fitting criteria calculated by the MIXED procedure. Fractional rate constants of in situ forage DM disappearance, lag time in hours, and B-fraction concentrations were calculated directly using a non-linear least squares regression method (NLIN procedure of SAS) for each steer and period combination (Coblentz, personal communication). The percentage DM remaining was regressed on incubation time using the model described in (Mertens and Loften, 1980) and (Galyean, 1997). Extent of in situ forage DM disappearance was taken from the LSMEANS estimates for each treatment and used in the NLIN model as the undegradable portion (C-fraction) of the in situ forage DM. Estimates of fractional DM disappearance rates, lag time, and A- and B-fractions were then subjected to ANOVA using the previously described methods for variables that were not repeated measures.

Results

Intake. Hay DM intake (Table 3) exhibited an interaction ($P < 0.01$) between corn and DIP. Added DIP resulted in quadratic increases ($P < 0.01$) in intake of DM from hay, both with and without supplemental corn grain. Because supplemental DM intake was maintained constant within each level of corn, total diet DM intake had the same pattern of responses as hay DM intake. An interaction ($P < 0.01$) occurred between corn and DIP for total DM intake, with quadratic ($P < 0.01$) increases in intake as DIP increased for supplements with or without added corn. Intake of NDF, ADF, hemicellulose, neutral detergent solubles, N and S exhibited interactions ($P < 0.02$)

between corn and DIP with quadratic increases ($P < 0.05$) as DIP increased in supplements with and without corn grain. Intake of NDF and ADF was similar ($P > 0.27$) when no supplemental DIP was added, and was greater ($P < 0.01$) for steers fed no supplemental corn at all three levels of supplemental DIP. However, hemicellulose intake was greater ($P < 0.01$) for corn-supplemented diets when fed with no supplemental DIP. When supplemental DIP requirements were met, hemicellulose intake was greater ($P < 0.05$) for supplements that did not contain corn. Neutral detergent solubles intake was greater ($P < 0.01$) at all levels of DIP for supplements with corn, as would be expected from feeding supplemental corn, which supplies a large amount of neutral detergent solubles. Intakes of N and S were greater ($P < 0.01$) for the corn diet when no supplemental DIP was fed, due to the large amount of N and S that were provided in the greater amount of supplement fed and the greater N and S concentrations of the corn-based supplement without added DIP. The N to S ratio also exhibited an interaction ($P < 0.01$) between corn and protein. Linear increases ($P < 0.01$) occurred in the N to S ratio as DIP increased for steers fed corn-based supplements. Quadratic increases ($P < 0.01$) were observed for the N to S ratio of steers fed supplements without added corn grain. Starch intake was not altered ($P > 0.22$) by increasing DIP, and was greater ($P < 0.01$) for steers fed supplements containing corn as would be expected. Diets without corn did not contain any starch, yet starch intake is reported. This is a result of the analytical procedure for starch detecting simple sugars as starch. In order to remain consistent with statistical analysis and data reporting, these values have been analyzed and included, but the starch intake values reported for diets fed without corn are not reflective of true starch

intake. A similar approach has been taken with starch digestibility and fecal output of starch.

Digestibility. Digestibility of NDF, ADF, hemicellulose, neutral detergent solubles, and N (Table 4) all exhibited interactions ($P < 0.01$) between corn and protein, whereas, S had a tendency ($P < 0.07$) for an interaction. Added DIP linearly increased ($P < 0.01$) digestibility of NDF, ADF, hemicellulose, N, and S by corn-supplemented steers, and NDF digestibility tended to linearly increase ($P < 0.06$) as DIP increased for steers fed supplements without added corn. Increasing intake of DIP did not appear to alter ($P < 0.13$) digestibility of ADF by steers fed supplements without corn. Digestibility of hemicellulose tended to respond quadratically ($P < 0.09$) and neutral detergent solubles digestibility tended to increase quadratically ($P < 0.07$) as DIP intake increased in supplements that did not contain corn. Digestion of N and S increased quadratically ($P < 0.01$) as DIP intake increased when supplements were fed without corn. Starch digestion in diets that contained corn was not altered ($P > 0.93$) by increasing DIP. As previously mentioned, the starch digestibility values reported for diets fed without corn are not reflective of true starch digestion.

Ruminal Acid Detergent Fiber Digestion Kinetics. Fractional rate of ruminal digestion of ADF (Figure 1) exhibited a tendency for an interaction ($P < 0.09$) between corn and protein. Steers fed supplements that contained corn had a linear increase ($P < 0.01$) in fractional rate of ruminal ADF digestion as degradable protein intake increased, whereas, when supplements were fed without corn, there was a quadratic response ($P < 0.01$). Steers fed supplements with corn had a decreased ($P < 0.01$) rate of ruminal ADF digestion than steers receiving supplements without corn at all levels of added DIP.

Ruminal ammonia-N exhibited an interaction ($P < 0.01$) between corn and DIP with a quadratic increase ($P < 0.01$) for steers supplemented with corn and a linear increase ($P < 0.01$) when no supplemental grain was fed.

Fecal Output. Fecal output of DM, NDF, ADF, hemicellulose, neutral detergent solubles, N, neutral detergent insoluble nitrogen (NDIN; fiber-bound N which would be assumed to represent only true feed N), and S (Table 5) exhibited interactions ($P < 0.01$) between corn and protein. As DIP intake increased, fecal output of DM, hemicellulose, neutral detergent solubles, N, and S responded quadratically ($P < 0.04$) and NDF output tended to respond quadratically ($P < 0.07$) when steers were fed supplements with added corn. Fecal output of ADF decreased linearly ($P < 0.01$) as DIP increased, whereas output of NDIN was not different ($P > 0.37$) due to DIP intake in supplements that contained corn. When steers were fed supplements without corn, fecal output of DM and NDF responded quadratically ($P < 0.01$), output of neutral detergent solubles, N, and S increased quadratically ($P < 0.01$), output of NDIN tended to increase quadratically ($P < 0.07$) and hemicellulose output increased linearly ($P < 0.01$) as DIP increased. Fecal N to S ratio did not exhibit an interaction ($P > 0.34$) between corn and DIP. Yet, it did increase quadratically ($P < 0.01$) with increasing DIP, and was greater ($P < 0.01$) for diets fed corn than for steers not receiving any grain. Increasing DIP intake did not alter ($P > 0.36$) fecal starch output. As would be expected, fecal starch output was greater ($P < 0.01$) for diets supplemented with corn than for diets fed without corn. Fecal starch output values reported for diets fed without corn are not reflective of true starch output.

Metabolic Fecal Nitrogen. Fecal output of neutral detergent soluble N (Table 6), which may be assumed to be MFN (NRC, 1985; Van Soest, 1994), exhibited an

interaction ($P < 0.02$) between corn and DIP. Output of MFN as measured from neutral detergent soluble N increased quadratically ($P < 0.01$) for diets with and without added corn. When the intercept of the regression of apparent N digestibility on dietary N concentration was used to predict MFN, an interaction ($P < 0.01$) was observed between corn and DIP. As DIP increased, output of MFN increased linearly ($P < 0.01$) for diets fed with corn and quadratically ($P < 0.01$) for supplements without corn. When MFN was predicted using the equations in the NRC (1978; 1984; and 1985), there was an interaction ($P < 0.01$) between corn and DIP and they all predicted quadratic increases ($P < 0.02$) in diets with and without corn as DIP intake increased. The software provided with the current NRC (1996) appeared to predict a similar response, unfortunately, we were unable to statistically analyze those equations because they require measures that were not taken in this study.

Fecal Nitrogen Concentration and Nitrogen Intake. Fecal N concentration (Figure 2) exhibited an interaction ($P < 0.02$) between corn and DIP. When steers were fed supplements with corn, fecal N concentration linearly increased ($P < 0.01$), even though N intake increased in a quadratic manner. Whereas, when steers were supplemented without corn, fecal N concentration increased in a quadratic manner ($P < 0.01$), just as N intake did. Concentration of fecal N was similar ($P > 0.40$) at the 0 additional supplemental DIP level, even though N intake differed ($P < 0.01$) between levels of corn. Fecal N differed ($P < 0.04$) between levels of corn at the 33 and 100 levels of DIP, although N intake was similar ($P > 0.22$) between levels of corn at those two levels of degradable protein.

In Situ Disappearance and Kinetics of Forage DM. Fractional rate of in situ DM disappearance, lag time, A-fraction, B-fraction, and C-fraction (Table 7) all exhibited interactions ($P < 0.01$) between corn and DIP. No interaction was detected ($P > 0.27$) between corn and DIP for effective ruminal degradation of forage DM. Increasing intake of degradable protein resulted in quadratic responses ($P < 0.01$) in fractional rates, A-fraction, and B-fraction for supplements with and without corn. Steers fed supplements with corn had a linear decrease ($P < 0.01$) in lag time and C-fraction as DIP increased, whereas steers fed supplements without corn had quadratic declines ($P < 0.02$) with increasing protein intake. Fractional rate of disappearance of forage DM from in situ bags was greater ($P < 0.01$) for steers fed supplements without corn at all levels of DIP except when no added soybean mean was supplemented. When no added DIP was included in the supplement, supplements with corn had faster ($P < 0.01$) fractional rates than steers fed supplements that did not contain corn. Steers fed corn supplements had longer ($P < 0.01$) lag times and decreased ($P < 0.04$) B-fraction than steers not receiving corn. Corn-supplemented steers also had greater ($P < 0.01$) C-fractions at the 0 and 33 levels of DIP and tended ($P < 0.09$) to be greater at the highest quantity of DIP. Across levels of corn, increasing DIP tended to quadratically increase ($P < 0.07$) effective ruminally degraded forage DM and, across levels of DIP, supplements with corn resulted in lesser ($P < 0.01$) concentrations of effective ruminally degraded forage DM.

In Situ Kinetics and Estimates of Forage UIP and DIP. The fractional rates of fiber-bound N (NDIN, Table 8) degradation of the basal prairie hay did not exhibit an interaction ($P = 0.16$) between corn and DIP. Across supplements with and without corn, increasing intake of ruminally degraded protein tended to linearly increase ($P < 0.08$)

fractional rate of forage protein degradation in the rumen, and supplements fed with corn had decreased ($P < 0.01$) rates of forage protein degradation in the rumen than steers that received no supplemental corn.

Concentrations of degradable and UIP in the prairie hay basal forage, expressed as a percentage of either DM or CP, did not exhibit interactions ($P = 0.13$) between corn and DIP. If the value of $P < 0.13$ is accepted as a tendency for an interaction between corn and protein then UIP concentration of the forage, expressed as a percentage of DM or CP, linearly increased ($P < 0.04$) and forage DIP decreased ($P < 0.04$) in diets fed with corn, as intake of degradable protein increased. There was a tendency for supplements that contained corn to have greater UIP and lesser DIP concentrations ($P < 0.08$) in the basal prairie hay than steers fed supplements without corn. The basal forage contained more UIP and less DIP ($P < 0.05$) when steers were fed supplements containing corn and had either 33 or 66% of the NRC (1996) requirement for DIP added to the diet.

Evaluation of the NRC (1996) Model. The NRC (1996) software over predicted forage and total DM intake (Table 9) when supplements were fed without corn or any added DIP, but under predicted forage and total DMI when soybean meal was added. When corn supplements were fed with no added DIP the model accurately predicted forage and total DM intake. Yet, when corn supplements were fed with added DIP the software predicted forage and total DM intake to be lesser than was observed. The model did not predict the observed response of increased DM intake with increasing degradable intake protein for these protein deficient diets. The model predicted energy intake to be similar to observed levels (comparing predicted TDN intake to observed digestible OM intake) when supplements without corn were fed, but, it predicted energy intake to be

greater than actual levels when DIP was deficient in the corn diets (0 to 66% levels). The addition of supplemental DIP quadratically increased ($P < 0.01$) intake of digestible OM for supplements with or without corn, which is similar to the predicted TDN intake pattern. Steers fed supplements with corn had greater ($P < 0.01$) digestible OM intake than steers fed supplements without corn, which also agrees with model predictions. However, observed energy intake for corn-supplemented steers was lesser than predictions for the 0 to 66% DIP levels, whereas predicted energy intake for corn-supplemented steers was greater than what was actually observed for the 0 to 66% DIP levels. Observed OM digestibility was greater than predicted by the NRC (1996) software (comparing observed OM digestibility to predicted dietary TDN concentration) of the diets without corn, whereas, actual OM digestibility was lesser than predicted values when corn was fed without any added degradable protein, yet it predicted total diet OM digestibility to be greater than observed values when corn was fed without any added degradable protein. The model predicted total diet OM digestibility for diets supplemented with corn and added DIP to be similar to observed values. The addition of DIP to diets with or without corn increased total diet OM digestibility in a linear ($P < 0.01$) manner. The increase in digestibility was greater for corn-supplemented diets than for supplements that did not contain corn. The NRC (1996) software predicted the opposite effect. Ruminal pH was predicted to be a constant value of 6.46 across all eight diets by the model. The predicted particulate passage rates were greater than the observed rates for all diets, and the predicted fluid passage rates for the supplements without corn were also greater than observed. Conversely, corn-supplemented diets had greater observed passage rates than the model predicted. The predicted DIP and MP

balances were reflective of our experimental design, as would be expected, since the model was used to formulate the experimental treatments. The software (NRC, 1996) predicted fecal N output coming from feed to be between two and four g/d, which was lesser than observed, and therefore, predicted true N digestibility to a constant value of 98% across all diets, which was greater than observed. It predicted MFN output (Table 6) to be between 56 and 85 g/d, whereas our observed values were from 19 to 44 g/d. As a result, the software predicted total fecal N output to be between 58 and 89 g/d when it actually ranged from 32 to 67 g/d. Consequently, the model predicted apparent N digestibilities to range from –21% to 51% when the observed range was 32% to 64%.

Discussion

When supplemental corn was fed to steers consuming low-quality prairie hay, the addition of graded levels of DIP increased intake of forage DM and fiber constituents in the total diet in a quadratic manner. This agrees with our previous research (Bodine et al., 2000; Bodine et al., 2001) as well as other previously reported studies (Beaty et al., 1994; Olson et al., 1999). It appears that the first increment of protein resulted in the greatest response in intake, and was followed by either a diminishing response to further increases in DIP, or a plateau at the two intermediate levels of intake followed by a slight reduction in fiber intake at the highest level of DIP. This slight reduction may be an artifact of the data created by the experimental design. As DIP was increased from greater intake of soybean meal, the amount of cottonseed hulls in the supplements was decreased. Cottonseed hulls are a high-fiber feedstuff, and as the amount of supplemental soybean meal was increased (Table 2), approximately one kg of cottonseed hulls was removed from the supplement. This resulted in a reduction in fiber intake from

supplement of up to 750 g (Table 1). This amount is greater than the observed decline in total fiber intake at the highest level of DIP. Supplements that did not contain corn had a similarly large response to the first addition of degradable protein. This response has been documented before (Beaty et al., 1994; Guthrie and Wagner, 1988; Sunvold et al., 1991). Supplements without corn had a slight decrease in fiber intake as DIP increased after the first increment, even though they had a greater reduction in supplemental fiber intake with increasing soybean meal inclusion. Yet, the quadratic effect and decrease in total fiber intake at the greater levels of DIP was less for these supplements than it was for steers fed corn-based supplements. If decreasing fiber intake from supplemental cottonseed hulls was the only cause of the decline in fiber intake, it should occur in both supplement types. The greater decline in fiber intake at the highest level of DIP intake for corn-supplemented steers indicates that the response differed between supplements with and without corn. If fiber intake truly presented a limit to, or control of, total DM intake, it would be expected that a plateau in intake such as this would be observed. It is entirely possible that the observed plateau in total dietary fiber intake may represent a maximum level of intake for this class of animal on this forage type. This also may be linked to a similar plateau noted in fecal output of fiber. It has been suggested that fecal output of fiber may represent a potential control of intake (Owens et al., 1991). In our study, fecal output of DM and fiber were relatively constant among all supplement treatments and fecal DM output averaged 1.1% of BW. Still, it is difficult to suggest a cause and effect relationship between measures that are as interrelated as these two. Another possible explanation for the increase, plateau, and subsequent decline in intakes may have been that as degradable protein intake increased, digestibility increased. The

increased DM intakes and digestibility that were observed resulted in greater energy intake from digestible OM (Moore et al., 1999). This increase in digestible OM intake has been reported in many studies (Beaty et al., 1994; Heldt et al., 1999a; Olson et al., 1999). The greater decline in fiber intake for steers fed supplements with corn could suggest that at the highest level of DIP, digestible OM intake was at a level where energy intake may have played a role in control of intake. When corn supplements were fed with adequate DIP, digestibility of the diet was within the range where it has been suggested that control of intake changes from physical limitations such as passage, digestibility, and output, to being largely controlled by physiological factors (Conrad et al., 1963). In a related study, in which a similar corn and soybean meal supplement was fed to steers grazing dormant winter native tallgrass prairie range, similar steers had an ADG of 0.73 kg/d (Bodine and Purvis, II, 2003), suggesting a relatively large energy intake for this class of forage. Given the levels of supplement intake, forage intake, digestibility, and kinetics that occurred within the current study, it is most likely that multiple mechanisms served to limit intake (Forbes, 1996; Illius and Jessop, 1996) across all supplements.

Additionally, it is interesting to note that although our study included a negative control treatment, we did not observe any substitution of supplement for forage, even though we did observe the classical negative associative effect of starch depressing forage and fiber digestibility. While some researchers have observed substitution to occur when low-quality forages were supplemented with corn (Heldt et al., 1999a; Sanson and Clanton, 1989), a lack of substitution is also commonly reported (Heldt et al., 1999b; Olson et al., 1999; Stafford et al., 1996). In related studies we have observed both

a lack of substitution (Bodine et al., 2000; Bodine et al., 2001), as well as substitution effects (Bodine and Purvis, II, 2003). In the study where we observed substitution, a similar corn-based supplement decreased forage intake of steers grazing dormant native tallgrass prairie (Bodine and Purvis, II, 2003). This may have been due to factors such as level of feeding, inaccuracies due to the use of markers to estimate intake of grazing animals, or that dietary selection allowed for higher quality forage to be consumed. Previous research has suggested that as forage quality increases, substitution is more likely to occur (Bowman and Sanson, 1996; Caton and Dhuyvetter, 1997; Horn and McCollum, 1987). Additional reasons for this apparent lack of agreement include differences in class of animal or between confined and pastured animals. The lack of substitution in the current study may be a result of the very low forage intake that occurred when this forage was fed without any added protein.

When supplements did not contain corn, increases in fiber digestion were minimal (from ~ 60% to 64%). The limited response in fiber digestibility may be a result of increased intake and more rapid passage rates decreasing total tract retention time. Owens et al. (1991) stated that retention time is the single largest factor determining digestibility in any compartment in the gastrointestinal tract. This response to protein supplementation has been seen extensively in previous research (McCollum and Galyean, 1985; McCollum and Horn, 1990; Owens et al., 1991). The slight increase observed in fiber digestion followed by the plateau in fiber intake, combined to create a small decline in fecal output of fiber. This is based on the observation that the first increment of protein resulted in the greatest increase in fiber intake, along with a small increase in fecal output. As DIP intake increased, no further increase in fiber intake occurred, fiber

digestibility slightly increased, and fecal output slightly decreased. Whereas, when corn was supplemented, very large increases in fiber digestion were observed (~50% increase) from the least to greatest levels of added DIP. When corn supplements were fed with adequate DIP, fiber digestion increased to similar levels of total tract fiber digestion as supplements fed without grain. The ability of ruminally degradable protein to recover fiber digestion is well documented (Crabtree and Williams, 1971; el-Shazly et al., 1961; Heldt et al., 1999a). We believe the ability of DIP to recover fiber digestibility of starch-supplemented rations to levels similar to diets fed without starch is indicative of the elimination of negative associative effects. The elimination of negative associative effects of starch on fiber digestibility by feeding added DIP indicates that the primary mechanism through which starch-based supplements depress fiber digestibility is to create a ruminal ammonia deficiency (el-Shazly et al., 1961; Erdman et al., 1986). Nonetheless, this is not a new suggestion. In two separate papers, Burroughs et al. (1949; 1950) suggested that increasing levels of starch in the ration would result in an increased need for dietary protein in order to maintain digestibility. Other researchers have also suggested that better utilization of supplementary energy will occur with increased protein (Clanton and Zimmerman, 1970). The large increase in digestibility observed when corn supplements were fed with the highest level of DIP, along with the plateau in fiber intake, may explain the decline in fecal output of fiber.

Increasing degradable protein intake for both supplement types resulted in increased rates of in situ forage DM disappearance, B-fractions, and effective ruminal degradability of DM, whereas it decreased lag time and C-fractions (Bodine et al., 2001; Erdman et al., 1986; Heldt et al., 1998). Starch-based supplements had greater lag times and C-fractions

with each level of DIP than did supplements fed without corn. (Hoover, 1986) also observed starch to increase lag time and C-fractions; yet, they did not see an effect on rate of digestion, whereas we found starch to decrease disappearance rates of DM. The increase in effective ruminal degradability of forage DM occurred even though passage rates also increased greatly in this study due to increasing protein. The increased rate of in situ DM disappearance was mirrored by an increase in the rate of forage protein degradation in the rumen. However, corn-supplemented diets had decreased DIP and increased UIP concentrations of the basal forage. One possible explanation for this may be due to the decreased rate of ruminal fiber disappearance and digestion observed in this study, which has been previously reported (Bodine et al., 2001; Chase, Jr. and Hibberd, 1987; Heldt et al., 1998).

When the rate of ruminal ADF digestion was plotted against ruminal NH_3-N values, we were surprised to discover that six of the diets in this study could be paired into three levels of ruminal NH_3-N concentrations. Yet, those similar ruminal NH_3-N levels did not occur at similar levels of DIP between diets supplemented with and without corn. Diets supplemented with corn required an additional increment of DIP in order to reach a similar ruminal NH_3-N level as diets without corn (No corn, 0% DIP paired with Corn, 33% DIP; No corn, 33% DIP paired with Corn, 66% DIP; and No corn 66% DIP paired with Corn, 100% DIP). A second additional increment of DIP was required to increase the rate of ruminal ADF digestion to a level similar to diets not supplemented with corn. As can be seen from the plot of rate of ruminal ADF digestion and ruminal NH_3-N concentration, diets supplemented with corn and a greater level of DIP than supplements without corn still had decreased rates of ADF digestion in the rumen. This occurred even

though these paired treatments had similar ruminal NH_3-N concentrations. It appears that increasing ruminal NH_3-N concentration to equal levels by increasing DIP did not completely eliminate ruminal negative associative effects. This is supported by the fact that corn-fed steers had decreased fractional rates of ruminal ADF digestion, decreased in situ DM disappearance rates, and increased lag times at similar ruminal NH_3-N levels vs. steers fed supplements without grain. Despite similar ruminal NH_3-N levels, steers fed the corn-based supplement with adequate soybean meal may not have reached sufficient levels of ruminal NH_3-N to allow similar rates of ruminal fiber digestion. Although the NRC (1996) predicted that we met DIP requirements, it is possible that we did not feed sufficient DIP to meet ruminal requirements. Since ruminal NH_3-N is a measure of supply and demand, it is possible that DIP supply was inadequate to fully meet the demand of ruminal fermentation of both forage and supplement. If carbohydrate digestion in the rumen is the predominant factor controlling microbial protein synthesis (NRC, 1996), and therefore DIP requirements, it is highly likely that the use of a single microbial efficiency across all of these diets was inappropriate. In several recent studies, we have shown that microbial efficiency of TDN conversion to microbial crude protein will range from 8.9 to 9.6% for unsupplemented prairie hay diets, and up to 12.8% for steers fed prairie hay and ruminally infused with casein (Basurto, 2003). When added corn was fed, greater amounts of ruminally available carbohydrates were consumed, and we may have underestimated the value for efficiency of TDN conversion to bacterial protein. This would increase the amount of DIP required to meet ruminal requirements, and could partially explain the lowered ruminal fiber digestibility rates observed for steers supplemented with corn. Had we fed additional levels of DIP to the corn-

supplemented steers, it is possible that we could have further increased rate of ruminal ADF digestion. However, since total tract fiber digestion, rates of passage and disappearance of OM and ADF, and ruminal mass and concentration of ADF in the rumen were all similar (Bodine and Purvis, II, 2003) between levels of corn, it suggests that even if extent of ruminal ADF digestion was decreased in the rumen by corn supplementation, the remainder of the ADF was being digested in the lower tract on the corn-supplemented diets. Another possible explanation for the decreased rates of ruminal ADF digestion is that ruminal fiber digestion was decreased by factors other than ruminal NH_3-N availability alone as has been previously suggested (Hoover, 1986; Russell, 1998). Even though rate of ruminal ADF digestion was increased by increasing DIP, the addition of DIP at NRC (1996) adequate levels did not completely alleviate all negative associative effects on rate of ruminal fiber digestibility, and fiber intake, yet it did eliminate differences in total tract fiber digestibility.

