Abstract

Mohney, Kathryn. Synchronization of Carbohydrate and Protein Metabolism by Ruminal Microbes in Continuous Culture.

A major factor in maximizing microbial protein synthesis is the availability of energy and protein in the diet. Our objective was to determine the effect of fermentable carbohydrate and protein on microbial fermentation. Diets were formulated using three ingredients, soybean meal (SBM), ground corn (GC) and soybean hulls (SBH). Corn and SBH were used in ratios of 60:20, 40:40 or 20:60, respectively to prepare high, medium or low non-fibrous carbohydrate (NFC) diets. Soybean meal was included either unextruded (control) or extruded at low, medium or high temperature. Degradability of the N fractions in the control, low, medium and high soybean meal were 97, 80, 80 and 60%