The N to S ratio of all diets was within range of 10-15 to 1 suggested as acceptable in order to insure adequate S for ruminal microbes to synthesize S-containing amino acids by (Puls, 1990). Nitrogen and S intake, digestibility, and output appear to be related, as increasing DIP intake increased intake of both N and S, increased N and S digestibility, and increased fecal N and S output. On these diets, a significant source of S may have been S-containing amino acids (NRC, 1996), thereby explaining the strong relationship between N and S observed in this study. This is a relationship we have observed previously in masticate samples taken from various native forages in Oklahoma collected across the calendar year (Reuter, 2000). Increasing apparent N digestibility with increasing dietary N intake is a well-documented occurrence in both ruminants and

non-ruminants alike (Basurto, 2003). This may be a result of increasing N intake levels reducing the impact of microbial contribution to fecal N output by increasing ruminal digestion and decreasing lower tract digestion. In other recent research we have reported similar ranges and an increase in apparent protein digestibility due to the ruminal infusion of casein to steers fed prairie hay (Basurto, 2003). The fecal excretion of bacterial cells that contain both N and S may also confound the values for apparent N and S digestion and fecal output. When NDIN in the feces was used to estimate fecal output of true feed N, true N digestibility ranged from 73% to 87%, which approaches the value of 90% suggested by the NRC (1985). Increasing true N digestibility was a response to increasing N intake of a protein of a higher quality than the basal diet. Concentration of N in the feces has been suggested as a possible method to estimate N intake, and thereby N status of grazing cattle (Lyons and Stuth, 1992; Ward et al., 1982; Wofford et al., 1985). Steers fed supplements without corn had quadratic increases in both fecal N output and in N intake. We have shown similar ranges and levels of fecal N output for prairie hay-fed steers, and that fecal N output will increase when casein is ruminally infused (Basurto, 2003). This was primarily the result of the very low N intake when supplements without corn were fed without added soybean meal. Although fecal N concentrations of the no corn, no DIP steers were similar to the corn, no DIP cattle, the corn-supplemented animals had a 70% greater intake of N. When DIP was added at the intermediate levels, fecal N appeared to adequately predict N intake. However, as DIP intake increased, it appeared that the two lines were beginning to diverge, with corn-supplemented diets having a greater rate of increase in fecal N than supplements without corn even though N intakes were increasing at a greater rate for supplements fed without

corn. If additional increments of DIP had been fed, it would appear that steers supplements with corn would have had lesser N intake, and greater fecal N than steers not receiving corn. This may explain why we show agreement between fecal N and N intake on this data set, and in a previous study we have reported that fecal N did not correctly rank N intake and was not appropriate for assessing N status of steers fed energy supplements (Bodine and Purvis, II, 2003). In that paper the fecal N values of protein-supplemented, corn-supplemented, and corn plus soybean meal-supplemented steers were greater than steers fed similar supplements reported in this paper, whereas unsupplemented cattle in the grazing study had values similar to those observed in the present study. Differences between the two studies may be related to the observation that fecal N and N intake were beginning to diverge as protein intake increased, or the possibility that the opportunity for dietary selection by grazing steers resulted in a N intake that was actually greater than expected based on masticate samples. The possibility for dietary and behavioral differences between penned and grazing animals, as well as the lack of agreement between our data sets suggests that caution be used when applying the data in this study to a field setting.

Intake of neutral detergent solubles and starch were primarily a function of experimental design, with increases due to increased intake of corn, soybean meal, or both from the supplement. Still, we believe that the differences in digestibility and fecal output are suggestive of altered site of digestion and increased lower tract fermentation. The NRC (1985; 1996) suggests that from 7 to 47 % of total tract digestion can occur post-ruminally. The low fecal output of N and neutral detergent solubles for the diets supplemented without corn and no added soybean meal are suggestive of a severely

protein-deficient animal. While the ability of an animal in this physiological state to recycle N back to the gastrointestinal tract may be greatest, the total amount available to be recycled may be limited. The steers fed the supplement without corn or DIP had minimal intakes of N (48 g), starch (0 g), simple sugars (25 g) and relatively low intakes of neutral detergent solubles (1211 g). Therefore, even the presence of minor amounts of N, neutral detergent solubles (cell contents), starch, and simple sugars of microbial origin in the feces could drastically reduce apparent digestibility measures. When DIP was added to the supplements, intakes and fecal excretion of N, neutral detergent solubles, and starch increased. However, the mathematical effect of increased fecal output on digestibility was decreased by increased intake, greater output of fecal DM of feed origin, and decreased lower tract fermentation occurring, thereby reducing the impact of microbial contribution to apparent digestibility values. The increase in digestibility of neutral detergent solubles in diets fed without corn, after the initial increment of DIP was fed, indicates increased ruminal digestibility of fiber, decreased intestinal digestion of fiber, decreased fecal output of neutral detergent solubles from bacteria, and increased apparent neutral detergent solubles digestion. The depressed neutral detergent solubles intake in the negative control diet would not be expected to have such a large impact on true digestion of neutral detergent solubles, thereby suggesting that microbial contribution to fecal output may have lowered apparent digestibility of this treatment. A similar trend can be observed in both supplement types for apparent N and S digestibility as well. The negative values for starch digestion and the fecal output of starch on the diets fed without corn are also suggestive of this. These diets did not contain a source of starch, and any of the simple sugars detected in the feed as starch by the assay would be

expected to be rapidly fermented by the microbes in the rumen. The detection of starch in the feces of these diets suggests that simple sugars were present in the feces at levels considerably greater than present in the feed, and can be attributed as to microbial sources, as bacteria can contain from 3 to 6% carbohydrate.

In our study, increasing DIP, and as a result, increasing fiber intake and digestion, did not alter starch digestion. Possibly, levels of corn fed in this study were such that it was possible for all of the starch to be fermented in the rumen, digested in the small intestine, or both (Huntington, 1997), thereby preventing any effect that fiber might have had on starch. From this result we can conclude that supplement digestibility did not vary across treatments, and the calculation of forage digestion using fecal output minus indigestible supplement amounts would be valid.

Output of MFN increased with increasing N intake, and made up more than 60% of total fecal N output. Research summarized in the NRC (1985) suggests that 38 to 74% of fecal N can be attributed to MFN of microbial origin. However, the source of origin of the microbes cannot necessarily be attributed to increased large intestinal fermentation. Yet, in a related study with similar supplements, feeding corn decreased fecal pH by one pH unit (Bodine and Purvis, II, 2003), which can be assumed to be primarily a result of increased large intestinal fermentation. Estimates of MFN have been made using many methods (NRC, 1984; 1985; 1996). Using the intercept of the regression of apparent N digestibility on dietary N concentration resulted in an average over prediction of 70% compared to observed values. Using the equation based on fecal DM output found in the dairy cattle NRC (1978) and reported in the beef cattle NRC (1984), MFN was fairly accurately predicted, on average, to within 20% of measured values. The majority of the

error occurred when no supplemental DIP was fed. When the equation developed in the beef cattle NRC (1984, which is based on DMI, was evaluated, MFN output was over predicted by an average of 30% vs. observed values, with most of the error being attributable to the diets supplemented with no added DIP. Yet, when the more complicated equation based on indigestible DM output from the Ruminant Nitrogen Usage publication (NRC, 1985) was tested, it predicted MFN to be more than 60% greater than the measured amounts. The NRC (1996) states that this equation has a tendency to over predict MFN of forage-fed cattle. When the data from each treatment of the current study were entered into the current beef cattle NRC (1996) software, it over predicted MFN from 100% to 200%. It predicted MFN output to be 45% greater than observed total fecal N output actually was on this study, and it predicted output of MFN alone to be greater than total N intake for one of our experimental treatments. Even though the NRC (1996) software predicted fecal N output to be greater than N intake, no treatments in this study resulted in a negative apparent N digestibility. The calculations that the model uses resulted in MFN making up from 95% to 97% of total fecal N output, even though the NRC (1985) itself suggests that a range of 38 to 74% of total fecal N can be attributed to MFN. In the text of the NRC (1996), it is stated that the values for MFN generated by the 1985 NRC equations are unrealistically high for forage-fed beef cows. Unfortunately, it appears that use of the equations in the current NRC (1996) will result in even higher estimates for forage-fed beef cattle than previous versions.

The model was unable to predict the effects of protein supplementation on intake, passage rates, under predicted OM digestibility for protein-supplemented diets, over predicted OM digestibility of corn-supplemented diets. This is not surprising, as the

model is not designed to incorporate associative effects into its predictions, although this has been an issue about which there has been much discussion. The difficulties of modeling associative effects have been previously mentioned (Horn and McCollum, 1987) even though there are efforts to overcome these challenges (Moore et al., 1999). Using the software provided with the current NRC (1996), we were unable to predict the observed reductions in ruminal pH due to feeding corn, or to increasing DIP, and the resultant increase in ruminal fermentation and VFA. Possibly the most bothersome result was that it under predicted feed N in the feces by 10-fold, and as a result, over predicted true N digestibility. It over predicted total fecal N output by 50%, and as a result, under predicted apparent N digestion. If this software is used to build models for N excretion, N loading, and N balance by beef cattle operations, it will not be appropriate for forage-fed animals. Overall, the software program associated with the current beef cattle NRC (1996) does not incorporate associative effects into the modeling outputs. The data generated in this research study was primarily based on the expected associative effects observed when low-quality forages are fed with either supplemental energy or protein. The model incorporates some very advanced theories, and while it can explain a great deal of the results observed in previous research, it is clearly a tool for advanced users who are already aware of the effects of various nutrient imbalances.

Implications

Supplementing degradable intake protein increased fiber intake and digestion. When low-quality prairie hay was supplemented with corn, more degradable intake protein was necessary to increase fiber intake and digestibility to values similar to steers fed supplements without corn. Supplemental corn negatively impacted ruminal digestion of

forage and fiber. However, adding degradable intake protein to meet ruminal requirements eliminated the total tract negative associative effects of starch on fiber digestibility and intake. Alterations in ruminal measures were largely a result of deficiencies in ruminal NH_3-N due to supplementing ruminally fermentable organic matter without added ruminally degradable protein necessary to balance microbial fermentation. The inclusion of degradable intake protein in grain-based energy supplements fed to cattle consuming low-quality forages will alleviate the negative associative effects typically observed in previous research.

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Table 1. Chemical composition of prairie hay and supplements with or without corn and increasing amounts of degradable intake protein (DIP) fed to beef steers

Chemical composition, % of DM	Prairie hay, % of DM	Corn, % of BW	Amount of supplemental DIP, as a % of requirement[a]			
			0	33	66	100
DM	92.1	0	90.2	89.7	89.6	89.3
		0.75	88.2	88.1	88.1	88.0
OM	93.7	0	92.3	90.1	89.0	88.0
		0.75	96.2	95.7	95.3	94.9
NDF	74.6	0	85.3	48.8	30.9	12.5
		0.75	38.5	31.8	25.1	18.2
ADF	44.7	0	65.3	36.1	21.6	6.9
		0.75	22.7	17.3	11.9	6.4
Starch	0.8	0	0.4	1.1	1.4	1.8
		0.75	51.1	51.1	51.1	51.2
CP	6.1	0	4.0	27.3	38.6	50.4
		0.75	7.8	12.1	16.4	20.9
DIP	4.1	0	2.0	17.4	25.0	32.7
		0.75	3.5	6.4	9.3	12.2
TDN[b]	48.0	0	40.0	61.6	72.1	83.1
		0.75	75.7	79.6	83.6	87.7
DIP, g/100 g of TDN[b]	8.6	0	5.1	28.3	34.6	39.5
		0.75	4.7	8.1	11.1	14.0

[a]Requirements calculated from NRC (1996) software, Level 1 model.

[b]Calculated from tabular (NRC, 1996) TDN and DIP values for corn (90% of DM and 45% of CP), soybean meal (87% and 65%), and cottonseed hulls (42% and 50%). The TDN value of prairie hay was estimated from measured hay OM digestibility (48%) in a preliminary study, which was identical to tabular values, and hay DIP (70%) estimated from a previous study (Bodine et al., 2001).

Table 2. Ingredient and chemical component intake of supplements with or without corn and increasing amounts of degradable intake protein (DIP) fed to beef steers consuming prairie hay

Item	Corn, % of BW	Amount of supplemental DIP, as a % of requirements[a]			
		0	33	66	100
Ingredient, kg of DM					
Corn	0	0	0	0	0
	0.75	2.3	2.3	2.3	2.3
Soybean meal, 49% CP	0	0	0.6	0.9	1.2
	0.75	0	0.3	0.6	0.9
Cottonseed hulls	0	1.2	0.6	0.3	0
	0.75	0.9	0.6	0.3	0
Mineral mix	0	0.1	0.1	0.1	0.1
	0.75	0.1	0.1	0.1	0.1
Supplement	0	1.3	1.3	1.3	1.3
	0.75	3.3	3.3	3.3	3.3
Intake from supplement, g/kg of BW					
Supplement, DM	0	3.7	3.9	3.8	3.9
	0.75	10.2	10.2	10.2	10.4
DIP[b]	0	0.1	0.7	1.0	1.3
	0.75	0.4	0.7	1.0	1.3
TDN[b]	0	1.5	2.4	2.7	3.2
	0.75	7.7	8.1	8.5	9.2
Starch	0	0.0	0.0	0.1	0.1
	0.75	5.2	5.2	5.2	5.3

[a]Requirements calculated from NRC (1996) software, Level 1 model.

[b]Calculated from tabular (NRC, 1996) TDN and DIP values for corn (90% and 45%), soybean meal (87% and 65%), and cottonseed hulls (42% and 50%). The TDN value of prairie hay was estimated from measured hay OM digestibility (48%) in a preliminary study, which was identical to tabular values and hay DIP (70%) estimated from a previous study ((Bodine et al., 2001).

Table 3. Daily intake of selected chemical components by beef steers fed prairie hay and supplemented with two levels of corn (0 or 0.75% of BW) and four levels of degradable intake protein (DIP; as a percentage of NRC (1996) requirements)

Intake, g	Corn, % of BW	Amount of supplemental DIP, % of requirements[a] 0	33	66	100	SEM[c]	Probability Level[b] Corn	DIP	INT	L	Q
Forage DM	0	4,159	6,762[u]	7,062[u]	7,284[u]	352	0.01	0.01	0.01	0.01	0.01
	0.75	4,195	5,427[v]	5,729[v]	5,666[v]					0.01	0.01
Total diet DM	0	5,348[u]	7,982[w]	8,271[w]	8,530[y]	381	0.01	0.01	0.01	0.01	0.01
	0.75	7,416[v]	8,658[x]	8,966[x]	8,987[z]					0.01	0.01
Neutral detergent fiber	0	4,138	5,641[u]	5,643[u]	5,592[u]	268	0.01	0.01	0.01	0.01	0.01
	0.75	4,375	5,078[v]	5,087[v]	4,833[v]					0.04	0.01
Acid detergent fiber	0	2,652	3,459[u]	3,415[u]	3,339[u]	159	0.01	0.01	0.02	0.01	0.01
	0.75	2,607	2,983[v]	2,944[v]	2,743[v]					0.36	0.01
Hemicellulose	0	1,486[u]	2,182	2,228	2,253[w]	108	0.75	0.01	0.01	0.01	0.01
	0.75	1,768[v]	2,095	2,143	2,090[x]					0.01	0.01
Neutral detergent solubles	0	1,211[u]	2,340[u]	2,628[u]	2,938[u]	123	0.01	0.01	0.01	0.01	0.01
	0.75	3,043[v]	3,581[v]	3,879[v]	4,155[v]					0.01	0.03
Nitrogen	0	48[u]	121	146	175	4.7	0.01	0.01	0.01	0.01	0.01
	0.75	83[v]	118	144	171					0.01	0.05
Sulfur	0	5.6[u]	11.0	12.5	14.1	0.5	0.01	0.01	0.01	0.01	0.01
	0.75	8.3[v]	10.9	12.5	13.8					0.01	0.01
N to S ratio	0	8.6[u]	11.1[y]	11.7	12.4	0.1	0.01	0.01	0.01	0.01	0.01
	0.75	10.0[v]	10.9[z]	11.6	12.4					0.01	0.53
Starch intake[d]	0[d]	25[d]	66[d]	72[d]	79[d]	42.5	0.01	0.22	0.81	ND	ND
	0.75	1,685	1,692	1,698	1,743						

[a]Requirements calculated from NRC (1996) software, Level 1 model.
[b]Corn = main effect for amount of corn; DIP = main effect for amount of degradable intake protein; INT = interaction between corn and DIP; L = linear; Q = quadratic response across amounts of degradable intake protein; ND = Not Determined.
[c]SE = Standard error of the means, n = 8.
[d]Starch was not present in diets without corn, therefore, these values represent simple sugars detected by the starch assay.
[u,v]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.01$) between levels of corn.
[w,x]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.05$) between levels of corn.
[y,z]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.10$) between levels of corn.

Table 4. Apparent total tract digestibility of selected chemical components by beef steers fed prairie hay and supplemented with two levels of corn (0 or 0.75% of BW) and four levels of degradable intake protein (DIP; as a percentage of NRC (1996) requirements)

	Corn, % of	Amount of supplemental DIP, % of requirements[a]					Probability Level[b]				
Apparent digestibility, %	BW	0	33	66	100	SEM[c]	Corn	DIP	INT	L	Q
Neutral detergent fiber	0	60.5[u]	61.2[y]	64.0[u]	64.0	2.2	0.01	0.01	0.01	0.06	0.80
	0.75	45.1[v]	57.5[z]	55.3[v]	64.1					0.01	0.23
Acid detergent fiber	0	59.1[u]	58.6	61.3	62.9	3.1	0.01	0.01	0.01	0.13	0.61
	0.75	43.6[v]	57.6	55.4	63.7					0.01	0.16
Hemicellulose	0	62.1[u]	65.3[u]	68.0[u]	65.5	3.9	0.01	0.01	0.01	0.10	0.09
	0.75	46.6[v]	57.3[v]	55.1[v]	64.6					0.01	0.70
Neutral detergent solubles	0	26.8[u]	40.1[u]	44.8[u]	48.8[u]	2.7	0.01	0.01	0.01	0.01	0.07
	0.75	58.8[v]	60.7[v]	58.9[v]	63.3[v]					0.29	0.59
Nitrogen	0	32.2[u]	53.8	59.6[w]	63.9	2.0	0.18	0.01	0.01	0.01	0.01
	0.75	44.6[v]	54.6	53.7[x]	63.5					0.01	0.95
Sulfur	0	30.9[w]	41.8[y]	46.3	47.4	2.5	0.08	0.01	0.07	0.01	0.01
	0.75	36.5[x]	46.4[z]	42.5	50.7					0.01	0.65
Starch[d]	0[d]	-69.8[d]	-21.3[d]	-16.7[d]	-24.8[d]	12.2	0.01	0.05	0.04	ND[d]	ND[d]
	0.75	89.8	87.6	87.2	87.7					0.94	0.93

[a]Requirements calculated from NRC (1996) software, Level 1 model.

[b]Corn = main effect for amount of corn; DIP = main effect for amount of degradable intake protein; INT = interaction between corn and DIP; L = linear; Q = quadratic response across amounts of degradable intake protein; ND = Not Determined.

[c]SE = Standard error of the means, n = 8.

[d]Starch was not present in diets without corn, therefore, starch digestibility was not statistically analyzed within those diets, and these values represent simple sugars detected by the starch assay.

[e]Estimated from nitrogen intake and output of neutral detergent insoluble nitrogen (fiber-bound nitrogen).

[u,v]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.01$) between levels of corn.

[w,x]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.05$) between levels of corn.

[y,z]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.10$) between levels of corn.

Table 5. Fecal output of selected chemical components by beef steers fed prairie hay and supplemented with two levels of corn (0 or 0.75% of BW) and four levels of degradable intake protein (DIP; as a percentage of NRC (1996) requirements)

Fecal output, g	Corn, % of BW	Amount of supplemental DIP, % of requirements[a] 0	33	66	100	SEM[c]	Probability Level[b] Corn	DIP	INT	L	Q
Total DM	0	2,531[u]	3,619	3,488[u]	3,530[y]	244	0.01	0.01	0.01	0.01	0.01
	0.75	3,648[v]	3,569	3,885[v]	3,264[z]					0.10	0.02
Neutral detergent fiber	0	1,665[u]	2,210	2,033[w]	2,028[u]	168	0.01	0.01	0.01	0.01	0.01
	0.75	2,413[v]	2,153	2,284[x]	1,735[v]					0.01	0.07
Acid detergent fiber	0	1,114[u]	1,452[w]	1,330	1,253[u]	125	0.43	0.01	0.01	0.27	0.01
	0.75	1,464[v]	1,259[x]	1,304	991[v]					0.01	0.36
Hemicellulose	0	548[u]	759[w]	703[u]	775	92	0.01	0.09	0.01	0.01	0.12
	0.75	951[v]	894[x]	981[v]	744					0.01	0.04
Neutral detergent solubles	0	862[u]	1,408	1,456[y]	1,503	92	0.01	0.01	0.01	0.01	0.01
	0.75	1,237[v]	1,417	1,601[z]	1,529					0.01	0.03
Nitrogen	0	32.1[u]	56.1	59.0[u]	63.0	3.5	0.01	0.01	0.01	0.01	0.01
	0.75	45.5[v]	53.8	67.1[v]	62.4					0.01	0.01
Neutral detergent insoluble N (feed N)	0	13.8[u]	20.9	20.5[y]	23.0	2.4	0.01	0.01	0.01	0.01	0.07
	0.75	21.6[v]	22.1	23.4[z]	21.8					0.74	0.37
Sulfur	0	3.9[u]	6.4[w]	6.7[y]	7.4[w]	0.37	0.17	0.01	0.01	0.01	0.01
	0.75	5.3[v]	5.9[x]	7.2[z]	6.8[x]					0.01	0.03
Fecal N to S ratio	0	8.5	8.8	8.8	8.5	0.19	0.01	0.01	0.34	0.04	0.01
	0.75	8.7	9.2	9.4	9.2						
Starch[d]	0[d]	61[d]	80[d]	83[d]	98[d]	17.2	0.01	0.36	0.83	ND	ND
	0.75	197	211	216	211						

[a]Requirements calculated from NRC (1996) software, Level 1 model.
[b]Corn = main effect for amount of corn; DIP = main effect for amount of degradable intake protein; INT = interaction between corn and DIP; L = linear; Q = quadratic response across amounts of degradable intake protein; ND = Not Determined.
[c]SE = Standard error of the means, n = 8.
[d]Starch was not present in diets without corn, therefore, these values represent simple sugars detected by the starch assay.
[u,v]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.01$) between levels of corn.
[w,x]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.05$) between levels of corn.
[y,z]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.10$) between levels of corn.

Table 6. Output of metabolic fecal nitrogen by beef steers fed prairie hay and supplemented with two levels of corn (0 or 0.75% of BW) and four levels of degradable intake protein (DIP; as a percentage of NRC (1996) requirements) estimated using various methods

	Corn,	Amount of supplemental DIP, % of requirements[a]					Probability Level[b]				
Metabolic Fecal N, g	% of BW	0	33	66	100	SEM[c]	Corn	DIP	INT	L	Q
Neutral detergent soluble N (measured)	0	18.6[w]	35.2	38.5[w]	40.0	2.0	0.08	0.01	0.02	0.01	0.01
	0.75	24.3[x]	31.7	43.7[x]	40.6					0.01	0.01
Regression of N digestion on dietary N	0	32.2[u]	53.8	59.6[w]	63.9	2.4	0.18	0.01	0.01	0.01	0.01
	0.75	44.6[v]	54.7	53.7[x]	63.5					0.01	0.95
1978 NRC equation	0	27.5[u]	39.4	38.0[w]	38.4[y]	2.7	0.01	0.01	0.01	0.01	0.01
	0.75	39.7[v]	38.9	42.3[x]	35.5[z]					0.10	0.02
1984 NRC equation	0	28.6[u]	42.7[w]	44.3[w]	45.6[y]	2.0	0.01	0.01	0.01	0.01	0.01
	0.75	39.7[v]	46.3[x]	48.0[x]	48.1[z]					0.01	0.01
1985 NRC equation	0	38.8[u]	55.3	53.7[w]	54.3	3.4	0.01	0.01	0.01	0.01	0.01
	0.75	55.9[v]	55.6	59.3[x]	51.4					0.16	0.02
1996 NRC software	0	56.4	80.3	81.6	82.4	ND	ND	ND	ND	ND	ND
	0.75	72.3	83.7	85.1	83.3						

[a]Requirements calculated from NRC (1996) software, Level 1 model.

[b]Corn = main effect for amount of corn; DIP = main effect for amount of degradable intake protein; INT = interaction between corn and DIP; L = linear; Q = quadratic response across amounts of degradable intake protein; ND = Not Determined.

[c]SE = Standard error of the means, n = 8.

[u,v]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.01$) between levels of corn.

[w,x]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.05$) between levels of corn.

[y,z]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.10$) between levels of corn.

Table 7. In situ kinetics of prairie hay on diets supplemented with two levels of corn (0 or 0.75% BW) and four levels of degradable intake protein (DIP; as a percentage of NRC (1996) requirements) and fed to beef steers

Item	Corn, % of BW	Amount of supplemental DIP, % of requirements[a]				SEM[c]	Probability Level[b]				
		0	33	66	100		Corn	DIP	INT	L	Q
Kd, %/h	0	2.02[u]	2.94[u]	3.65[u]	3.65[u]	0.26	0.01	0.01	0.01	0.01	0.01
	0.75	2.32[v]	2.12[v]	2.40[v]	2.88[v]					0.01	0.01
Lag time, h	0	11.9[u]	8.0[u]	8.3[u]	6.3[u]	0.78	0.01	0.01	0.01	0.01	0.02
	0.75	13.4[v]	12.7[v]	10.4[v]	10.2[v]					0.01	0.44
A-fraction, % of DM	0	20.0	20.6	22.2[u]	20.2[u]	0.47	0.02	0.01	0.01	0.01	0.01
	0.75	20.3	20.7	20.7[v]	22.3[v]					0.01	0.01
B-fraction, % of DM	0	38.9[u]	51.2[u]	50.0[w]	53.4[u]	0.47	0.01	0.01	0.01	0.01	0.01
	0.75	30.0[v]	45.7[v]	49.5[x]	49.1[v]					0.01	0.01
C-fraction, % of DM	0	41.2[u]	28.2[u]	27.9	26.4[y]	1.31	0.01	0.01	0.01	0.01	0.01
	0.75	49.8[v]	33.7[v]	29.8	28.6[z]					0.01	0.12
Effective ruminal degradability, % of DM	0	38.5	46.7	48.9	46.6	1.54	0.01	0.01	0.27	0.61	0.07
	0.75	33.7	39.2	42.2	42.7						

[a]Requirements calculated from NRC (1996) software, Level 1 model.

[b]Corn = main effect for amount of corn; DIP = main effect for amount of degradable intake protein; INT = interaction between corn and DIP; L = linear; Q = quadratic response across amounts of degradable intake protein.

[c]SE = Standard error of the means, n = 8.

[u,v]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.01$) between levels of corn.

[w,x]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.05$) between levels of corn.

Table 8. Concentrations of undegradable (UIP) or degradable (DIP) intake protein in prairie hay as determined by in situ neutral detergent insoluble nitrogen (NDIN) analysis from prairie hay diets supplemented with two levels of corn (0 or 0.75% BW) and four levels of degradable intake protein (DIP; as a percentage of NRC (1996) requirements) and fed to beef steers

Item	Corn, % of BW	Amount of supplemental DIP, % of requirements[a]				SEM[c]	Probability Level[b]				
		0	33	66	100		Corn	DIP	INT	L	Q
NDIN, Kd, %/h[d]	0	1.09	2.06^{u}	2.00^{w}	2.11^{u}	0.28	0.01	0.04	0.16	0.01	0.07
	0.75	0.83	0.98^{v}	1.31^{x}	0.82^{v}					0.78	0.18
UIP as a % of DM	0	1.35	1.29^{w}	1.39	1.38^{w}	0.14	0.08	0.17	0.13	0.74	0.86
	0.75	1.27	1.63^{x}	1.35	1.77^{x}					0.04	0.82
UIP as a % of CP	0	20.9	20.0^{w}	21.6	21.4^{w}	2.1	0.08	0.17	0.13	0.74	0.86
	0.75	19.7	25.3^{x}	21.0	27.4^{x}					0.04	0.82
DIP as a % of DM	0	5.10	5.15^{w}	5.05	5.07^{w}	0.14	0.08	0.17	0.13	0.74	0.86
	0.75	5.17	4.81^{x}	5.09	4.68^{x}					0.04	0.82
DIP as a % of CP	0	79.1	80.0^{w}	78.4	78.6^{w}	2.1	0.08	0.17	0.13	0.74	0.86
	0.75	80.3	74.7^{x}	79.0	72.6^{x}					0.04	0.82

[a]Requirements calculated from NRC (1996) software, Level 1 model.

[b]Corn = main effect for amount of corn; DIP = main effect for amount of degradable intake protein; INT = interaction between corn and DIP; L = linear; Q = quadratic response across amounts of degradable intake protein; ND = Not Determined.

[c]SE = Standard error of the means, n = 8.

[d]NDIN, Kd = rate of ruminal degradation of forage protein determined from in situ NDIN (Mass et al., 1999).

[w,x]Within each variable of interest and level of DIP, means without a common superscript differ ($P < 0.05$) between levels of corn.

Table 9. Comparison of observed values and 1996 software predictions from prairie hay diets supplemented with two levels of corn (0 or 0.75% BW) and four levels of degradable intake protein (DIP; as a percentage of NRC (1996) requirements) fed to beef steers

	% BW Corn	0				0.75			
Item	% DIP[a]	0	33	66	100	0	33	66	100
Observed forage DM intake, kg		4.16	6.76	7.06	7.29	4.20	5.43	5.72	5.66
Predicted forage DM intake, kg		5.68	5.92	6.04	6.06	4.17	4.17	4.18	4.07
Observed total DM intake, kg		5.37	7.98	8.27	8.54	7.42	8.66	8.96	8.98
Predicted total DM intake, kg		6.89	7.14	7.25	7.31	7.39	7.39	7.42	7.39
Observed digestible OM intake, kg		2.66	4.15	4.56	4.77	3.62	4.85	4.91	5.52
Predicted TDN intake, kg[b]		2.56	4.19	4.51	4.84	4.51	5.29	5.61	5.83
Observed OM digestibility, %		54.1	56.8	59.8	60.7	52.0	60.3	58.9	65.6
Predicted TDN, %[b]		47.6	52.6	54.6	56.6	60.7	61.0	62.6	64.9
Observed rumen pH		7.07	6.94	6.84	6.81	7.04	6.73	6.57	6.50
Predicted rumen pH		6.46	6.46	6.46	6.46	6.46	6.46	6.46	6.46
Observed particulate passage rate, %/h		1.96	3.01	3.31	3.55	2.63	3.10	3.29	3.86
Predicted particulate passage rate, %/h[b]		3.65	4.00	4.03	4.07	3.92	4.08	4.11	4.12
Observed fluid passage rate, %/h		6.1	8.8	8.5	9.8	9.9	9.8	10.4	11.0
Predicted fluid passage rate, %/h[b]		7.62	9.25	9.40	9.56	8.88	9.63	9.78	9.81
Predicted DIP balance, g		-84	31	100	181	-213	-155	-84	-3
Predicted MP balance, g		-25	-33	-30	-26	-187	-203	-199	-197
Observed feed N in feces, g[c]		13.8	20.9	20.5	23.0	21.6	22.1	23.4	21.8
Predicted feed N in feces, g[b]		1.6	2.6	2.9	3.3	3.4	3.9	4.2	4.6
Observed true N digestibility, %[c]		72.4	82.9	86.0	86.8	73.9	81.4	84.0	87.3
Predicted true N digestibility, %[b]		96.7	97.9	98.0	98.1	95.9	96.7	97.1	97.3
Observed total N in feces, g		32.1	56.1	59.0	63.0	45.5	53.8	67.1	62.4
Predicted total N in feces, g[b]		58.0	82.8	84.5	85.8	75.7	87.5	89.3	87.9
Observed apparent N digestibility, %[c]		32.2	53.8	59.6	63.9	44.6	54.6	53.7	63.5
Predicted apparent N digestibility, %[b]		-20.8	31.6	42.1	51.0	8.8	25.9	38.0	48.6

[a]Requirements calculated from NRC (1996) software, Level 1 model.

[b]Calculated from Level 2 model software, NRC (1996).

[c]Estimated from neutral detergent insoluble nitrogen output.

Figure 1. Ruminal rate of ADF digestion plotted against increasing ruminal NH_3-N concentrations by beef steers fed prairie hay and supplemented with two levels of corn (0 or 0.75% of BW) and four levels of degradable intake protein (DIP; as a percentage of NRC (1996) requirements). Level of DIP fed in the supplement is shown on the figure next to each data point, along with ruminal ammonia-N concentration and rate of ADF digestion, respectively listed as (x,y) coordinates.

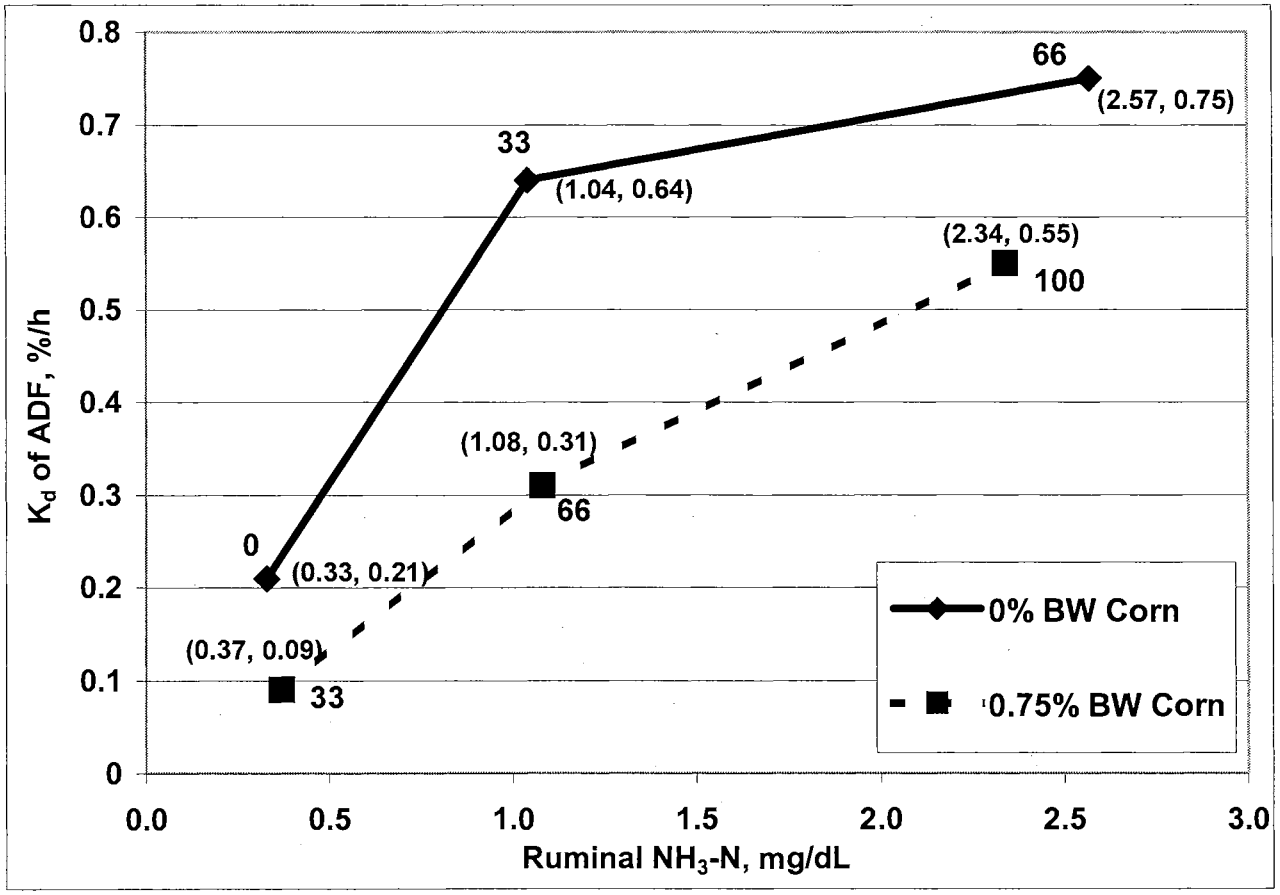
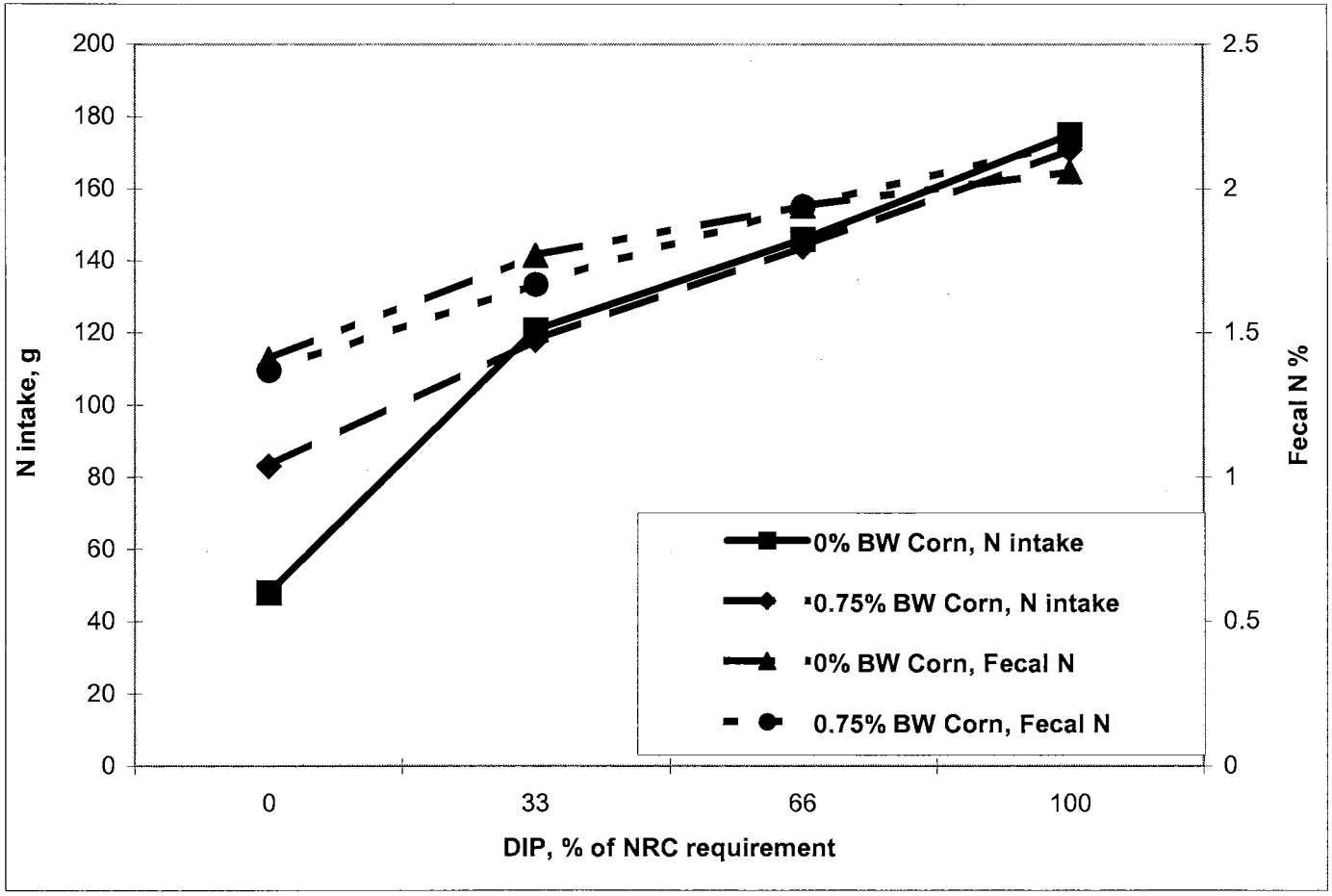


Figure 2. Fecal nitrogen concentration (% of OM) and total dietary nitrogen intake by beef steers fed prairie hay and supplemented with two levels of corn (0 or 0.75% of BW) and four levels of degradable intake protein (DIP; as a percentage of NRC (1996) requirements).



Chapter V

Effects of corn and(or) soybean meal supplements fed to steers consuming low-quality prairie hay on intake, ruminal measures, and ruminal microbial ecology[1,2]

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Abstract: To evaluate the effects of corn and/or soybean meal supplements on ruminal microbial ecology, we designed a study utilizing sixteen ruminally cannulated steers (586 ± 54 kg) with two periods. In the 10-d pre-treatment period, steers had ad libitum access to chopped prairie hay. During the 14-d treatment period, steers were fed prairie hay and adapted to, and then fed, supplemental treatments. Treatments were: **1)** no supplemental feed (**NEG**); **2)** soybean meal, **SOY**; **3)** cracked corn, **CORN**; or **4)** corn and soybean meal, **CRNSOY**. Steers consumed 0, 2.0, 5.3, or 7.1 g of DM/kg of BW, respectively, of supplement daily. Ruminal samples were collected at three times from each steer on the final day of both periods, and genomic DNA was extracted from whole ruminal contents. A portion of the bacterial 16S rDNA gene was amplified using PCR to assess changes in ruminal microbial ecology using terminal restriction fragment length polymorphism analysis and DNA sequencing of a clone library. Feeding supplemental protein increased ($P < 0.01$) hay DM intake and total fiber intake, whereas feeding corn tended to decrease ($P < 0.05$) hay intake. Supplemental protein increased ($P < 0.05$) ruminal ammonia-N and total VFA, whereas all supplements decreased ($P < 0.01$) ruminal pH. Greater ($P < 0.01$) amounts of DNA were extracted from ruminal contents when protein was supplemented. Although amount of DNA varied, estimates of bacterial diversity did not differ ($P > 0.55$) among treatments. Principal components analysis, an indirect gradient

analysis technique explained only 14% of the variation in the data set along the first axis. Analysis of the ruminal bacterial community by redundancy analysis, a direct gradient analysis method, revealed temporal shifts in the ruminal bacterial community due to supplementation ($P < 0.01$) that were supported by hierarchical cluster analysis. Feeding corn or corn plus soybean meal shifted bacterial communities in different directions, whereas soybean meal supplementation did not greatly change bacterial communities. There were clear relationships revealed between several measured ruminal variables, groups of restriction fragments, and experimental treatments. The large number of peaks discovered, and the few known matches indicate that there is considerable bacterial species diversity in the rumen, and much of it may be from previously unknown species. It also appears that shifts in ruminal bacterial species composition play a major role in negative associative effects.
Key Words: Beef Cattle, Intake, Microbial Ecology, Rumen, Species Composition, Supplementation

Introduction

Reviews of the effects of energy (Horn and McCollum, 1987; Bowman and Sanson, 1996; Caton and Dhuyvetter, 1997) or protein supplementation (McCollum and Horn, 1990; Owens et al., 1991) of forage diets have suggested many explanations for associative effects. Ruminal ammonia levels (Chase, Jr. and Hibberd, 1987), competition for substrates (el-Shazly et al., 1961), ruminal pH (Russell and Wilson, 1996), kinetics (Mertens and Loften, 1980), a "carbohydrate effect" (Hoover, 1986), changes in microbial species numbers and functions (White et al., 1999), protein to energy ratios (Egan and Moir, 1965), as well as a variety of other factors. In previous studies (Bodine

et al., 2000; Bodine et al., 2001; Bodine and Purvis, II, 2003) we have shown that supplying additional degradable intake protein can eliminate the reduction in total tract fiber digestion observed when rapidly fermentable carbohydrate sources are supplemented with protein-deficient forage. However, in Chapter 4, we reported that achieving similar ruminal ammonia levels did not result in similar ruminal fiber digestion in corn-supplemented and unsupplemented steers. Decreased ruminal fiber digestion with similar ruminal ammonia, pH, and kinetics, may be due to treatments altering the bacterial community structure and(or) function. It has been postulated that different microbial species will predominate when the carbon source is predominantly cellulose vs. starch (Wells and Russell, 1996), in a manner that is dependent on ruminal ammonia availability (Russell, 1998). Yet, extensive studies of the ruminal microbial ecosystems response to protein and carbohydrates have not been previously performed. The objectives of this study were to determine the changes in ruminal bacterial species composition when prairie hay was supplemented with protein, starch, or the combination.

Materials and Methods

Animals, Housing, Care, and Prior Treatments. All protocols were approved by the Oklahoma State University Institutional Animal Care and Use Committee. At trial initiation, sixteen ruminally cannulated (i.d. 10 cm) steers (586 ± 54 kg; Angus and Angus × Hereford) were weighed, randomly assigned to treatments, housed in individual indoor 3 × 4 m pens with slatted floors, and provided with ad libitum access to fresh water. Steers had been on three different studies previously, using either low-quality forage, high concentrate feedlot rations, or grazing wheat pasture. Following these studies, all steers were placed on a diet of prairie hay and a soybean meal-based protein

supplement with monensin for a minimum of two weeks. Then a protein supplement without monensin was fed for two weeks. One week before the start of the first period, all steers were given ad libitum access to prairie hay without any protein supplementation.

Treatments, Experimental Periods, and Adaptation to Treatments. Prairie hay from a single source with primary species composition of big bluestem (*Andropogon gerardii*), little bluestem (*Schizachyrium scoparium*), indiangrass (*Sorghastrum nutans*) and switchgrass (*Panicum virgatum*) was fed with ad libitum access by providing each steer with the greater of either 7 kg of hay/d or 2.27 kg more hay than had disappeared the previous day.

Treatments were one of four supplements fed to steers with ad libitum access to chopped prairie hay. Supplements consisted of: 1) no supplement, NEG; 2) soybean meal, SOY; 3) cracked corn, CORN; or 4) corn and soybean meal, CRNSOY. Each day, steers consumed 0, 2.0, 5.3, or 7.1 g of DM/kg of BW, respectively, of each supplement. All steers were fed 85 g of a trace mineralized salt mixture daily. Treatments were fed at 0800, prior to the daily offering of hay. Amounts of hay and supplement offered and hay refused were weighed and disappearance of hay was recorded daily as the difference between amount offered and refused. Steers were given approximately one hour to consume supplements. Supplement refusals were dosed via ruminal cannula. Chemical composition of forage and supplements as well as supplement ingredient DM intake are shown in Table 1. The trial consisted of two experimental periods. During the pre-treatment period (May 12-21; d 1-10), steers had ad libitum access to chopped prairie hay for 10 d, before the initiation of supplementation

treatments. The treatment period involved adapting steers to supplements over 4 d (May 22-26; d 11-14). This was accomplished by feeding 50% of the corn and all of the soybean meal at 0800 on d 1, 50% of the corn and all of the soybean meal at 0800 and 25% of the corn at 1600 on d 2, 50% of the corn and all of the soybean meal at 0800 and 50% of the corn at 1600 on d 3, and 75% of the corn and all of the soybean meal at 0800 and 25% of the corn at 1600 on d 4. The remainder of the treatment period (May 27-June 4; d 15-24) involved feeding the full amount of all supplements for 10 d.

Sample Collection. Feed samples (~ 454 g) were collected daily; prairie hay samples were composited across days for the 10-d pre-treatment period and prairie hay and supplement (corn and soybean meal) samples were composited across days for the 14-d supplement treatment period. All feed samples were ground to pass a 2-mm screen in a Wiley mill (Thomas Scientific, Philadelphia, PA) for determination of dry matter, organic matter, neutral detergent fiber, acid detergent fiber, carbon, and nitrogen.

Grab samples of ruminal contents were collected from steers on the last day of the pre-treatment (d 10) and treatment (d 24) periods at three times (0800, 1600, 2400). At each sampling time, ruminal contents were taken from the cranial, medial, and caudal regions (400 mL from each site) of the ruminal mat, mixed, and divided into two equal portions (600 mL each). One portion was frozen (-20°C) and reserved for compositing within steer and period. The other was strained through eight layers of cheesecloth and pH was measured using a portable, combination electrode pH meter (Corning 314i pH/mV/temperature portable pH meter with an ion selective field effect transistor electrode, Corning, NY). Following pH measurement at each sampling time, 100 mL of

ruminal fluid was acidified with 1 mL of 7.2 N H_2SO_4, 5 mL was placed into a 15 mL centrifuge tube, and stored frozen (-20°C) for compositing within steer and period.

Nutrition Laboratory Analyses. Dry matter and ash content of supplement, prairie hay, and ruminal content samples were determined by oven drying at 105°C for 24 h, followed by ashing at 500°C for 6 h in a muffle furnace, respectively. A combustion method (Leco NS2000, St. Joseph, MI; AOAC, 1996) was utilized to determine nitrogen and carbon content of supplement and prairie hay. Neutral detergent fiber (procedure A, without sodium sulfite) and acid detergent fiber concentrations of supplement and prairie hay were determined as described by Van Soest et al. (1991). Ruminal fluid samples were thawed, centrifuged (10,000 × g; 10 min), and subsampled for ammonia and volatile fatty acid determination. Subsamples for ammonia and volatile fatty acid analysis were composited across time within steer and period. Ruminal ammonia concentration was determined colorimetrically using the phenol-hypochlorite procedure (Broderick and Kang, 1980). Ruminal volatile fatty acid concentrations were determined by deproteinizing 5 mL of ruminal fluid with 1 mL of 25% metaphosphoric acid (Erwin et al., 1961) and centrifuging at 20,000 × g for 15 min. Individual VFA were separated by gas chromatography (Perkin Elmer Autosystem, 9000 series, Norwalk, CT) with 8 mL/min flow rate of ultra-high purity helium as a carrier gas and 2-ethylbutyric acid as an internal standard.

Molecular Laboratory Analyses

DNA Extraction Methods. Frozen ruminal contents (1.2 L) were thawed at 4°C, mixed with 400 mL of cold (4°C) ddH_2O, and homogenized in a Waring blender (Waring Laboratory Science, Torrington, CT) at maximum speed for two minutes total with a one-

minute period separating each blending period to reduce heat build-up. Homogeneized samples were aliquoted into individual sterile 1.5 mL or 2.0 mL micro-centrifuge tubes for extraction of DNA and into individual sterile 50 mL centrifuge tubes for further analyses, while the remainder was reserved and all samples were stored frozen (-20°C).

Nucleic acids were extracted from ruminal contents using two methods. The first method used a commercial kit (QIAamp DNA stool Mini Kit, Qiagen Inc., Valencia,CA) according to the manufacturer's protocol, with the following modifications; an initial 5-min incubation at 95°C and 0.35 g (wet weight) of ruminal contents was used. In brief, the procedure involved incubation at 95°C in a lysis buffer, binding of inhibitors with a pellet, followed by a proteinase K incubation with lysis buffer at 70°C. Nucleic acids were then captured in a spin-column, washed, and eluted in buffer. The second method was similar to the first, with the following modifications. One gram of pooled (by treatment) ruminal contents was added to 0.5 g of 0.1 mm zirconium beads, the tubes were filled with Qiagen lysis buffer (from the DNA stool Mini kit), subjected to two one-min cycles separated by a one-min pause on a Mini-BeadBeater (Bio-Spec Products Inc., Bartlesville, OK) at maximum speed. Following that, the extraction procedure followed the Qiagen protocol.

Size and purity of genomic DNA extracts were determined by agarose gel electrophoresis (1.0% agarose, 1X TBE, and 0.5 μg/mL ethidium bromide) followed by visualization under UV transillumination and documentation with a GDAS-1200 (UVP BioImaging Systems, Upland, CA) system. Concentration and purity of genomic DNA extracts were determined by UV absorbance at 260 and 280 nm. Genomic DNA extracts were adjusted to concentrations of 100 ng/μL and 25 ng/μL and stored frozen (-20°C).

All dilutions, reagents, and PCR reaction mixtures utilized destilled deionized water (**ddH_2O**), that had been autoclaved and treated with UV light (365 nm) for at least 30 min to minimize background bacterial DNA contamination.

Evaluation of Extraction Protocol. Subsamples of DNA extracted by each method from ruminal contents collected from steers during the treatment period were pooled within treatment and compared. Comparison of ruminal microbial communities between the two extraction protocols involved two fingerprinting techniques utilizing PCR amplified sequences of the bacterial 16S rRNA gene (16S rDNA). The first technique utilized temporal temperature gradient gel electrophoresis (TTGE). The TTGE procedure allows separation of similar size PCR products in a polyacrylamide gel based on sequence-dependent melting behavior, which is similar to denaturing gradient gel electrophoresis (DGGE; Muyzer et al., 1993). Gradient gel electrophoresis techniques (DGGE, TGGE, TTGE) separate similar-sized fragments based on the melting behavior of the PCR product, which is affected by differences in the internal sequence information of the fragment, as opposed to size-dependent traditional electrophoresis. Pooled genomic DNA extracted from rumen samples were used in PCR amplification with the forward primer GCBacT0341F (5'– CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGGCCTACGGGAGGCAGCAG –3') which has a 40 bp GC clamp, and corresponds to bases 341-357 in the *E. coli* 16S rRNA numbering system (Brosius et al., 1978), the reverse primer BacT0534R (5' – ATTACCGCGGCTGCTGG – 3'; bases 518-534 in *E. coli*), and results in a 233 bp PCR product that includes the variable V3 region of *Eubacterial* microbes (Muyzer et al., 1993). Reaction mixtures included 0.1 μg/μL bovine serum albumin, 1X PCR buffer (Promega, Madison, WI), 2.0 mM $MgCl_2$, 200

μM of each dNTP, 500 nM of each primer, 1.25 U of *Taq* polymerase, and 100 ng of genomic DNA extract brought to a total volume of 50 μL with ddH_2O. Amplification conditions were initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min, followed by 5 min at 72°C. Amplicons were evaluated by agarose gel electrophoresis (2% agarose, 1X TBE, and 0.5 μg/mL ethidium bromide). TTGE was performed using a DCode system (Bio-Rad Laboratories, Inc., Hercules, CA) at 130V with a temperature range from 48 to 54°C for 5h, resulting in a temperature ramp rate of 1.2°C/h. Fifteen μL of PCR product and 5 μL of loading dye was applied to a 7% (w/v) polyacrylamide gel (acrylamide:N,N'-methylene bisacrylamide, 37.5:1 [w/w], 7M urea, 20% formamide, 1.25X TAE). After completion of electrophoresis, the gel was stained with ethidium bromide (0.5 μg/mL) in 1.25X TAE for 20 min, destained in 1.25X TAE for 10 min, and visualized and documented under UV transillumination.

Ruminal microbial communities were also compared between the two extraction protocols using terminal restriction fragment length polymorphism (**T-RFLP**) analysis. This fingerprinting technique measures the length of the terminal restriction fragment polymorphism of a PCR amplified marker, in this case the 16S rRNA gene, created with a primer fluorescently labeled at the 5' end (Marsh, 1999). In this technique, PCR products are digested with a restriction enzyme, the fragments are separated using a high-resolution polyacrylamide sequencing gel, and the terminal fragments (fluorescently labeled) are detected using laser-induced fluorescence with an automated DNA sequencer. Size estimates are highly accurate due to the use of a fluorescently labeled internal size standard in each lane. The second PCR reaction was performed using the

forward primer FAMBacT0008F (5'– 6-FAM-AGAGTTTGATCCTGGCTCAG –3'; bases 8-27 in *E. coli*), which was 5' end labeled with phosphoramidite fluorochrome 5-carboxyfluorescein (5' 6-FAM) and the reverse primer BacT1510R (5' – GGTTACCTT GTTACGACTT – 3'; bases 1492-1510 in *E. coli*), and resulted in a 1503 bp PCR product that includes the majority of the *Eubacteria* 16S rRNA gene. Reaction mixtures included 0.1 μg/μL bovine serum albumin, 1.25% DMSO, 1X PCR buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 200 μM dNTP, 100 nM of each primer, 1.5 U of *Taq* polymerase, and 25 ng of genomic DNA extract brought to a total volume of 25 μL with ddH_2O. Amplification conditions were initial denaturation at 95°C for 5 min followed by 36 cycles of 95°C for 30 sec, 48°C for 30 sec, and 72°C for 90 sec, followed by 5 min at 72°C. PCR products were evaluated by electrophoresis in an agarose gel (1.2% agarose, 1X TBE, and 0.5 μg/mL ethidium bromide) followed by visualization and documentation under UV transillumination.

PCR products were purified using Microcon YM-100 centrifugal filters (Millipore Corp., Bedford, MA) and 5 μL of purified PCR product was digested with 15 μL of a master mix containing 2.5 U of Rsa I (Invitrogen Life Technologies, Carlsbad, CA), 1X buffer, ddH_2O, and 0.1 μg/μL of BSA at 37°C for 3 h and 65°C for 25 min. Three μL of digested PCR product was mixed with 0.5 μL of Genescan 1000 ROX size standard (Applied Biosystems Instruments, Foster City, CA), 0.5 μL of loading dye, and 2.5 μL of deionized formamide, the mixture was heated to 95°C for 5 min, and placed on ice. Restriction enzyme digested PCR products were separated by size on an ABI 377 automated sequencer using 6% polyacrylamide (Long Ranger, Applied Biosystems Instruments, Foster City, CA) 8.25M urea, and 1X TBE, and run at 3 kV for 8 h. The 5'

terminal fragments were detected at 520 nm by excitation of the 6-FAM molecule attached to the forward primer. The gel image was captured using Genescan 3.1 analysis software.

Terminal Restriction Fragment Length Polymorphism. To determine which restriction enzymes revealed the greatest amount of bacterial species diversity, four restriction enzyme digestions were compared. Genomic DNA extracted from rumen samples taken from three steers before and after the supplement treatments were fed, *E. coli* cells, and a ddH_2O negative control were amplified using PCR with the FamBacT0008F and BacT1510R primer set using the previously reported conditions. Amplicons were purified using Microcon YM-50 centrifugal filters (Millipore Corp., Bedford, MA) and 5 μL of purified PCR product was digested with 5 μL of a master mix containing 2.5 U of one of the restriction enzymes Hae III, Hha I, Msp I, or Rsa I, 1X of the supplied buffer for that enzyme, ddH_2O, and 0.1 μg/μL of BSA for Hha I and Msp I (Promega, Madison, WI), and without BSA for Hae III (Gibco/BRL, Carlsbad, CA) and Rsa I (Invitrogen Life Technologies, Carlsbad, CA), at 37°C for 3 h and at 65°C for 25 min. 2.5 μL of digested PCR product was mixed with 0.6 μL of Genescan 1000 ROX size standard (Applied Biosystems Instruments, Foster City, CA), 0.6 μL of loading dye, and 2.5 μL of deionized formamide, the mixture was heated to 95°C for 5 min, and placed on ice. Restriction enzyme digested PCR products were separated by size on an ABI 373 automated sequencer using 6% polyacrylamide (Long Ranger, Applied Biosystems Instruments, Foster City, CA), 8.25M urea, 1X TBE, and run at 30W for 16 h. The 5' terminal fragments were detected at 540 nm by excitation of the 6-FAM

molecule attached to the forward primer. The gel image was captured using Genescan 2.1 analysis software.

To compare the response of the ruminal microbial community to experimental treatments, genomic DNA extracted from rumen samples along with *E. coli* cells and a ddH_2O negative control were amplified using PCR with the FamBacT0008F forward primer and the reverse primer BacT0805R (5' – GGACTACCAGGGTATCTAATCC – 3'; bases 784 - 805 in *E. coli*), and results in a 798 bp PCR product. This fragment was chosen for T-RFLP analysis because it represented the portion of the 16S rDNA molecule that was used to create a random clone library of 16S sequences that were subsequently subjected to DNA sequence analysis. Reaction mixtures included 0.1 μg/μL bovine serum albumin, 1X PCR buffer (Promega, Madison, WI), 2.0 mM $MgCl_2$, 200 μM dNTP, 300 nM of each primer, 2.5 U of *Taq* polymerase, and 10 ng of genomic DNA extract brought to a total volume of 50 μL with ddH_2O. Prior to the addition of dNTP's and primers, the master mix was exposed to UV light (365 nm) for 30 min to minimize trace amounts of nucleic acids present in the DNA polymerase. Amplification conditions were initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 60 sec, followed by 7 min at 72°C.

Amplicons were purified via electrophoresis in 1.0% low-melting point agarose in 1X TBE, bands of the appropriate size were excised, and PCR products were purified by phenol-chloroform extraction and ethanol precipitation. Five μL of purified PCR product was digested with 5 μL of a master mix containing 2.5 U of either restriction enzymes Hae III or Rsa I, 1X of the supplied buffer for that enzyme, and ddH_2O, at 37°C for 3 h and 65°C for 25 min. 1.5 μL of digested PCR product was mixed with 0.55 μL of

Genescan 1000 ROX size standard (Applied Biosystems Instruments, Foster City, CA), 0.45 μL of the provided 2X agarose loading buffer, 0.5 μL of formamide loading dye (Amersham Pharmacia Biotech Inc.), and 2.0 μL of deionized formamide, and the mixture was heated to 95°C for 5 min and placed on ice. Restriction enzyme digested PCR products (4 μL/well) were separated by size on an ABI 373 automated sequencer using 6% polyacrylamide (Long Ranger, Applied Biosystems Instruments, Foster City, CA), 8.25M urea, 1X TBE, and run at 30W for 16 h. The 5' terminal fragments were detected at 540 nm by excitation of the 6-FAM molecule attached to the forward primer. The gel image was captured using Genescan 2.1 analysis software.

16S rDNA fragments for cloning were created using the BacT0008F forward primer and the BacT0805R reverse primer set. Reaction mixtures included 0.1 μg/μL bovine serum albumin, 1X PCR buffer (containing 2.0 mM $MgCl_2$), 200 μM dNTP, 300 nM of each primer, 2.5 U of *Pfu* polymerase (Stratagene, La Jolla, CA), and 10 ng of genomic DNA extract brought to a total volume of 50 μL with ddH_2O. Amplification conditions were initial denaturation at 95°C for 5 min followed by 34 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 105 sec, followed by 10 min at 72°C. PCR products were purified via electrophoresis in 1.0% low-melting point agarose in 1X TBE, bands of the appropriate size were excised, and PCR products were purified by phenol-chloroform extraction and ethanol precipitation. Purified PCR products were ligated into pCR-Blunt vector and transformed into TOP10 competent cells according to the manufacturers protocols (Zero Blunt PCR Cloning Kit, Invitrogen Life Technologies, Carlsbad, CA). Transformed cells were grown on plates of YT agar medium containing kanamycin, colonies were picked into 96-well u-bottom culture plates containing 150 μL/well of YT

broth with kanamycin and 8% glycerol and grown overnight at 37°C. Five μL of overnight growth was added to 95 μL of ddH_2O in 96-well PCR plates, and cells were lysed using one cycle of 96°C for 6 min on a thermocycler. One μL was used for PCR with M13 forward and reverse primers. Reaction mixtures included 1X PCR buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 200 μM dNTP, 500 nM of each primer, 1.5 U of *Taq* polymerase, brought to a total volume of 25 μL with ddH_2O. Amplification conditions were 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, followed by 5 min at 72°C. PCR products were evaluated by electrophoresis in an agarose gel (1.2% agarose, 1X TBE, and 0.5 μg/mL ethidium bromide) followed by visualization and documentation under UV transillumination. Clones with inserts of the expected size were then submitted for DNA sequencing. Clones were submitted to the Advanced Center for Genome Technology (ACGT) for sequence analysis.

Calculations. Intakes of selected dietary components were calculated as DMI multiplied by the DM concentration of that component as determined from lab analyses. Ruminal ammonia-N values were calculated by multiplying ammonia concentrations by 0.8225 (to adjust ammonia to ammonia-N). Concentrations of individual VFA were expressed as a proportion of total VFA by dividing each VFA by the sum of all measured VFA. Amount of DNA recovered from extracted ruminal samples was corrected for amount of starting DM and expressed as μg of DNA/g of DM. To determine the changes in variables between the pre-treatment and the treatment periods, mean values for each steer in the treatment period were subtracted from mean values for that steer from the pre-treatment period. Bacterial species richness was calculated as the number of unique

restriction fragment peaks from each lane. Bacterial species diversity was estimated using the Shannon-Weaver index, which was calculated as $-\sum(p_i)(\ln p_i)$, where the summation is within each lane, where each unique restriction fragment i is expressed as p_i, which is relative to the sum of all fragments. Species evenness was calculated as the Shannon-Weaver index divided by the natural log of species richness.

Experimental Design and Statistical Analyses. Experimental design was a completely randomized design. Supplemental dietary treatment was included in the model as a fixed effect (Littell et al., 1996). We analyzed response variables for intake and ruminal measures using PROC MIXED (SAS Inst. Inc., Cary, NC), calculated means using least squares means, and separated the means using least significant differences methods only when the overall F-value was < 0.05. Means for differences between pre-treatment and treatment variables were analyzed to determine if they differed from zero using Student's T-test. To evaluate species richness, diversity, and evenness measures among replicate gel lanes we used a model with treatment, replicate gel lane, and their interaction as fixed effects and analyzed measures determined from each enzyme separately. Least squares means for each steer, period, and enzyme were then calculated and species richness, diversity, and evenness means were analyzed for effect of treatment similarly to intake and ruminal measures. To determine treatment effects on pH, a repeated measures in time analysis was tested using an analysis of covariance method. Treatment, sampling time, and their interaction were included in the model along with the interaction of pre-treatment pH values and treatment, which was included as a covariate. Kenward-Roger degrees of freedom approximation techniques were used. Steer × period combinations defined the subjects on which the repeated measures were taken and the

covariance structure was modeled using variance components. Because the covariate term was non-significant, treatment effects on pH values were analyzed using a model with treatment, sampling time, and their interaction as fixed effects.

Terminal Restriction Fragment Length Polymorphism Analysis. The Genescan 2.1 analysis software was set to eliminate all peaks with a peak height less than 50 relative fluorescent units (**RFU**). Peaks detected by the ABI 373 were removed from the data set if the predicted size was less than 50 bp (to eliminate noise at the electrophoretic front), or if they did not have an assigned size (in bp). Peaks were then manually aligned, and terminal restriction fragment peaks (**T-RF**) with only one occurrence, and cumulative peak height of that occurrence of a T-RF of less than 100 RFU were removed from the data set. The aligned data set was standardized by dividing by the cumulative peak heights of each sample by the sample with the lowest cumulative peak height. The data set was subsequently converted to relative peak height by expressing each peak height in each sample as a proportion of the standardized cumulative peak height of that sample. Statistical methods of comparing the relationships between T-RFLP profiles used included hierarchical clustering with the unweighted-pair group method using arithmetic averages (UPGMA; Jobson, 1992). The cophenetic correlation was calculated for dendrograms using a SAS PROC IML algorithm. The SAS PROC IML code was obtained from Dr. Chris Blackwood, USDA-ARS Sustainable Agriculture Systems Lab, incorporating the conclusions reported in Blackwood et al. (2003). Evaluation of clustering errors was performed using dendrograms showing the hierarchical relationships between T-RFLP profiles determined from the clustering procedure. An error was counted when replicates of the same sample were clustered into different

groups. Data sets that had been standardized and adjusted to relative peak height were exported and used for redundancy analysis.

Bacterial Species Community Analysis using Canoco. Detrended correspondence analysis was performed to evaluate the length of the longest gradient to determine if a linear or unimodal model was appropriate (ter Braak and Šmilauer, 1998) using Canoco for Windows (Microcomputer Power, Ithaca, NY). To evaluate the bacterial community species composition for the presence of an environmental gradient, principal components analysis (**PCA**; Gauch, Jr., 1982), an indirect gradient analysis technique was utilized. We used redundancy analysis (**RDA**; ter Braak and Šmilauer, 1998; Legendre and Legendre, 1998), a direct gradient analysis method, to analyze variation between replicate gel lanes, due to steer, due to the environmental variables of intake and ruminal measures, and due to experimental treatments over time. Indirect gradient analysis is a technique utilized where the species composition data is used to infer an environmental gradient when the gradient is unknown *a priori*. Direct gradient analysis is a method used when environmental gradients are known and measured, and the variables are displayed as arrows representing the direction of maximum correlation. To determine change in species composition as a function of experimental treatment, variables were coded for each treatment as categorical (dummy, 0 or 1) variables and analyzed the study as a replicated BACI (Before, After, Control, Impact) design with a factorial treatment in time arrangement using the data collected from the pre-treatment and treatment sampling periods (ter Braak and Šmilauer, 1998). Sampling period (time) was included as a continuous variable to allow use of the interaction between the selected treatment variables and time as explanatory variables and time and steers as covariables. The

resulting temporal trajectory in species composition in response to experimental treatments was then plotted using CanoDraw and images were imported into CanoPost 1.0 for presentation. The number of T-RF plotted on the ordination diagram was restricted by only plotting those T-RF that explained an amount of the variation greater than or equal to the first axis (ter Braak and Šmilauer, 1998). Monte Carlo permutation tests of significance (499) for all canonical axes were utilized to assess the statistical significance of changes in the ruminal bacterial community due to treatment over time (ter Braak and Šmilauer, 1998).

Results and Discussion

Intakes. Intake of hay DM (Table 2) was similar ($P > 0.65$) during the pre-treatment period. Intake of forage by NEG steers was similar ($P > 0.38$) between periods. Feeding supplements with added degradable intake protein (SOY, CRNSOY) increased ($P < 0.001$) intake of forage DM by steers from the pre-treatment to the treatment period. Steers supplemented with corn alone had decreased ($P < 0.05$) forage DMI during the treatment period compared with their pre-treatment forage intake. During the treatment period, steers fed either supplement with soybean meal had similar ($P > 0.84$) hay DMI, as did steers fed CORN and unsupplemented steers ($P > 0.25$). Steers receiving either supplement with added soybean meal tended to have greater ($P = 0.06$) forage DMI than unsupplemented or CORN supplemented steers. Total DMI was greater ($P < 0.001$) for all supplemented diets during the treatment period than pre-treatment values. When steers were fed treatments, supplementing with corn alone resulted in a similar ($P > 0.46$) total DMI as unsupplemented steers. Steers fed either supplement with soybean meal tended to have greater ($P = 0.07$) total DMI than steers fed CORN or those not

consuming any supplement. When CRNSOY was fed, steers tended to have greater ($P < 0.06$) total DM intake than steers supplemented with soybean meal. Intake of carbon was similar ($P > 0.65$) among supplements during the pre-treatment period, similar ($P > 0.35$) for unsupplemented steers between pre-treatment and treatment periods, and increased ($P < 0.001$) for all supplement treatments from the pre-treatment to treatment periods. During the treatment period, unsupplemented steers and CORN-fed cattle had similar ($P > 0.45$) intakes of carbon. Feeding supplemental soybean meal by itself increased ($P < 0.02$) carbon intake compared to unsupplemented steers and tended to be greater ($P < 0.08$) than for CORN-supplemented cattle. Supplementing steers with corn and soybean meal resulted in greater ($P < 0.002$) carbon intake than unsupplemented and CORN steers, and tended to result in greater ($P < 0.06$) intakes of carbon than animals supplemented with soybean meal alone. Intake of fiber (ADF, NDF, and hemicellulose) was similar ($P > 0.66$) for all treatments during pre-treatment. Fiber intakes for unsupplemented and CORN-supplemented steers were similar ($P > 0.28$) between pre- and treatment periods. Feeding either supplement with soybean meal increased ($P < 0.001$) fiber intake from the pre-treatment to treatment periods. When steers were being supplemented, unsupplemented cattle and steers fed CORN had similar ($P > 0.39$) fiber intakes. Steers receiving either supplement containing soybean meal had similar ($P > 0.51$) intakes of fiber. Feeding supplemental soybean meal alone increased ($P < 0.02$) fiber intake vs. CORN-fed cattle, and tended to increase ($P < 0.06$) fiber intake compared to unsupplemented steers. Supplementing animals with corn and soybean meal resulted in greater ($P < 0.02$) fiber intakes than CORN-fed steers and greater ($P < 0.004$) fiber intakes than unsupplemented steers. Before the initiation of supplement feeding, all

steers had similar ($P > 0.65$) intakes of neutral detergent solubles, and unsupplemented cattle maintained a similar ($P > 0.69$) intake between experimental periods. Supplementation increased ($P < 0.001$) intakes of neutral detergent solubles from the pre-treatment to treatment periods. During the treatment period, supplementing with soybean meal alone, with corn alone, or with the combination of corn and soybean meal, increased intakes of neutral detergent solubles compared to unsupplemented steers. Feeding supplements with either only soybean meal or only corn resulted in similar ($P > 0.14$) intakes of neutral detergent solubles, whereas supplementing with the combination increased ($P < 0.001$) intake compared to either one alone. Feeding protein increased forage and fiber intake, feeding corn decreased forage and fiber intake, and feeding both corn and soybean meal increased intake of forage and fiber. This increased forage and fiber intake resulted in greater total DM intake. This agrees with our previously published reports (Bodine et al., 2000; Bodine et al., 2001; Bodine and Purvis, II, 2003). We attribute this increase to the greater intake of protein, specifically degradable intake protein for the diets fed with soybean meal. The greater intake of neutral detergent solubles for the supplements fed with corn would be expected from experimental design. However, the similarity of neutral detergent solubles intake between SOY and CORN supports the large increase in forage intake caused by protein supplementation and the replacement of neutral detergent solubles intake from forage with that coming from corn.

Prior to supplements being fed, intake of nitrogen, crude protein and degradable intake protein were similar ($P > 0.65$) for all treatments. Intake of nitrogen, CP, and DIP was similar ($P > 0.13$) for unsupplemented steers between pre-treatment and treatment periods. However, all supplemented steers had greater ($P < 0.0001$) nitrogen, CP, and

DIP intakes during the supplemental feeding period vs. their respective pre-treatment intakes. Supplemental feeding increased ($P < 0.03$) CP intake vs. unsupplemented steers, SOY steers had greater ($P < 0.001$) CP intake than CORN or NEG cattle, and were lesser ($P < 0.01$) than CRNSOY steers, which had the greatest CP intake. Unsupplemented steers and animals consuming supplemental corn alone had similar ($P > 0.23$) intakes of DIP. When soybean meal was fed, SOY and CRNSOY steers had greater ($P < 0.001$) DIP intakes than either unsupplemented or CORN cattle, with CRNSOY steers consuming more ($P = 0.05$) DIP than animals supplemented with only soybean meal. This would be expected due to experimental design and the amounts of protein contained in the supplements.

Ruminal Measures. During pre-treatment, ruminal pH measures (Table 3) did not exhibit an interaction ($P > 0.83$) between treatment and time, were similar ($P > 0.83$) among treatments, but did have a tendency ($P < 0.07$) to differ among sampling times. Supplement feeding did not result in an interaction ($P > 0.63$) between treatment and sampling time, however, ruminal pH differed ($P < 0.001$) among treatments, and had a tendency to differ ($P < 0.09$) among sampling times. Unsupplemented steers had similar ($P > 0.77$) ruminal pH between pre-treatment and treatment periods, whereas supplementation decreased ($P < 0.001$) ruminal pH from the pre-treatment to treatment periods. During supplemental feeding, all supplements had decreased ($P < 0.01$) pH compared to unsupplemented steers. Feeding either supplement with soybean meal resulted in a similar ($P >0.12$) ruminal pH. Feeding supplements with either corn or soybean meal only also resulted in similar ($P > 0.23$) pH values. When soybean meal was added to corn-based supplements, observed ruminal pH values decreased ($P < 0.01$)

vs. CORN-supplemented steers. In Chapter 4 we reported decreased fiber digestion in the rumen caused by feeding corn, and yet again, we show that it cannot be linked to ruminal pH (Bodine et al., 2000; Bodine et al., 2001), which was similar across all three treatments fed in this study. el-Shazly et al. (1961) reported inhibition of cellulose digestion even when pH was maintained by continuous culture and (Stern et al., 1978) noted decreased in vitro ADF and cellulose digestion with no changes in pH, indicating that depressed fiber digestion was not caused by pH reductions. The lack of connection between decreased fiber digestion and ruminal pH is widely accepted and has been extensively covered in many reviews (Horn and McCollum, 1987; Bowman and Sanson, 1996; Caton and Dhuyvetter, 1997).

Ruminal ammonia-N levels (Table 3) tended to differ ($P < 0.10$) among treatments during the pre-treatment period, however, the biological significance of this difference is negligible. Unsupplemented and CORN-fed steers had similar ruminal ammonia-N between experimental periods, and steers fed supplements with soybean meal had increased ($P \leq 0.03$) values from pre-treatment to treatment periods. During the treatment period, unsupplemented cattle and steers fed CORN supplements had similar ammonia levels. Steers supplemented with the combination of corn and soybean meal had similar ($P > 0.18$) ruminal ammonia-N values as did cattle fed either corn alone or no supplement. Feeding soybean meal by itself resulted in greater ($P < 0.004$) ruminal ammonia-N concentrations than either unsupplemented or CORN-fed animals, and greater ($P < 0.04$) values than cattle supplemented with the combination of corn and soybean meal. The increase in DIP intake resulted in greater ruminal ammonia-N, a vital nutrient for ruminal microbes, especially fibrolytic species (Russell et al., 1992).

Unsupplemented steers and steers fed CORN were severely deficient in ruminal ammonia-N. In Chapter 4, we reported that when steers were fed supplements with corn, they had decreased rates of ruminal fiber digestion, even when fed greater levels of DIP. The deficiency of available substrate for survival and growth of fibrolytic microbes would be expected to limit their ability to degrade the fibrous component of the diet, as Russell et al. (1992) suggested that efficiency of microbial protein growth would be decreased by imbalances between N and fermentable energy.

Concentrations of total VFA, proportions of individual VFA and the branched-chain VFA, and the ratio of acetate to propionate (Table 3) were similar ($P > 0.26$) during the pre-treatment period. During the treatment period, total VFA and branched-chain VFA were similar ($P > 0.20$) among treatments, however, proportions of individual VFA and the ratio of acetate to propionate differed ($P > 0.05$) among supplements. Feeding either supplement with soybean meal increased ($P < 0.001$) total VFA concentration from the pre-treatment to treatment periods, whereas similar ($P > 0.22$) values were observed between periods for unsupplemented and CORN-fed cattle. Steers consuming the CORN supplement tended to have increased ($P < 0.10$) propionate, had decreased ($P < 0.001$) acetate, and tended to have decreased ($P < 0.07$) acetate to propionate ratios than they did during the pre-treatment period. Acetate, propionate, and the acetate to propionate ratio were similar ($P > 0.13$) between periods for steers receiving NEG, SOY, and CRNSOY treatments. During the treatment period, unsupplemented steers and ones fed SOY had similar ($P > 0.88$) acetate levels that were greater ($P < 0.03$) than the either the CORN or CRNSOY treatments. When the corn-based supplements were fed with soybean meal, steers had greater ($P < 0.04$) acetate than cattle supplemented with only corn.

Supplementing with CORN increased ($P < 0.03$) the propionate levels of steers during the treatment period compared to all other supplements, which had similar ($P > 0.45$) values of propionate. Steers receiving CORN had the least ($P < 0.04$) acetate to propionate ratio. When steers received CRNSOY they had a similar ($P > 0.12$) ratio as unsupplemented steers, but tended to have a decreased ($P < 0.07$) ratio compared to SOY-fed cattle. Supplements without corn had similar ($P > 0.76$) acetate to propionate ratios during the supplementation period. Feeding either supplement with soybean meal increased ($P < 0.001$) butyrate compared to pre-treatment concentrations, whereas unsupplemented and SOY-fed steers had similar ($P > 0.45$) butyrate levels between periods. Unsupplemented steers and those fed SOY had similar ($P > 0.63$) butyrate concentrations, which were lesser ($P < 0.03$) than cattle receiving supplements with corn, which had similar ($P > 0.88$) butyrate values. Isobutyrate concentrations of CORN-fed steers were greater ($P < 0.002$) during treatment than pre-treatment, whereas all other supplements had similar ($P > 0.23$) values between periods. Steers fed CORN had the greatest ($P < 0.003$) isobutyrate during the treatment period, with all other treatments having similar ($P > 0.47$) values. All supplements increased ($P < 0.01$) valerate levels vs. pre-treatment values, with unsupplemented steers having similar ($P > 0.62$) values between periods. Feeding supplements resulted in similar ($P > 0.20$) valerate levels, which were greater ($P < 0.003$) than unsupplemented cattle. Steers receiving CORN had increased ($P < 0.01$) isovalerate and total branched-chain VFA, and steers fed CRNSOY tended to have greater ($P < 0.08$) isovalerate and had increased ($P < 0.05$) total branched-chain VFA than their pre-treatment values. Cattle receiving NEG and SOY treatments had similar ($P > 0.15$) isovalerate and total branched-chain VFA levels between treatment

periods. Feeding CORN resulted in greater ($P < 0.03$) isovalerate concentrations than unsupplemented or SOY-fed cattle and similar ($P > 0.12$) values as CRNSOY-supplemented steers, which were similar ($P > 0.18$) to NEG and SOY treatment levels. Increases in total VFA and alterations on the proportions of individual VFA due to supplementation have been reported previously (Hannah et al., 1991; Olson et al., 1999). When steers were fed soybean meal as the sole supplement, a numeric increase in acetate, a decrease in propionate, and an increased acetate to propionate ratio were observed. These alterations in ruminal VFA profile leads us to suggest that increased ruminal degradation of fiber by fibrolytic microorganisms was occurring, indicating that either greater numbers were present, or the microbes present had an increased rate of digestion. However, when steers received corn as the sole source of supplement, just the opposite phenomenon was observed. Acetate decreased, propionate increased, and the acetate to propionate ratio was dramatically decreased. This is suggestive of preferential digestion of starch coming from corn occurring in the rumen, along with concomitant reduction in fiber digestion. When the two supplemental feeds (corn and soybean meal) were fed together, the acetate and propionate concentrations were intermediate, as was the acetate to propionate ratio. This leads us to conclude that when steers received the CRNSOY supplements, that both the starch from the corn, and the fiber of that basal forage were being fermented, due to greater concentrations of ruminal ammonia. However, in Chapter 4, we reported that even when ruminal ammonia levels are increased, fiber digestion in the rumen may still be decreased. We believe that this is indicative of a shift in the composition of the microbial species present and/or predominant in the rumen that similar levels of ruminal ammonia cannot overcome.

Nucleic Acid Extraction and Enzyme Selection. The use of the considerably harsher lysis procedure that included bead-beating did not yield greater species diversity as assessed by either a greater number of bands from a TTGE gel or a greater number of peaks from T-RFLP analysis (data not shown). This agrees with previously published research (Gabor et al., 2003). Therefore, all subsequent DNA extractions were performed using the Qiagen kit without bead-beating. Comparison of T-RFLP peak patterns indicated little difference in revealed bacterial species diversity among *Hae* III, *Hha* I, and *Rsa* I. However, *Msp* I resulted in fewer peaks, and a greater amount of uncut PCR product (data not shown). The enzymes *Hae* III and *Rsa* I were chosen because they appeared to create the majority of T–RFLP peaks in different areas of the PCR product. Amount (μg) of nucleic acids extracted from a gram of DM of ruminal contents (Table 3) was similar ($P > 0.62$) among treatments during the pre-treatment period. Unsupplemented and CORN-fed steers had similar ($P > 0.41$) concentrations of extracted nucleic acids between pre- and treatment periods. Cattle supplemented with either treatment containing soybean meal had greater ($P < 0.001$) amounts of nucleic acids extracted from ruminal contents collected during the treatment period than from the pre-treatment period. During the supplemental feeding period, steers receiving supplements without corn had similar ($P > 0.43$) amounts of extracted nucleic acids, and steers fed supplements with soybean meal had similar ($P > 0.26$) values, which were greater ($P < 0.008$) than when supplements did not contain soybean meal. This leads us to suggest that there was a large change in ruminal microbes due to treatments, and that this alteration may be due to changes in the ruminal microorganism community, as well as in numbers of microbes when protein supplements were fed. We are unaware of any other

researchers who have reported the quantity of DNA extracted from ruminal contents.

Ruminal Bacterial Species Richness, Diversity, and Evenness. Following manual alignment of T-RFLP peaks generated from *Hae* III digestion of 16S rDNA PCR amplicons, 161 unique T-RF were detected, 155 unique T-RF resulted from digestion with *Rsa* I, and DNA sequence analysis of a clone library resulted in 136 different putative matches. However, further analysis of DNA sequences from the clone library will reveal further unique sequences, as only a small proportion of the putative matches can be considered to be definitive matches (> 97% sequence similarity). Of the 136 unique putative matches, 80 occurred only once in the clone library, 20 occurred twice, 33 occurred between 3 and 9 times, and only four occurred 10 or more times. This indicates the incredible amount of diversity presence in the bacterial community of the rumen. Only 4 out of 136 putatively matched bacterial species occurred more than four times from the same steer, and only 10 occurred more than twice from an individual steer. Out of 136 sequences, only 36 sequences matched sequences in GenBank with > 97% similarity. Of those 36 definitive matches, 3 matched known species and 2 were identified at the family taxonomic level. The following species (along with the number of times the species occurred out of 324 sequences) *Butyrivibrio fibrisolvens* (3 strains, 5 occurences), *Ruminococcus flavefaciens* (2), and *Succinivibrio dextrinosolvens* (1) were identified. An uncultured *Clostridiaceae* (1) and a *Lachnospiraceae* bacterium (1) were also definitively identified. These organisms have all been described as inhabitants of the bovine rumen (Tajima et al., 1999). Sequences that putatively matched (< 98% sequence similarity) known species (and the number of strains, the number of times the species occurred out of 324 sequences, and the percentage similarity), are as follows:

Butyrivibrio fibrisolvens (4 strains, 7 occurrences, 95 to 97% similarity); *Ruminococcus flavefaciens* (2 strains, 4 occurrences, 93 to 97% similarity); *Fibrobacter succinogenes* (1 strain, 2 occurrences, 96 to 97% similarity); *Clostridium proteoclasticum* (1 strain, 3 occurrences, 96 to 97% similarity); *Prevotella genomosp.* C1 (1 strain, 2 occurrences, 96% similarity); *Eubacterium oxidoreducens* (1 strain, 1 occurrence, 94% similarity). The remaining 119 out of 136 unique putative matches had sequence similarities from 83 to 100% and matched sequences reported in previous research on the bacterial community of the rumen, including sequences labeled as "uncultured rumen bacterium" (Tajima et al., 2000), "unidentified rumen bacterium" (Whitford et al., 1998; Tajima et al., 1999), and "uncultured bacterium" (Koike et al., 2003), as well as "uncultured bacterium" detected from the gastrointestinal tract of pigs (Leser et al., 2002). The large diversity and low redundancy that we observed has been reported by these researchers, as well as others working with rumen (White et al., 1999) and other environmental samples (Theron and Cloete, 2000; Gabor et al., 2003). The relatively small number of sequences that match sequences in GenBank, as well the very minor fraction that match known sequences again illustrates how little we know of the species, the diversity and function of rumen bacterial community.

Species richness, diversity, and evenness of bacteria in the rumen did not differ ($P > 0.24$) between replicate lanes for any treatment or either enzyme (data not shown). Using the enzyme *Hae* III, no differences ($P > 0.55$) between treatments were detected in species richness, diversity, or evenness (Table 4) during the pre-treatment or treatment periods, or the difference between the two periods, with the exception of a tendency for an increase ($P = 0.09$) in species richness of CORN-supplemented cattle from pre-

treatment to treatment periods. When T-RF were generated with *Rsa* I, no differences ($P > 0.21$) among treatments were observed for species richness or diversity in the treatment period, and evenness was similar ($P > 0.17$) during the pre-treatment period. However, differences ($P < 0.02$) among treatments were observed for species richness and diversity in the pre-treatment period, whereas evenness differed ($P < 0.04$) during the treatment period. There was a tendency for species diversity to decrease ($P = 0.10$) for NEG steers and to increase ($P = 0.09$) for CORN-fed cattle from pre-treatment to treatment periods and for evenness of CORN-supplemented animals to be greater ($P < 0.007$) in the treatment period than during pre-treatment. The differences revealed in richness, diversity and evenness in the pre-treatment period are due to one steer, whose *Rsa* I generated T-RFLP profile had a considerable number of peaks unique to that animal and one other steer in the treatment period. The differences in species evenness during the treatment period were due to the steer housed next to the previously discussed animal having a significant number of peaks that were unique to this animal in the treatment period and to previously discussed animal in the pre-treatment period. We are at a loss to explain this. The lack of any differences in the *Hae* III data set, and the minimal differences during the treatment period in the *Rsa* I data set was unexpected, based on our observations of greater ruminal DNA recovery for the protein-supplemented treatments. However, increased DNA would not necessarily result in greater species richness or diversity. Yet, we feel that the inability of the richness, diversity, and evenness estimates to detect differences is a result of the complexity of the ruminal ecosystem, and the difficulties to express changes in such a complicated data set in a single number. We reported species richness, diversity, evenness, although T-RFLP is not capable of guaranteeing that each

unique T-RF is a unique bacterial species, or that a unique bacterial species will not produce more than one T-RF. Even though T-RFLP actually produces peaks that are considered to represent operation taxonomic units, we believe that the calculation of diversity measures is acceptable, because all bacterial species had the opportunity to be represented. Additionally, a recent paper (Crosby and Criddle, 2003) showed T-RFLP to be an acceptable technique when limiting bias due to operon copy number of 16S rDNA is a concern. However, we do not believe it to be appropriate to calculate diversity indices on species determined from the sequenced clone library, because a relatively small number of clones (24) from each steer were sequenced. Yet, due to concerns with using rDNA based community analysis techniques such as T-RFLP (Crosby and Criddle, 2003), we are reporting species richness calculated from our clone library to allow comparison with the species richness revealed by T-RFLP. Based on putative matches, steers on the NEG and SOY treatments had 47 unique species, CORN had 44, and CRNSOY had 62. We expect these numbers to increase as we move from putative identification to final determination of sequence similarity. Once again, because the clone library was constructed from a very small random sample from each steer, these numbers are only reported as a point of reference.

Ruminal Microbial Ecology. Detrended correspondence analysis revealed a relatively short gradient (gradient lengths less than 1.5 for *Hae* III and less than 2.25 for *Rsa* I); therefore linear models of species response were selected for subsequent analyses. This would be expected by the similarity of ruminal measures among treatments that were observed, which would result in relatively narrow environmental gradients.

Principal components analysis of the *Hae* III data was performed to determine if

ruminal bacterial species were responding to a dominant, primary environmental gradient. Principal components analysis is an indirect gradient analysis technique that uses the changes in species composition to determine the presence of an environmental gradient in the data. The output from PCA is plotted as an ordination bi-plot. Ordination diagrams have X- and Y-axes, an origin, and arrows representing variables. Each arrow is pointing in the direction of maximum correlation, and the length of the arrow is related to the strength of the correlation. Our PCA analysis of the *Hae* III data set showed the first and second axes explained only 13.6% and 10.9% of the variation in the species. These low correlations indicate that there was not a primary gradient that can be used to explain the response of ruminal bacterial species. The major environmental variables correlated with the first and second axes were the CRNSOY treatment, ruminal ammonia-N, pH, total VFA, steer BW, intake, and period, with correlations between 0.30 and 0.47. This shows that individual measures routinely taken of the ruminal environment in many nutrition studies can each explain from one-third to one-half of the variation associated with a gradient that explains less than 14% of the species response in the rumen. Possibly, the measures collected in this study do not describe the environmental factors that the ruminal bacterial species were responding to, or that the narrow range of environmental conditions made detection of a gradient difficult. Correlations of individual steers with the axis ranged from < 0.01 to 0.32, which leads to the conclusion that individual steer to steer variation in ruminal bacterial communities may be almost as large of a factor as many of the individual environmental measures we routinely collect. Replicate gel lanes had correlations less than 0.03. The low correlation of replicates, along with the correct clustering analysis (Appendix Figures 9-14), and no

differences in diversity indices, all indicate that the T-RFLP is highly repeatable technique. There was a noticeable grouping of steers by pre-treatment and treatment periods, with relatively few outliers (Figure 1). The same general pattern was detected in the cluster analysis (Appendix Figures 9-12). There also appears to be two separate groups of T-RF, each responding to steers in either the pre-treatment group or the treatment group. The relationship of the treatments with the highest intakes (SOY, CRNSOY), with intake, total VFA, rumen ammonia-N, and decreasing pH can be seen in Figure 2. There is also a group of T-RF associated with these variables, whereas the remainder of T-RF are relatively scattered. This is supported by our ANOVA analysis, in which steers fed these supplements had the greatest intakes, total VFA, and ruminal ammonia concentrations, and the lowest ruminal pH. The appearance of two major groups of steers in pre-treatment and treatment periods indicate that PCA analysis, supported by the hierarchical cluster analysis, revealed changes in the bacterial community of the rumen due to treatments or sampling time, a conclusion not detected in the diversity indices. This may be due to the difficulty in expressing the changes in a complex community with a single number in a univariate approach as opposed to the multivariate ordination methods.

Redundancy analysis of microbial species composition did not differ ($P > 0.99$) between replicates. This is in agreement with hierarchical clustering analysis (Appendix Figures 9-14), which detected only one clustering error of replicate gel lanes. The only clustering error of replicate gel lanes occurred in the *Rsa* I data set. Clustering analysis (Appendix Figures 9-14) supported the grouping of steers in the pre-treatment period and treatment periods determined from redundancy analysis (Appendix Figures 3 and 4).

Redundancy analysis is a direct, or constrained, gradient analysis technique, where species responses are fitted to a known, measured, environmental gradient.

Redundancy analysis of T-RF generated using *Hae* III digestions of PCR products generated from samples collected from steers during the treatment period revealed that the measured environmental variables accounted for 11% of the variation along Axis 1 and an additional 10% of the variation along Axis 2. However, the permutation test of the first axis ($P < 0.01$) supported the fit of the environmental variables with community data. Additionally, unconstrained ordination explained only 13.6%, therefore, when taken as a group, the measured environmental variables were able to explain 80% of the variation in the first axis from the PCA. However, that variation was less than 14% of the total variation. The ordination diagram (Figure 3) shows a relationship between neutral detergent solubles intake, propionate, butyrate and a group of T-RF peaks. These environmental variables had correlation values with axis 1 from –0.3 to –0.6, and with axis 2 from 0.4 to 0.5. A second relationship can be observed for increasing protein intake, ruminal ammonia-N, pH, acetate, and acetate to propionate ratio, and one T-RF peak. The branched-chain VFA isobutyrate and isovalerate were strongly negatively correlated with axis 1 (-0.80 and -0.73) and could be associated with a single T-RF. Both NDF intake and acetate to propionate ratio were positively correlated with axis 1, responded in the same general manner as DIP intake, ruminal ammonia-N, and increasing pH, and were loosely associated with three T-RF.

Redundancy analysis of treatments over time using a BACI model showed changes ($P < 0.01$) in community composition over time. Axis 1 and 2 explained 9.6% and 6%, respectively, of the variation in the data set. While this is a small amount of the total

variation, it is 70% of the dominant gradient that was revealed by PCA analysis. Correlations with axis 1 and 2 for the supplement treatments were NEG (-0.06, -0.80), SOY (0.17, 0.11), CORN (-0.78, 0.36), and CRNSOY (0.67, 0.15), respectively. While not displayed on the ordination diagram, the environmental variables measured in this study that had strong correlations with either axis 1 or 2 were intake which increased along both axis 1 and 2, agreeing with the greater intakes of the SOY and CRNSOY treatments, and pH, which increased down axis 2 with the NEG treatment, agreeing with our observations of steers on that treatment having the greatest pH. The ability of the ordination techniques to detect changes in ruminal bacterial community composition is supported by our observations of changes in measured ruminal environmental variables, greater amounts of DNA extracted for two treatments, and the hierarchical clustering analysis. Even though the estimates of species richness, diversity, and evenness did not detect changes, we have shown that supplementation strategy will alter the bacterial community of the rumens of forage-fed beef cattle. This indicates that there is a significant role of many bacterial species in terms of structure and function of the microbial community of the rumen, and that trying to broadly group individual members of that community into functional groups, may result in the overlooking of important community changes.

Although the bacterial community of NEG steers changed from pre-treatment to treatment periods, it was in the opposite direction as the other three supplements. This may indicate that a temporal trend was occurring in the rumen communities prior to the beginning of our study, and that trend was reversed by supplementation. Steers fed CRNSOY and SOY had changes in bacterial community composition that were similar in

direction, but a much greater change was observed for the CRNSOY steers. Steers supplemented with CORN had a large shift in bacterial community from the pre-treatment to treatment periods that was not similar to any of the other treatments. The shifts in the CORN and CRNSOY had similar correlations with axis 1, just in opposite directions. It can be seen in Figure 4 that supplementation appeared to be positively correlated with axis 2, and that the shift in the bacterial community of the NEG treatment was highly negatively correlated with axis 2.

Implications

Feeding supplemental energy in the form of high starch feedstuffs can reduce intake of low-quality forages, unless degradable intake protein is supplemented to meet the requirements of fermentation of the supplement and of the basal forage. Changes in the ruminal bacterial community can be partially related to environmental variables such as pH, ammonia, and intake. However, these measures explain very little of the changes in the bacterial communities. Using community analysis methods and a clone library, we have shown that the bacterial community in the rumen is incredibly diverse, little is known about the species that inhabit the rumen, and even less is known about how these species and consequently the entire community respond to nutritional changes. When corn supplements are fed the direction of change in the microbial community is dependent on the presence of DIP. Supplements with corn and soybean meal are more similar in bacterial community to soybean meal supplements than to corn supplements.

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Table 1. Chemical composition of prairie hay and supplements and intake of supplements fed to beef steers

Chemical	Forage	Supplement[a]		
Composition (% DM)	**Prairie Hay**	**SOY**	**CORN**	**CRNSOY**
Dry Matter	90.0	88.2	86.3	86.8
Organic Matter	94.9	90.5	98.7	96.6
Nitrogen	0.7	7.3	1.4	2.9
Crude protein	4.5	45.8	8.6	18.2
DIP	2.9	30.7	3.9	10.8
UIP	1.6	15.1	4.7	7.4
Carbon	46.4	44.1	46.5	45.9
Acid Detergent Fiber	42.7	6.9	4.5	5.1
Neutral Detergent Fiber	78.3	15.0	16.1	15.8
TDN:CP[b]	11.6	1.9	10.5	4.9
DIP:TDN[b]	5.6	34.9	4.3	12.1
Supplement Intake	**NEG**	**SOY**	**CORN**	**CRNSOY**
Corn, kg of DM	0	0.0	3.2	3.1
Soybean meal, kg of DM	0	1.1	0.0	1.1
g/(kg of BW·d)	0	2.0	5.3	7.2
g/(kg of $BW^{0.75}$·d)	0	9.6	26.2	35.1

[a] **NEG** = no supplement fed; **SOY** = 1.1 kg of DM/feeding of soybean meal; **CORN** =3.2 kg of DM/feeding of dry-rolled corn; **CRNSOY** = 1.1 kg of DM/feeding of soybean meal, plus 3.1 kg of DM/feeding of dry-rolled corn.

[b]Estimated using tabular TDN to approximate DOM, and measured CP values, similar to {938}, and using degradable intake protein values and tabular TDN values, similar to {4}.

Table 2. Intake of selected chemical components by steers consuming prairie hay and fed one of four supplements

Item	Supplement[a]				SEM[b]	*P*[c]
	NEG	SOY	CORN	CRNSOY		
Steers, #	4	4	4	4	---	
Trial BW, kg	585	579	601	580	30.6	0.94
Intake, g/kg of BW						
Hay DM, pre	10.0	8.2	7.9	10.4	1.7	0.65
Hay DM, treat	9.3^{qrtux}	$14.7^{ruvy***}$	6.2^{qtx*}	15.2^{rvy***}	1.9	0.01
Total DM, pre	10.0	8.2	7.9	10.4	1.7	0.65
Total, DM, treat	9.3^{qtx}	$16.7^{qruvy***}$	$11.4^{qtux***}$	22.4^{rvz***}	1.9	0.002
Nitrogen, pre	0.08	0.07	0.07	0.09	0.01	0.66
Nitrogen, treat	0.07^{qt}	0.28^{ru***}	0.13^{qv***}	0.36^{sw***}	0.02	0.001
Crude Protein, pre	0.52	0.43	0.41	0.54	0.09	0.65
Crude Protein, treat	0.45^{qt}	1.7^{ru***}	0.81^{qv***}	2.2^{sw***}	0.11	0.001
DIP, pre	0.29	0.24	0.23	0.30	0.05	0.66
DIP, treat	0.27^{qx}	1.0^{ry***}	0.38^{qx***}	1.2^{rz***}	0.02	0.001
Carbon, pre	4.7	3.8	3.7	4.8	0.79	0.65
Carbon, treat	4.3^{qtx}	$7.7^{qruvy***}$	$5.3^{qtux***}$	10.3^{rvz***}	0.88	0.002
ADF, pre	4.3	3.5	3.4	4.4	0.72	0.66
ADF, treat	4.0^{qrtuw}	6.4^{ruv***}	3.0^{qtw}	7.0^{rvx***}	0.80	0.01
NDF, pre	8.0	6.6	6.3	8.3	1.4	0.66
NDF, treat	7.4^{qrt}	12.0^{rsu***}	5.7^{qrt}	13.2^{su***}	1.5	0.01
Hemicellulose, pre	3.7	3.1	2.9	3.9	0.63	0.65
Hemicellulose, treat	3.4^{qrt}	5.6^{ru***}	2.7^{qt}	6.3^{ru***}	0.68	0.009
Neutral detergent solubles, pre	2.0	1.7	1.6	2.1	0.34	0.66
Neutral detergent solubles, treat	2.0^{q}	4.7^{r***}	5.7^{r***}	9.2^{s***}	0.43	0.001

[a]**NEG** = no supplement fed; **SOY** = 1.1 kg of DM/feeding of soybean meal; **CORN** =3.2 kg of DM/feeding of dry-rolled corn; **CRNSOY** = 1.1 kg of DM/feeding of soybean meal, plus 3.1 kg of DM/feeding of dry-rolled corn.

[b]SEM = Standard error of the means, n = 4.

[c]Probability of main effect of treatment.

[q,r,s]Within a row, means without a common superscript letter differ ($P < 0.01$).

[t,u,v,w]Within a row, means without a common superscript letter differ ($P < 0.05$).

[x,y,z]Within a row, means without a common superscript letter differ ($P < 0.10$).

[†,*,**,***]Means differ from pre-treatment values at $P < 0.10$, 0.05, 0.01, or 0.001, respectively.

Table 3. Ruminal pH, ammonia nitrogen, volatile fatty acid, and nucleic acid concentrations from ruminal contents of steers consuming prairie hay and fed one of four supplements

	Supplement[a]					
Item	**NEG**	**SOY**	**CORN**	**CRNSOY**	**SEM[b]**	***P*[c]**
Ruminal pH						
Average pH, pre	7.03	7.02	7.00	7.02	0.03	0.83
Average pH, treat	7.02^{q}	6.63^{rs***}	6.70^{r***}	6.55^{s***}	0.04	0.001
Ruminal ammonia-N, mg/dL						
Ammonia-N, pre	0.12	0.06	0.13	0.13	0.02	0.10
Ammonia-N, treat	0.31^{qt}	2.2^{ru***}	0.28^{qt}	1.0^{qrt*}	0.37	0.01
Ruminal VFA, mmol/L						
Total VFA, pre	68	60	67	59	7.4	0.65
Total VFA, treat	71	100^{***}	82	89^{***}	8.9	0.20
Individual VFA, mol/100 mol						
Acetate, pre	78.1	77.0	76.2	75.1	1.5	0.43
Acetate, treat	77.9^{rw}	78.2^{rw}	66.6^{qu***}	72.1^{qrv}	1.7	0.001
Propionate, pre	14.0	14.7	15.7	16.4	1.2	0.41
Propionate, treat	13.7^{qt}	13.5^{qt}	$19.6^{ru\dagger}$	15.0^{qrt}	1.3	0.02
Butyrate, pre	6.6	7.2	7.0	7.1	0.68	0.89
Butyrate, treat	7.3^{qrt}	6.6^{qt}	10.7^{ru***}	10.9^{ru***}	0.98	0.02
Isobutyrate, pre	0.67	0.65	0.68	0.69	0.07	0.97
Isobutyrate, treat	0.63^{q}	0.55^{q}	1.2^{r***}	0.66^{q}	0.10	0.002
Valerate, pre	0.10	0.0	0.0	0.14	0.07	0.26
Valerate, treat	0.06^{q}	0.39^{r**}	0.37^{r**}	0.49^{r**}	0.06	0.002
Isovalerate, pre	0.52	0.46	0.56	0.53	0.08	0.78
Isovalerate, treat	0.46^{qt}	0.67^{qrt}	1.5^{ru**}	$0.94^{qrtu\dagger}$	0.24	0.05
BCVFA[d], pre	1.3	1.1	1.2	1.4	0.18	0.71
BCVFA[d], treat	1.2^{qtx}	1.6^{qrtxy}	3.1^{ruz***}	2.1^{qrtuy*}	0.35	0.02
Acetate:Propionate, pre	5.66	5.26	5.10	4.64	0.53	0.50
Acetate:Propionate, treat	5.70^{ruyz}	5.86^{ruz}	$3.60^{qtx\dagger}$	4.82^{qruy}	0.38	0.004
Extracted DNA, μg/g of DM						
DNA, pre	228	209	229	252	22.7	0.63
DNA, treat	199^{q}	464^{r***}	240^{q}	404^{r***}	36.0	0.001

[a]**NEG** = no supplement fed; **SOY** = 1.1 kg of DM/feeding of soybean meal; **CORN** =3.2 kg of DM/feeding of dry-rolled corn; **CRNSOY** = 1.1 kg of DM/feeding of soybean meal, plus 3.1 kg of DM/feeding of dry-rolled corn.

[b]SEM = Standard error of the means, n = 4.

[c]Probability of main effect of treatment.

[d]BCVFA = Branched-chain VFA (isobutyrate + isovalerate + valerate).

[q,r,s]Within a row, means without a common superscript letter differ ($P < 0.01$).

[t,u,v,w]Within a row, means without a common superscript letter differ ($P < 0.05$).

[x,y,z]Within a row, means without a common superscript letter differ ($P < 0.10$).

[†,*,**,***]Means differ from pre-treatment values at $P < 0.10$, 0.05, 0.01, or 0.001, respectively.

Table 4. Ruminal bacterial species richness, diversity, and evenness of steers fed prairie hay, and one of four supplement treatments.

	Supplement[a]					
Item	**NEG**	**SOY**	**CORN**	**CRNSOY**	**SEM[b]**	***P*[c]**
Rsa I						
Richness, pre	30.4^{qx}	33.5^{qrx}	29.5^{qx}	39.0^{ry}	1.9	0.02
Richness, treat	27.8	30.8	28.3	35.9	3.6	0.40
Diversity, pre	2.74^{qrtuy}	2.78^{qruy}	2.55^{qtx}	3.02^{rvz}	0.07	0.01
Diversity, treat	$2.55^{\dagger}$	2.69	$2.74^{\dagger}$	2.90	0.11	0.21
Evenness, pre	0.804	0.798	0.758	0.824	0.020	0.17
Evenness, treat	0.774^{qtx}	0.785^{qtux}	0.825^{qvy**}	0.820^{quvy}	0.013	0.04
Hae III						
Richness, pre	47.5	51.8	40.4	45.5	6.1	0.62
Richness, treat	47.5	53.3	$47.8^{\dagger}$	42.6	5.2	0.56
Diversity, pre	3.18	3.20	2.98	3.02	0.14	0.56
Diversity, treat	3.10	3.23	3.13	2.98	0.12	0.55
Evenness, pre	0.825	0.815	0.810	0.803	0.014	0.70
Evenness, treat	0.806	0.814	0.815	0.803	0.017	0.94

[a]**NEG** = no supplement fed; **SOY** = 1.1 kg of DM/feeding of soybean meal; **CORN** =3.2 kg of DM/feeding of dry-rolled corn; **CRNSOY** = 1.1 kg of DM/feeding of soybean meal, plus 3.1 kg of DM/feeding of dry-rolled corn.

[b]SEM = Standard error of the means, n = 4.

[c]Probability of main effect of treatment.

[q,r,s]Within a row, means without a common superscript letter differ ($P < 0.01$).

[t,u,v,w]Within a row, means without a common superscript letter differ ($P < 0.05$).

[x,y,z]Within a row, means without a common superscript letter differ ($P < 0.10$).

[†,*,**,***]Means differ from pre-treatment values at $P < 0.10$, 0.05, 0.01, or 0.001, respectively.

Figure 1. Ordination diagram of Principal Components Analysis of T-RFLP peaks generated from *Hae* III digestion of PCR amplified rumen bacterial 16S rDNA with restriction fragment lengths of interest and samples collected from individual steers during the pre-treatment and treatment periods displayed.

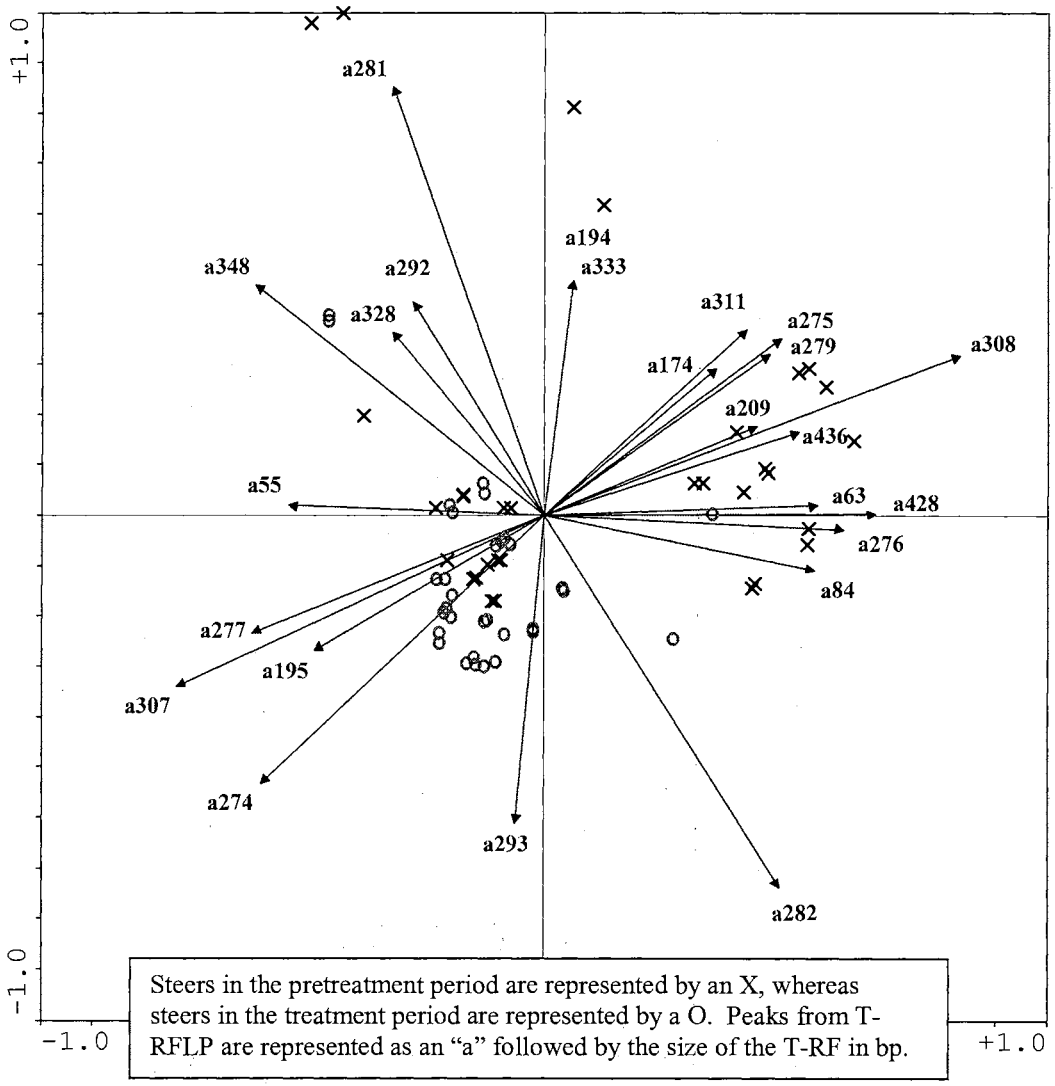
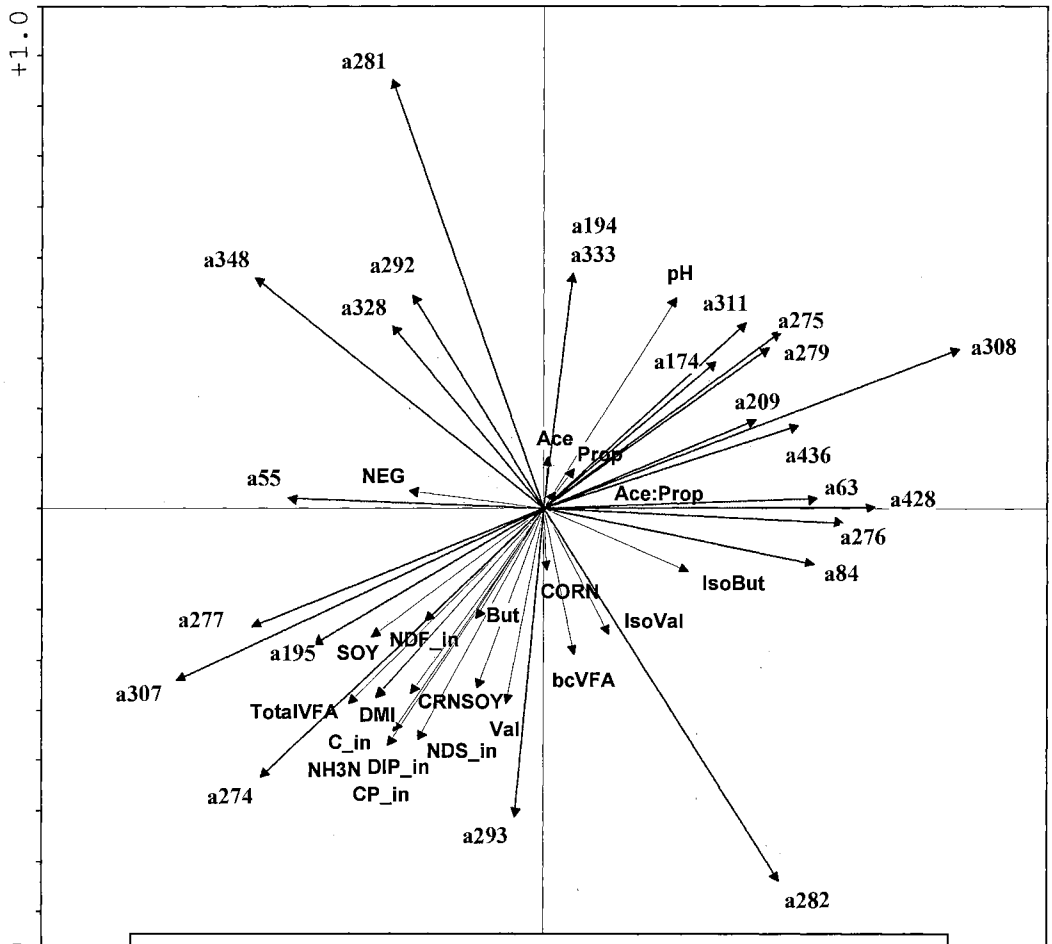
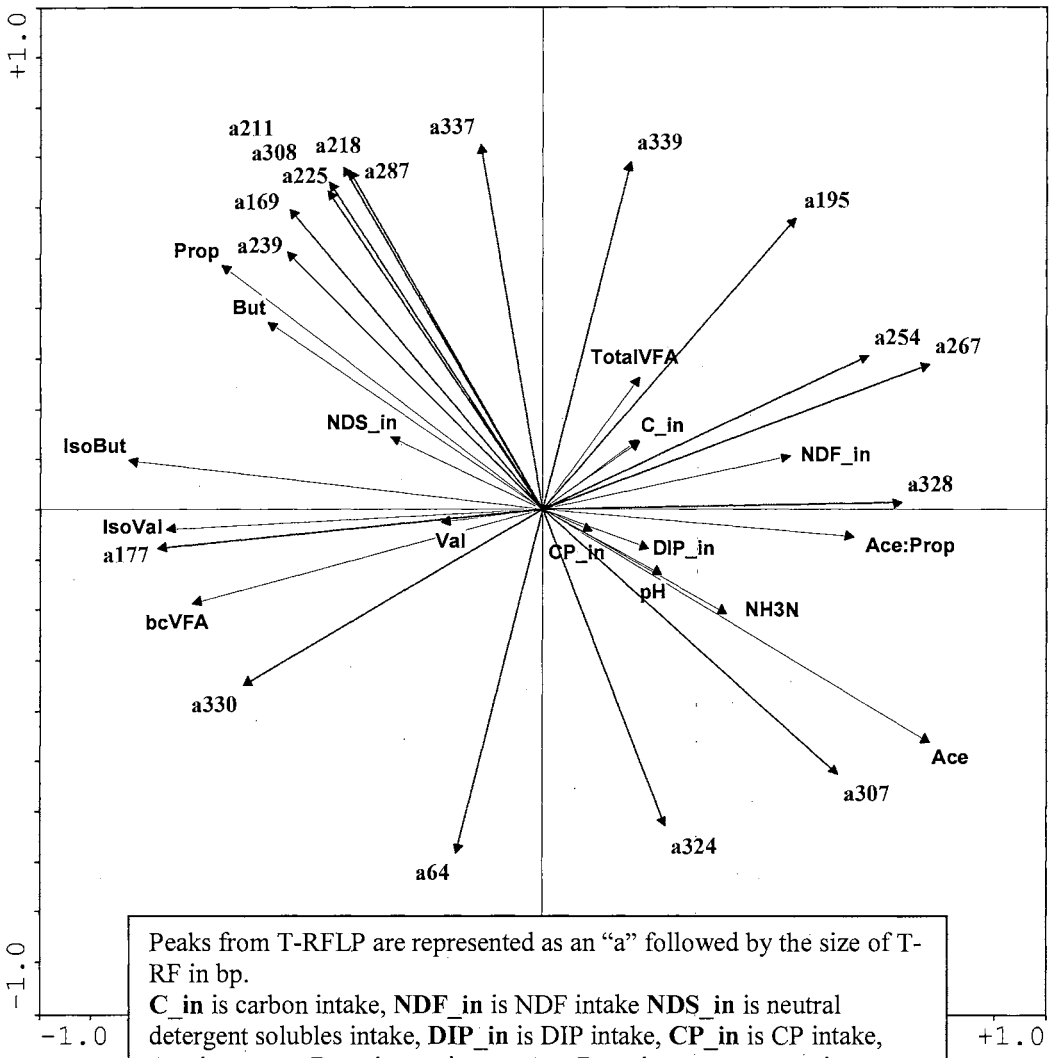


Figure 2. Ordination diagram of Principal Components Analysis of T-RFLP peaks generated from *Hae* III digestion of PCR amplified rumen bacterial 16S rDNA from samples collected during pre-treatment and treatment periods with restriction fragment lengths of interest and environmental measures taken from both periods displayed.



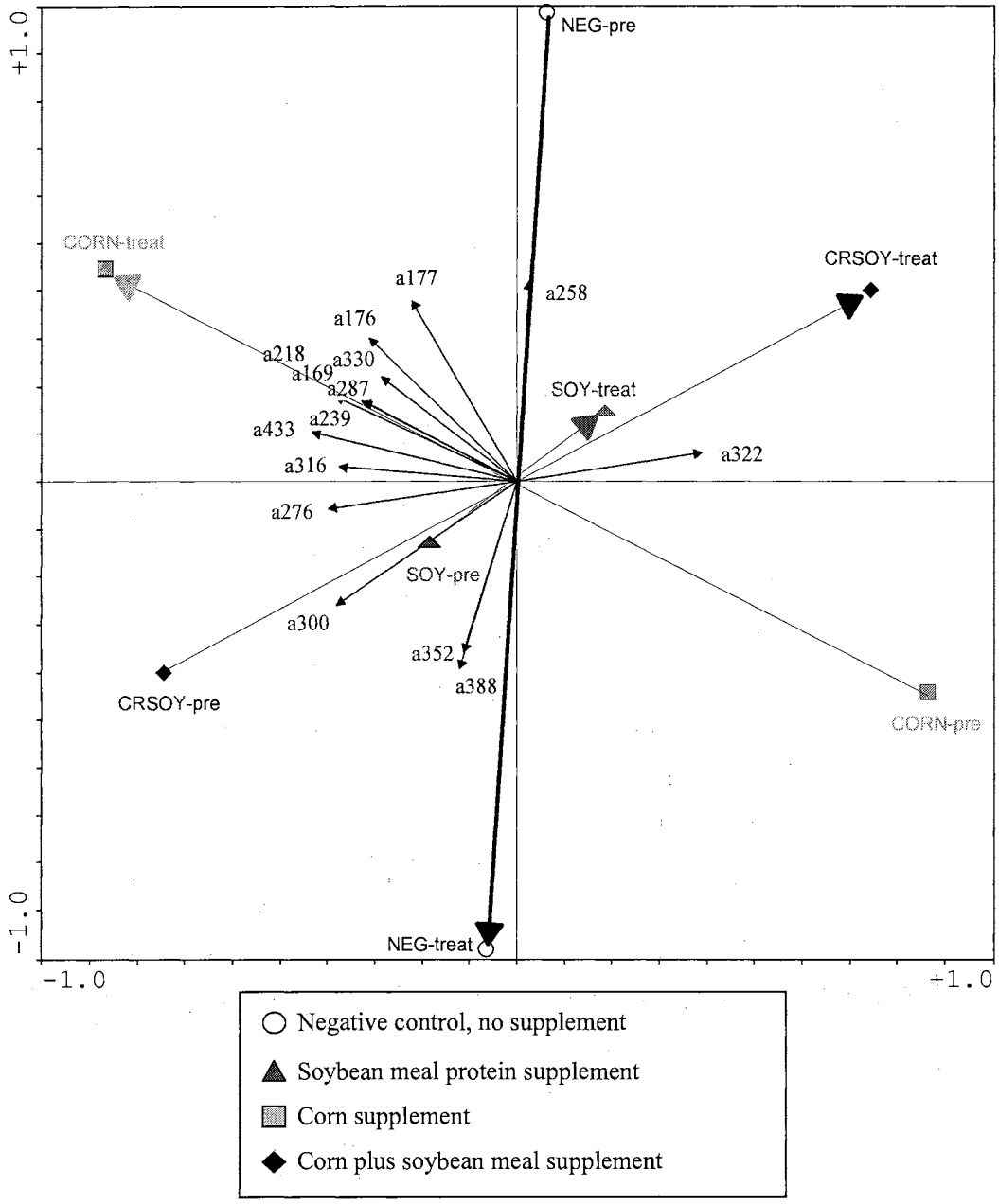
Peaks from T-RFLP are represented as an "a" followed by the size of T-RF in bp.
C_in is carbon intake, **NDF_in** is NDF intake **NDS_in** is neutral detergent solubles intake, **DIP_in** is DIP intake, **CP_in** is CP intake, **Ace** is acetate, **Prop** is propionate, **Ace:Prop** is acetate to propionate ratio, **But** is butyrate, **Val** is valerate, **IsoVal** is isovalerate, **IsoBut** is isobutyrate, **bcVFA** is branched-chain VFA.

Figure 3. Ordination diagram of Redundancy Analysis of T-RFLP peaks generated from *Hae* III digestion of PCR amplified rumen bacterial 16S rDNA from samples collected during the treatment period with restriction fragment lengths of interest and environmental measures displayed.



Peaks from T-RFLP are represented as an "a" followed by the size of T-RF in bp.
C_in is carbon intake, **NDF_in** is NDF intake **NDS_in** is neutral detergent solubles intake, **DIP_in** is DIP intake, **CP_in** is CP intake, **Ace** is acetate, **Prop** is propionate, **Ace:Prop** is acetate to propionate ratio, **But** is butyrate, **Val** is valerate, **IsoVal** is isovalerate, **IsoBut** is isobutyrate, **bcVFA** is branched-chain VFA.

Figure 4. Ordination diagram of Redundancy Analysis of T-RFLP peaks generated from *Hae* III digestion of PCR amplified rumen bacterial 16S rDNA showing temporal changes in ruminal bacterial communities due to dietary treatments from the pre-treatment period to the treatment period and restriction fragment lengths of interest displayed.



Chapter VI

Summary and Conclusion

Many beef cattle production systems are based on forage. Yet, forage alone is often inadequate to achieve desired levels of animal production. Consequently, cattle producers frequently find it necessary to provide supplements in an attempt to meet animal requirements. Animal requirements vary due to many reasons, and livestock production can be measured many ways. Rates of gain of stocker cattle, body condition of cows, and weaning live calves are all measures of productivity where animals have different requirements. The two most commonly limiting nutrients across many sectors of the beef industry are energy and protein. In addition to animal requirements, beef cattle nutritionists also have to meet the nutrient requirements of the microbial population associated with the rumen. An understanding of the interactions between dietary components, livestock behavior, and the mechanisms of digestion and metabolism is necessary for nutritionists to aid producers in achieving their desired production goals.

Two experiments were undertaken to evaluate the impact of inclusion of a source of ruminally degradable protein in the supplement when corn grain is used as a source of supplemental energy. A concern with using corn to supply energy to a ruminant consuming a forage-based diet is the presence of negative associative effects. While it is commonly accepted that protein supplementation will increase forage intake and consequently improve energy and protein status of the animal, there is not a clear consensus on the requirements for additional protein when corn is supplemented.

The first experiment was designed to compare corn, soybean meal, and corn plus soybean meal supplements fed to steers grazing dormant native tallgrass prairie (a low-

quality forage) pastures in the winter. Supplementing with corn reduced forage intake and digestibility, and grazing time, whereas soybean meal did not alter forage intake or digestion. Feeding corn plus soybean meal resulted in greater forage digestion than corn alone. Feeding supplemental energy plus protein resulted in the greatest average daily gain. Greater gains can be explained by the greater energy and protein intakes, which resulted in greater blood urea nitrogen and serum insulin levels. In this study, the software provided with the Nutrient Requirements of Beef Cattle (NRC, 1996), did not predict intake, digestibility, or animal performance adequately. The environmental option of the model should never be used under any circumstances.

The second experiment was designed to evaluate the effects of supplements with four levels of DIP from soybean meal and two levels of corn fed to ruminally cannulated steers consuming low-quality prairie hay diets. Feeding increasing levels of DIP increased intake of fiber, and when corn was included in the supplement, DIP increased both intake and digestion of fiber. Even though increasing DIP increased ruminal ammonia in both supplement types, when sufficient DIP was feed to achieve equal levels of ammonia, corn supplemented steers still had reduced ruminal fiber digestion, even though total tract digestion was similar. We conclude that feeding corn plus DIP resulted in greater ruminal digestion than without added DIP, but that site of digestion was still altered and that this may be due to changes in microbial species in the rumen. Fecal output of fiber was relatively similar across all treatments. Increasing intake of N resulted in increasing N digestibility, fecal N concentration, and metabolic fecal N. Evaluation of the 1996 NRC model once again did not result in accurate predictions for a

variety of measures, including intake, digestibility, passage rates, rumen pH, and fecal N and metabolic fecal N output.

The third study was designed to investigate the effect of similar corn, soybean meal, corn plus soybean meal supplements on intake of low-quality hay, ruminal measures and ruminal bacterial ecology. Rumen samples were taken, total genomic DNA was extracted, PCR was performed and the ruminal bacterial communities were compared using terminal restriction fragment length polymorphism analysis and redundancy analysis. As we have shown previously, protein increased intake, corn depressed intake, and corn plus protein increased intake. Feeding any source of protein increased ruminal ammonia and total VFA, as well as resulting in greater amounts of DNA being extracted from rumen samples. Redundancy analysis revealed temporal shifts in bacterial communities as a result of treatments, with the community of corn-fed and corn plus soybean meal-fed cattle being very different, and unique from either unsupplemented or protein supplemented steers.

Taken together, these results lead us to conclude that the primary effect of corn supplementation on depressing fiber digestion in the rumen is to create a ruminal ammonia deficiency. How this exactly affects the bacterial community is still unknown, yet we can say that the bacteria in the rumen respond very differently as a community to these supplements, and vastly different than would be predicted based on looking at only a few species. Corn, and other grains, can be used to supplement beef cattle consuming low-quality forages provided that adequate DIP to ferment not only the added grain, but especially to ferment the basal forage, is provided in the supplement.

Appendix

Figure 1. Putative Matches of 16S rDNA extracted from rumen contents reported from a nucleotide sequence search (blastN) of the GenBank database at NCBI performed on 12/30/03 along with similarity of the submnitted DNA sequence and the predicted match in GenBank.

dbj|AB009171.1| Unidentified rumen bacterium RFN12 gene for 16S ribosomal RNA,	0.98
dbj|AB009172.1| Unidentified rumen bacterium RFN13 gene for 16S ribosomal RNA,	0.99
dbj|AB009181.1| Unidentified rumen bacterium RFN23 gene for 16S ribosomal RNA,	0.93
dbj|AB009185.1| Unidentified rumen bacterium RFN27 gene for 16S ribosomal RNA,	0.98
dbj|AB009186.1| Unidentified rumen bacterium RFN28 gene for 16S ribosomal RNA,	0.95
dbj|AB009207.1| Unidentified rumen bacterium RFN50 gene for 16S ribosomal RNA,	0.94
dbj|AB009220.1| Unidentified rumen bacterium RFN72 gene for 16S ribosomal RNA,	0.94
dbj|AB009224.1| Unidentified rumen bacterium RFN76 gene for 16S ribosomal RNA,	0.83
dbj|AB009231.1| Unidentified rumen bacterium RFN84 gene for 16S ribosomal RNA,	0.97
dbj|AB009232.1| Unidentified rumen bacterium RFN85 gene for 16S ribosomal RNA,	0.89
dbj|AB009243.1| Unidentified rumen bacterium RFN96 gene for 16S ribosomal RNA,	0.98
dbj|AB034019.1| Uncultured rumen bacterium 4C0d-6 gene for 16S rRNA, partial	0.88
dbj|AB034020.1| Uncultured rumen bacterium 4C0d-7 gene for 16S rRNA, partial	0.97
dbj|AB034023.1| Uncultured rumen bacterium 4C0d-10 gene for 16S rRNA, partial	0.98
dbj|AB034034.1| Uncultured rumen bacterium 3C0d-19 gene for 16S rRNA, partial	0.97
dbj|AB034038.1| Uncultured rumen bacterium 5C0d-4 gene for 16S rRNA, partial	0.98
dbj|AB034044.1| Uncultured rumen bacterium 5C0d-11 gene for 16S rRNA, partial	0.96
dbj|AB034049.1| Uncultured rumen bacterium 5C0d-19 gene for 16S rRNA, partial	0.93
dbj|AB034109.1| Uncultured rumen bacterium 4C3d-20 gene for 16S rRNA, partial	0.94
dbj|AB034124.1| Uncultured rumen bacterium 4C28d-19 gene for 16S rRNA, partial	0.93
dbj|AB064785.1| Uncultured firmicute gene for 16S rRNA, partial sequence,	0.94
dbj|AB089046.1| Uncultured Clostridiaceae bacterium gene for 16S rRNA, partial	1
dbj|AB113692.1| Uncultured bacterium gene for 16S rRNA, partial sequence,	0.97
dbj|AB113693.1| Uncultured bacterium gene for 16S rRNA, partial sequence,	0.96
dbj|AB113731.1| Uncultured bacterium gene for 16S rRNA, partial sequence,	1
emb|AJ229189.1|UEA229189 Unidentified eubacterium from anoxic soil 16S rRNA gene clone	0.97
emb|AJ229214.1|UEA229214 Unidentified eubacterium from anoxic soil 16S rRNA gene clone	0.95
emb|AJ400239.2|UBA400239 Uncultured bacterium 16S rRNA gene, clone F16	0.98
emb|AJ400267.2|UBA400267 Uncultured bacterium 16S rRNA gene, clone F8	0.96
emb|AJ409005.1|UEU409005 Uncultured bacterium 16S rRNA gene, clone HuCB40	0.95
emb|AJ488078.1|UBA488078 Uncultured bacterium partial 16S rRNA gene, clone IB-18	0.97
emb|X89970.1|BF16SRRN1 B.fibrisolvens DNA for ribosomal RNA gene (strain NCDO 2221)	0.98
emb|X89972.1|BF16SRRN3 B.fibrisolvens DNA for ribosomal RNA gene (strain NCDO 2398)	0.96
emb|X89973.1|BF16SRRN4 B.fibrisolvens DNA for ribosomal RNA gene (strain NCDO 2432)	0.98
emb|X89974.1|BF16SRRN5 B.fibrisolvens DNA for ribosomal RNA gene (strain NCDO 2434)	0.97
emb|X89980.1|BF16SRR11 B.fibrisolvens DNA for ribosomal RNA gene (strain NCDO 2399)	0.99
emb|Y17600.1|SDE17600 Succinivibrio dextrinosolvens 16S rRNA gene, partial	0.99
gb|AF001692.1|AF001692 Unidentified rumen bacterium RC25 16S ribosomal RNA gene, partial	0.98

gb|AF001694.1|AF001694 Unidentified rumen bacterium RC2 16S ribosomal RNA gene, partial	0.98
gb|AF001695.1|AF001695 Unidentified rumen bacterium RC32 16S ribosomal RNA gene, partial	0.96
gb|AF001697.1|AF001697 Unidentified rumen bacterium RC4 16S ribosomal RNA gene, partial	0.98
gb|AF001700.1|AF001700 Unidentified rumen bacterium RC7 16S ribosomal RNA gene, partial	1
gb|AF001701.1|AF001701 Unidentified rumen bacterium RC9 16S ribosomal RNA gene, partial	1
gb|AF001702.1|AF001702 Unidentified rumen bacterium RC10 16S ribosomal RNA gene, partial	0.96
gb|AF001703.1|AF001703 Unidentified rumen bacterium RC11 16S ribosomal RNA gene, partial	0.94
gb|AF001707.1|AF001707 Unidentified rumen bacterium RC16 16S ribosomal RNA gene, partial	0.97
gb|AF001708.1|AF001708 Unidentified rumen bacterium RC17 16S ribosomal RNA gene, partial	0.98
gb|AF001709.1|AF001709 Unidentified rumen bacterium RC18 16S ribosomal RNA gene, partial	1
gb|AF001710.1|AF001710 Unidentified rumen bacterium RC20 16S ribosomal RNA gene, partial	0.96
gb|AF001716.1|AF001716 Unidentified rumen bacterium RC27 16S ribosomal RNA gene, partial	0.84
gb|AF001717.1|AF001717 Unidentified rumen bacterium RC28 16S ribosomal RNA gene, partial	0.97
gb|AF001718.1|AF001718 Unidentified rumen bacterium RC34 16S ribosomal RNA gene, partial	0.95
gb|AF001722.1|AF001722 Unidentified rumen bacterium RC36 16S ribosomal RNA gene, partial	0.97
gb|AF001733.1|AF001733 Unidentified rumen bacterium RC31 16S ribosomal RNA gene, partial	0.98
gb|AF001747.1|AF001747 Unidentified rumen bacterium RF15 16S ribosomal RNA gene, partial	0.97
gb|AF001760.1|AF001760 Unidentified rumen bacterium RF28 16S ribosomal RNA gene, partial	0.93
gb|AF001769.1|AF001769 Unidentified rumen bacterium RF38 16S ribosomal RNA gene, partial	0.99
gb|AF001776.1|AF001776 Unidentified rumen bacterium RFP18 16S ribosomal RNA gene,partial	0.94
gb|AF018443.1| Unidentified rumen bacterium JW12 16S ribosomal RNA gene, partial	0.92
gb|AF018445.1| Unidentified rumen bacterium JW16 16S ribosomal RNA gene, partial	0.94
gb|AF018449.1| Unidentified rumen bacterium JW30 16S ribosomal RNA gene, partial	0.93
gb|AF018465.1| Unidentified rumen bacterium 12-39 16S ribosomal RNA gene, partial	0.95
gb|AF018469.1| Unidentified rumen bacterium 12-51 16S ribosomal RNA gene, partial	0.87
gb|AF018479.1| Unidentified rumen bacterium 12-80 16S ribosomal RNA gene, partial	0.93
gb|AF018507.1| Unidentified rumen bacterium 30-10 16S ribosomal RNA gene, partial	0.97
gb|AF018525.1| Unidentified rumen bacterium 30-31 16S ribosomal RNA gene, partial	0.91
gb|AF018535.1| Unidentified rumen bacterium 30-41 16S ribosomal RNA gene, partial	0.96
gb|AF018545.1| Unidentified rumen bacterium JW5 16S ribosomal RNA gene, partial	0.98
gb|AF018552.1| Unidentified rumen bacterium JW23 16S ribosomal RNA gene, partial	0.96
gb|AF018553.1| Unidentified rumen bacterium JW24 16S ribosomal RNA gene, partial	0.92
gb|AF018554.1| Unidentified rumen bacterium JW25 16S ribosomal RNA gene, partial	0.98
gb|AF018556.1| Unidentified rumen bacterium JW28 16S ribosomal RNA gene, partial	0.98
gb|AF018559.1| Unidentified rumen bacterium JW33 16S ribosomal RNA gene, partial	0.95
gb|AF018566.1| Unidentified rumen bacterium 30-2 16S ribosomal RNA gene, partial	0.95
gb|AF030448.1|AF030448 Ruminococcus flavefaciens strain 4 16S ribosomal RNA, partial	0.93
gb|AF104841.1|AF104841 Ruminococcus flavefaciens strain AR72 16S ribosomal RNA gene,	0.99
gb|AF104846.1|AF104846 Ruminococcus flavefaciens strain Y1 16S ribosomal RNA gene, partial	0.97
gb|AF132250.1|AF132250 Uncultured bacterium adhufec236 16S ribosomal RNA gene, partial	0.95
gb|AF202258.1|AF202258 Eubacterium oxidoreducens strain DAS110 16S ribosomal RNA gene,	0.94

gb|AF349662.1|AF349662 Butyrivibrio fibrisolvens strain OB247 16S ribosomal RNA gene,	0.95
gb|AF371525.1| Uncultured bacterium clone p-2601-9F5 16S ribosomal RNA gene,	0.94
gb|AF371528.1| Uncultured bacterium clone p-3025-SwA5 16S ribosomal RNA gene,	0.94
gb|AF371529.1| Uncultured bacterium clone p-3133-SwA3 16S ribosomal RNA gene,	0.88
gb|AF371554.1| Uncultured bacterium clone p-214-o5 16S ribosomal RNA gene, partial	0.97
gb|AF371570.1| Uncultured bacterium clone p-416-o3 16S ribosomal RNA gene, partial	0.93
gb|AF371576.1| Uncultured bacterium clone p-247-o5 16S ribosomal RNA gene, partial	0.98
gb|AF371627.1| Uncultured bacterium clone p-2205-s959-3 16S ribosomal RNA gene,	0.94
gb|AF371655.1| Uncultured bacterium clone p-379-o3 16S ribosomal RNA gene, partial	0.98
gb|AF371676.1| Uncultured bacterium clone p-273-o5 16S ribosomal RNA gene, partial	0.95
gb|AF371748.1| Uncultured bacterium clone p-2031-s959-5 16S ribosomal RNA gene,	0.97
gb|AF371763.1| Uncultured bacterium clone p-594-a5 16S ribosomal RNA gene, partial	1
gb|AF371769.1| Uncultured bacterium clone p-2484-18B5 16S ribosomal RNA gene,	0.96
gb|AF371774.1| Uncultured bacterium clone p-878-a5 16S ribosomal RNA gene, partial	0.93
gb|AF371780.1| Uncultured bacterium clone p-2448-18B5 16S ribosomal RNA gene,	0.88
gb|AF371793.1| Uncultured bacterium clone p-249-o5 16S ribosomal RNA gene, partial	0.94
gb|AF371873.1| Uncultured bacterium clone p-2181-s959-3 16S ribosomal RNA gene,	0.89
gb|AF371875.1| Uncultured bacterium clone p-2190-s959-3 16S ribosomal RNA gene,	1
gb|AF371879.1| Uncultured bacterium clone p-1849-c3 16S ribosomal RNA gene,	0.88
gb|AF371910.1| Uncultured bacterium clone p-987-s962-5 16S ribosomal RNA gene,	0.94
gb|AF371912.1| Uncultured bacterium clone p-184-o5 16S ribosomal RNA gene, partial	0.93
gb|AF371913.1| Uncultured bacterium clone p-251-o5 16S ribosomal RNA gene, partial	0.98
gb|AF371921.1| Uncultured bacterium clone p-1026-a5 16S ribosomal RNA gene,	0.87
gb|AF385506.1| TM7 phylum sp. oral clone CW040 16S ribosomal RNA gene, partial	0.95
gb|AF407403.1| Uncultured bacterium clone RA13C3 16S ribosomal RNA gene, partial	0.93
gb|AF550610.1| Lachnospiraceae bacterium 19gly4 16S ribosomal RNA gene, partial	1
gb|AY006647.1| Uncultured rumen bacterium 16S ribosomal RNA gene, partial sequence	0.92
gb|AY006755.1| Uncultured rumen bacterium 16S ribosomal RNA gene, partial sequence	0.98
gb|AY006780.1| Uncultured rumen bacterium 16S ribosomal RNA gene, partial sequence	0.89
gb|AY006813.1| Uncultured rumen bacterium 16S ribosomal RNA gene, partial sequence	0.93
gb|AY167969.1| Swine manure bacterium 37-8 16S ribosomal RNA gene, partial	1
gb|AY179349.1| Uncultured bacterium clone WYSK 0002 16S ribosomal RNA gene,	0.96
gb|AY244881.1| Uncultured rumen bacterium clone BF3 16S ribosomal RNA gene,	0.96
gb|AY244886.1| Uncultured rumen bacterium clone BF8 16S ribosomal RNA gene,	0.88
gb|AY244902.1| Uncultured rumen bacterium clone BF24 16S ribosomal RNA gene,	0.91
gb|AY244903.1| Uncultured rumen bacterium clone BF25 16S ribosomal RNA gene,	0.99
gb|AY244904.1| Uncultured rumen bacterium clone BF26 16S ribosomal RNA gene,	0.96
gb|AY244905.1| Uncultured rumen bacterium clone BF27 16S ribosomal RNA gene,	0.91
gb|AY244919.1| Uncultured rumen bacterium clone BE1 16S ribosomal RNA gene,	0.93
gb|AY244921.1| Uncultured rumen bacterium clone BE4 16S ribosomal RNA gene,	0.96
gb|AY244922.1| Uncultured rumen bacterium clone BE5 16S ribosomal RNA gene,	0.97

gb|AY244923.1| Uncultured rumen bacterium clone BE6 16S ribosomal RNA gene,	0.96
gb|AY244925.1| Uncultured rumen bacterium clone BE8 16S ribosomal RNA gene,	0.98
gb|AY244933.1| Uncultured rumen bacterium clone BE15 16S ribosomal RNA gene,	0.94
gb|AY244952.1| Uncultured rumen bacterium clone BE36 16S ribosomal RNA gene,	0.96
gb|AY244963.1| Uncultured rumen bacterium clone BS9 16S ribosomal RNA gene,	0.97
gb|AY244965.1| Uncultured rumen bacterium clone BS11 16S ribosomal RNA gene,	0.95
gb|AY244966.1| Uncultured rumen bacterium clone BS12 16S ribosomal RNA gene,	0.95
gb|AY244968.1| Uncultured rumen bacterium clone BS14 16S ribosomal RNA gene,	0.92
gb|AY245489.1| Uncultured bacterium ML23_ANME11 16S ribosomal RNA gene, partial	0.95
gb|AY278624.1| Prevotella genomosp. C1 16S ribosomal RNA gene, partial sequence	0.96
gb|AY327235.1| Uncultured bacterium clone ZB18 16S ribosomal RNA gene, partial	0.93
gb|AY343216.1| Uncultured bacterium clone REC6M_80 16S ribosomal RNA gene, partial	0.92
gb|M62689.1|FIBRR16SH Fibrobacter succinogenes (strain HM2) 16S ribosomal RNA	0.97
gb|U37378.1|CPU37378 Clostridium proteoclasticum 16S ribosomal RNA gene	0.97
gb|U41168.1|BFU41168 Butyrivibrio fibrisolvens OB156 16S rRNA gene	0.96

Figure 1. Ordination diagram of Redundancy Analysis of samples collected during the pre-treatment period from individual steers generated from T-RFLP peaks obtained with *Hae* III digestion of PCR amplified rumen bacterial 16S rDNA plotted by previous dietary treatment.

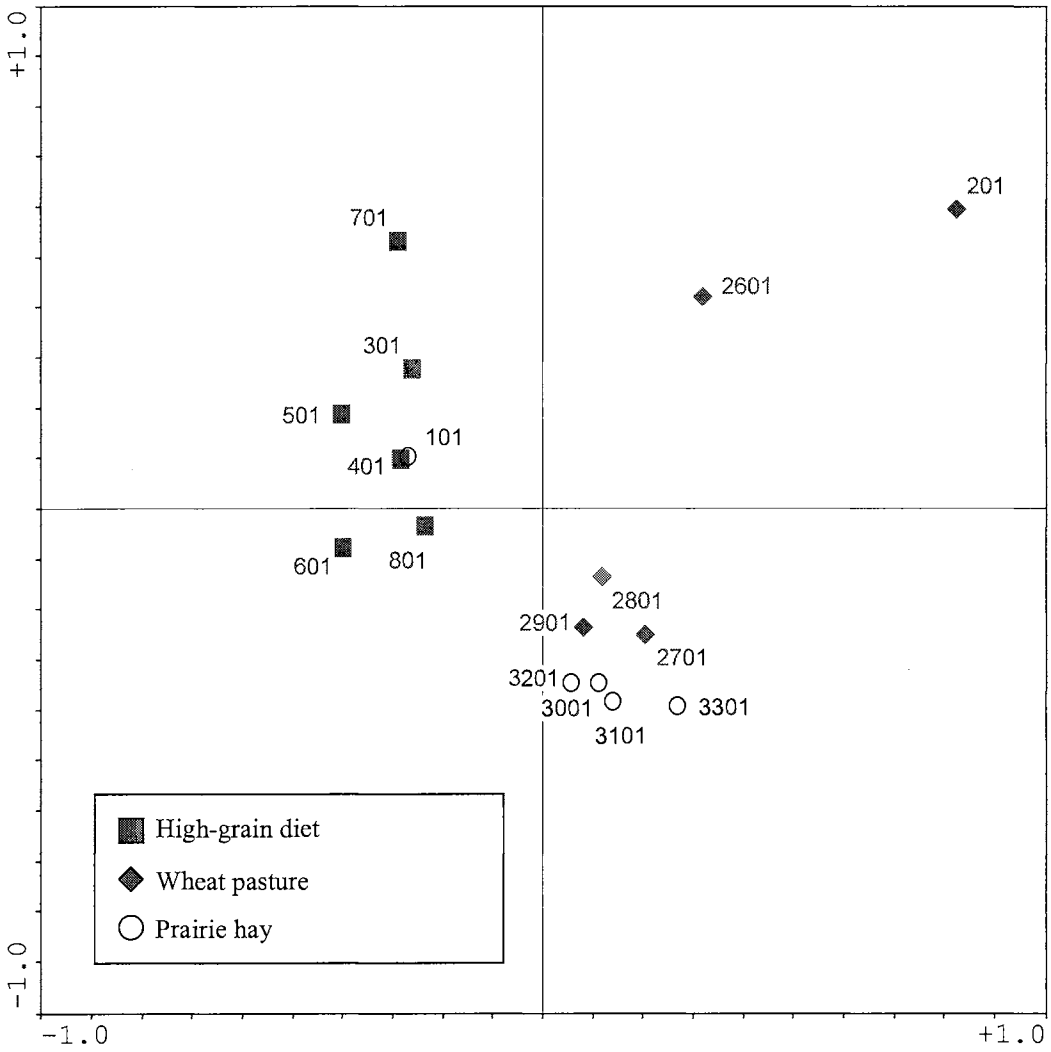


Figure 2. Ordination diagram of Redundancy Analysis of samples collected during the treatment period from individual steers generated from T-RFLP peaks obtained with *Hae* III digestion of PCR amplified rumen bacterial 16S rDNA plotted by experimental dietary treatment.

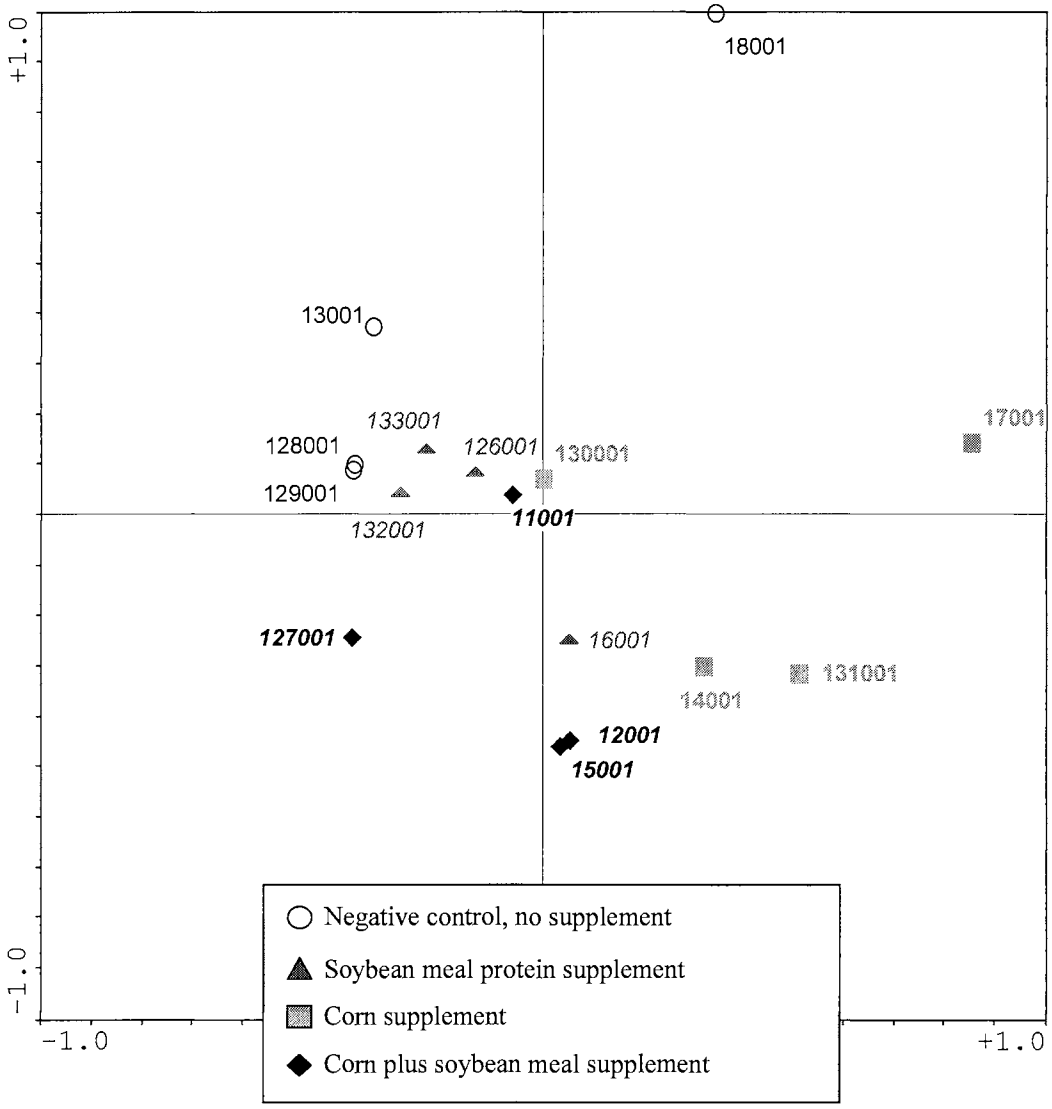


Figure 3. Ordination diagram of Principal Components Analysis of T-RFLP peaks generated from *Rsa* I digestion of PCR amplified rumen bacterial 16S rDNA with restriction fragment lengths of interest and samples collected from individual steers during the pre-treatment and treatment periods displayed.

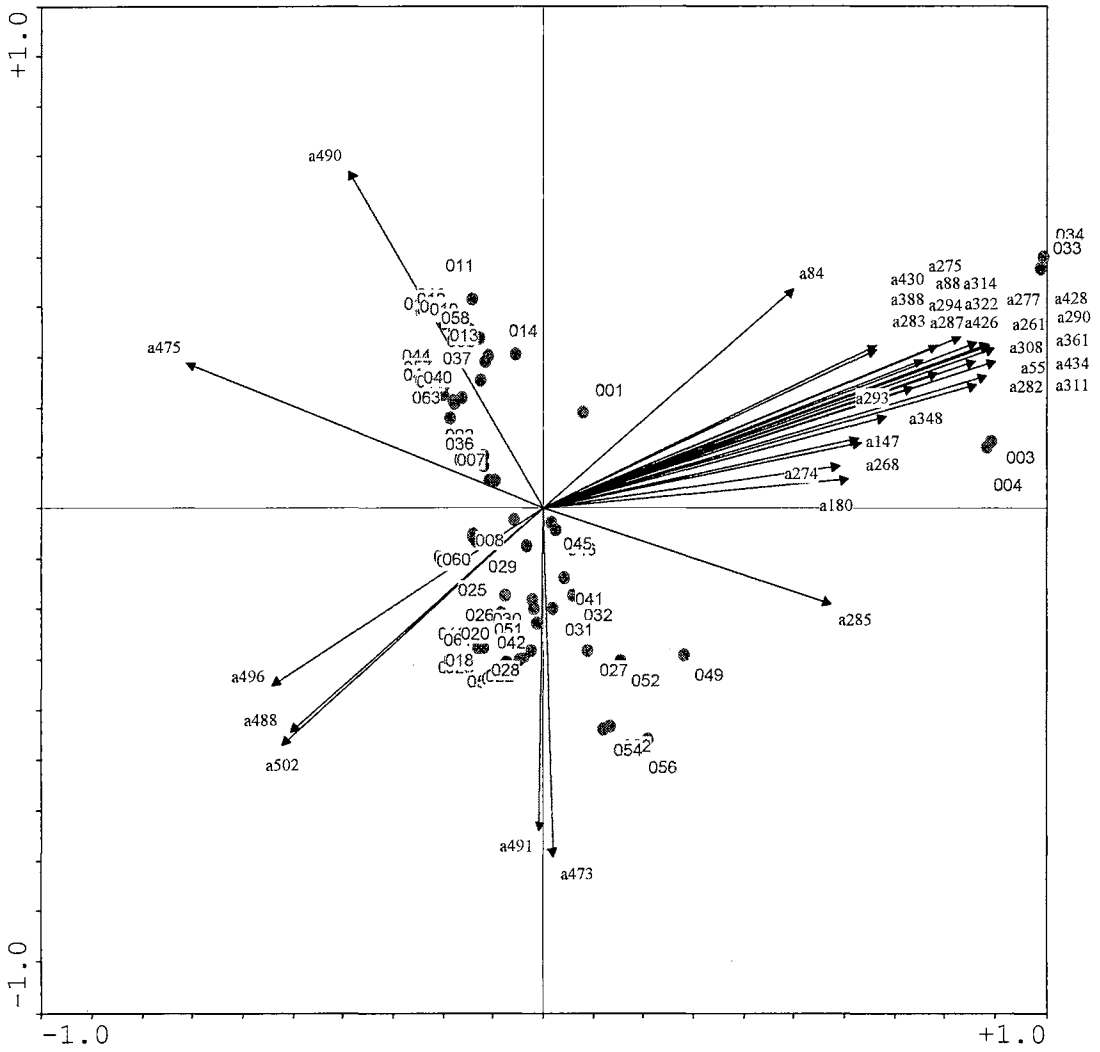


Figure 4. Ordination diagram of Principal Components Analysis of T-RFLP peaks generated from *Rsa* I digestion of PCR amplified rumen bacterial 16S rDNA from samples collected during pre-treatment and treatment periods with restriction fragment lengths of interest and environmental measures taken from both periods displayed.

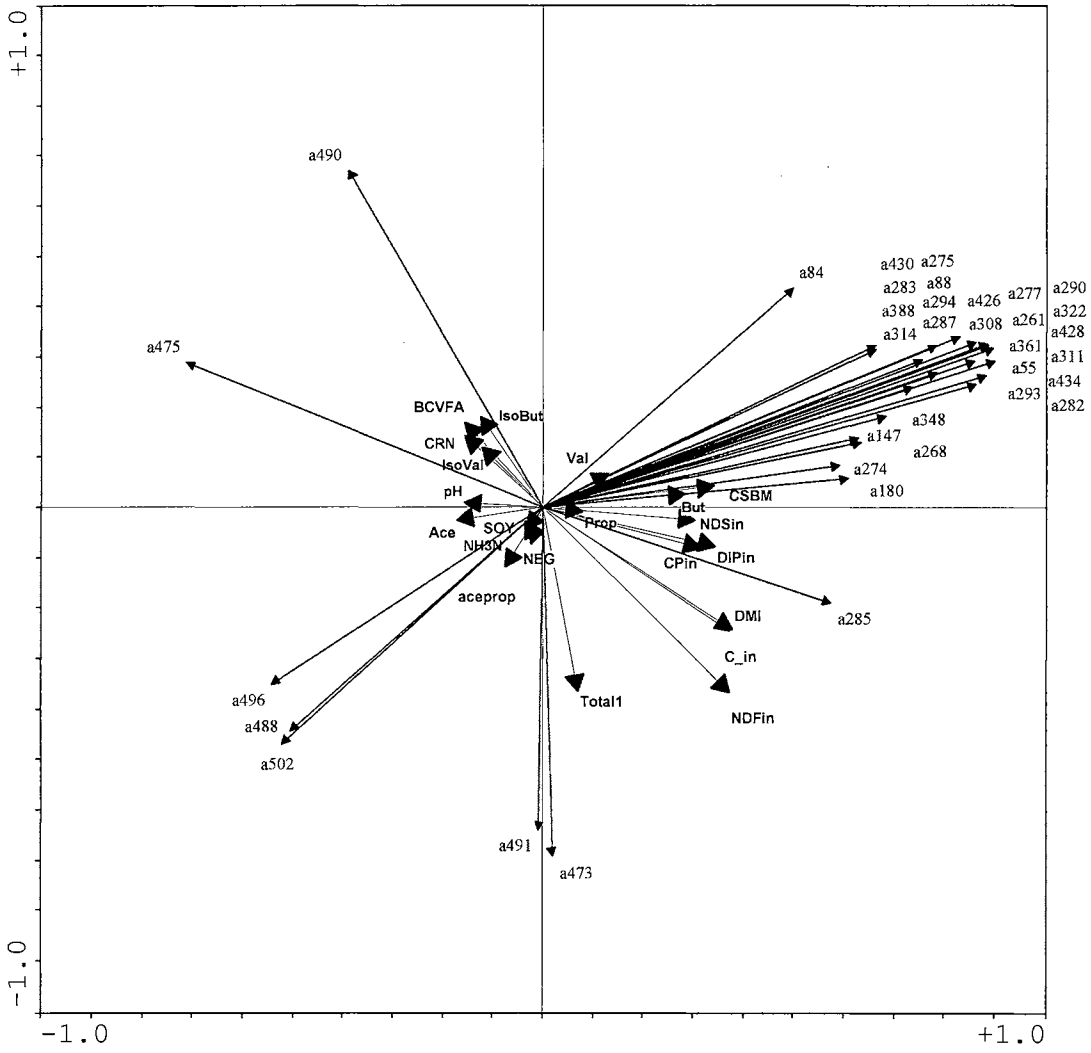


Figure 5. Ordination diagram of Redundancy Analysis of samples collected during the pre-treatment period from individual steers generated from T-RFLP peaks obtained with *Rsa* I digestion of PCR amplified rumen bacterial 16S rDNA plotted by previous dietary treatment.

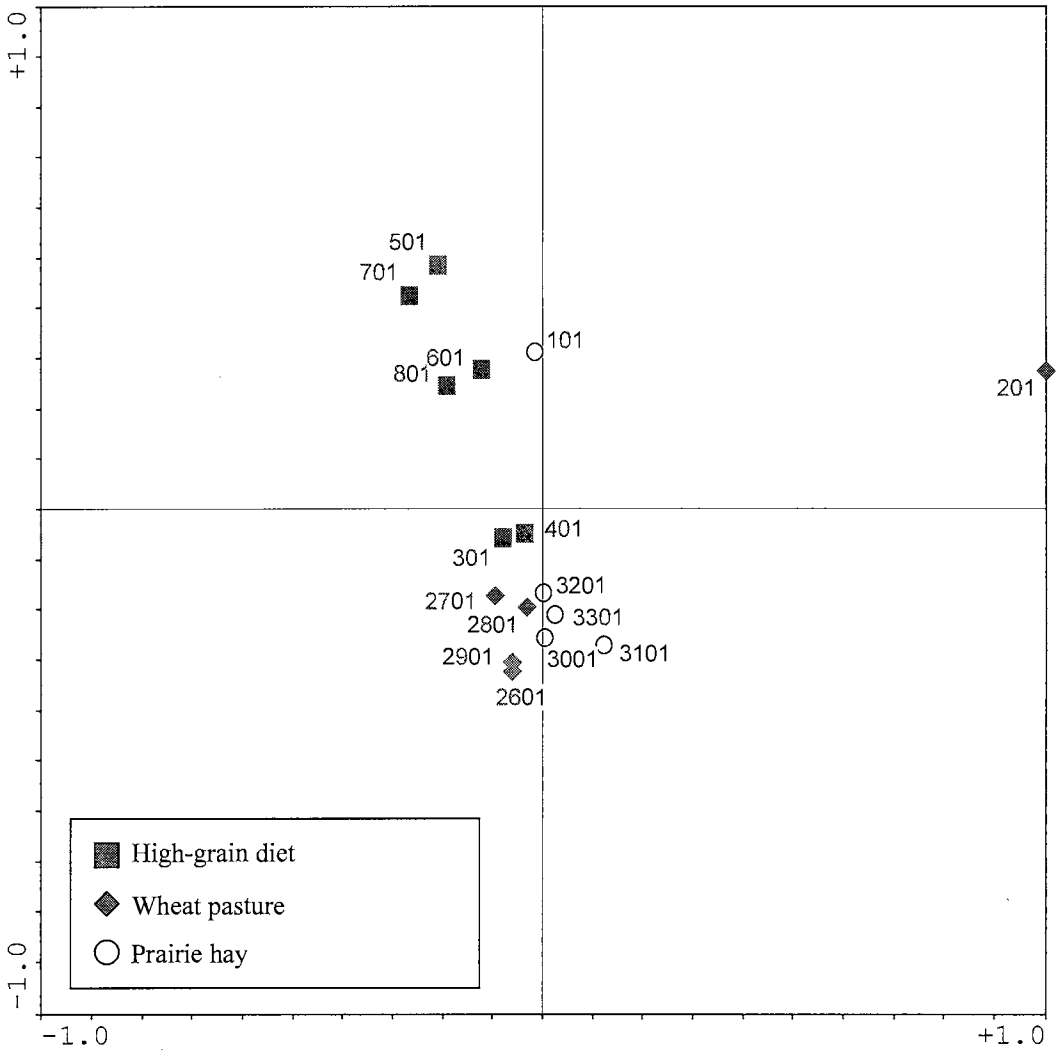


Figure 6. Ordination diagram of Redundancy Analysis of samples collected during the treatment period from individual steers generated from T-RFLP peaks obtained with *Rsa* I digestion of PCR amplified rumen bacterial 16S rDNA plotted by experimental dietary treatment.

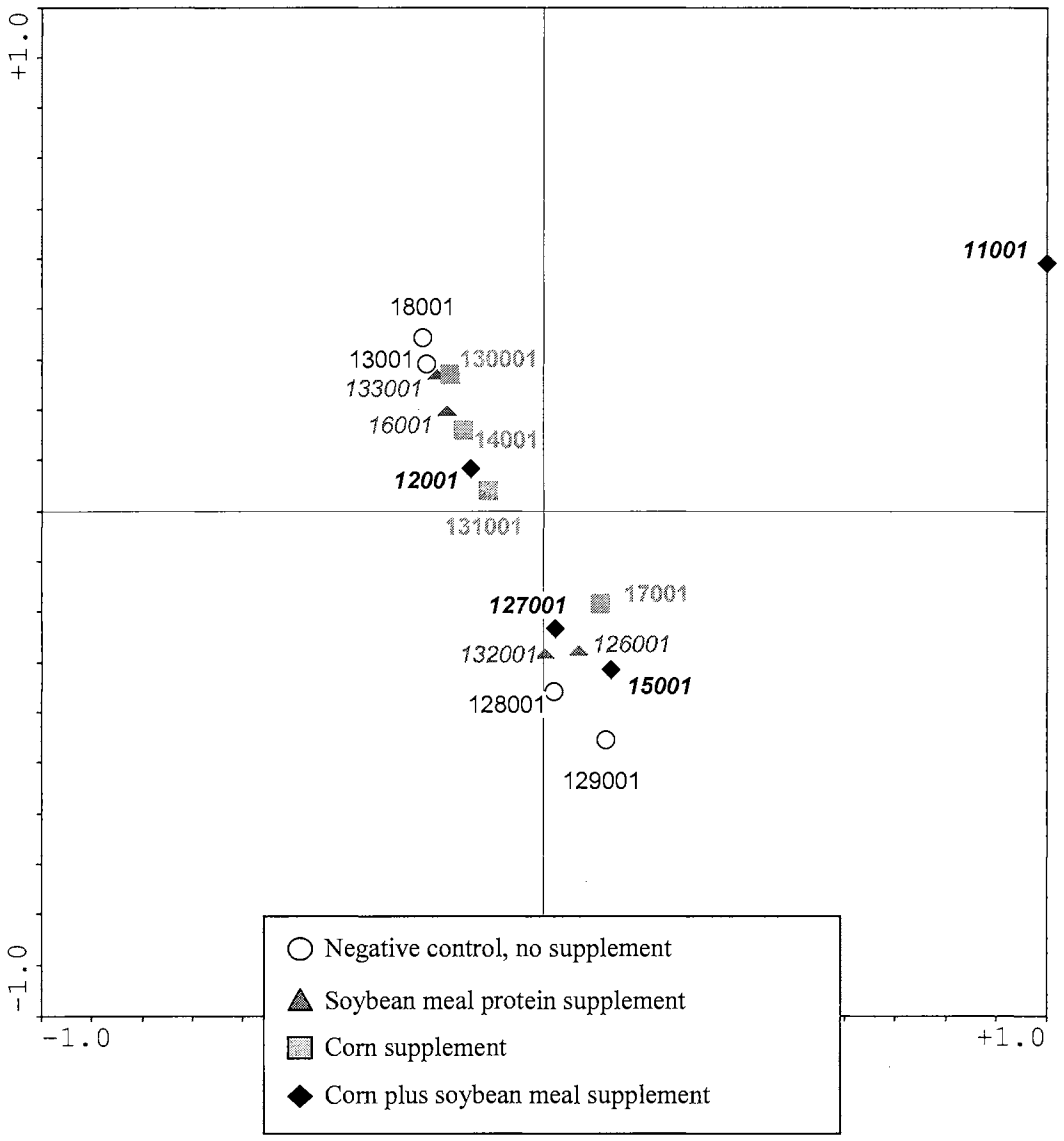


Figure 7. Ordination diagram of Redundancy Analysis of T-RFLP peaks generated from *Rsa* I digestion of PCR amplified rumen bacterial 16S rDNA from samples collected during the treatment period with restriction fragment lengths of interest and environmental measures displayed.

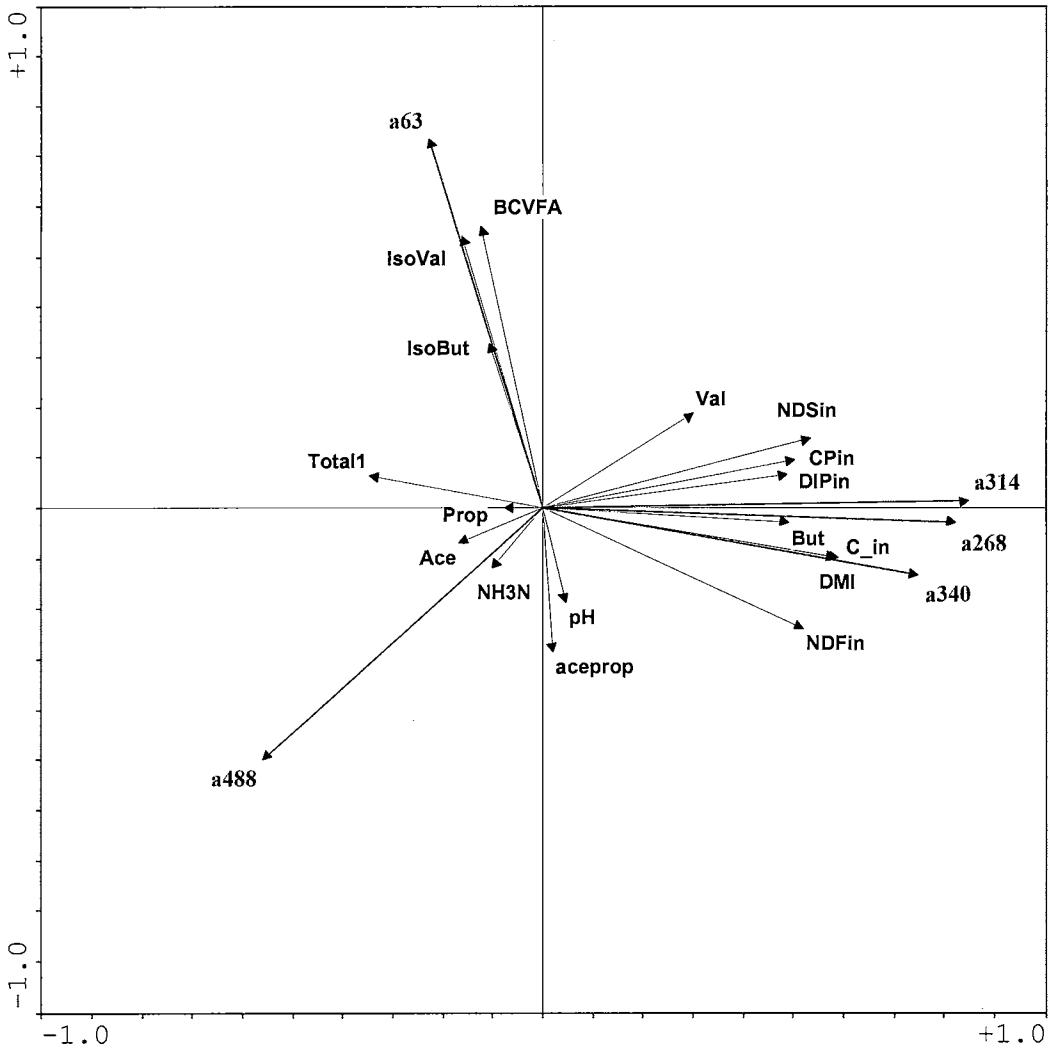


Figure 8. Ordination diagram of Redundancy Analysis of T-RFLP peaks generated from *Rsa* I digestion of PCR amplified rumen bacterial 16S rDNA showing temporal changes in ruminal bacterial communities due to dietary treatments from the pre-treatment period to the treatment period and restriction fragment lengths of interest displayed.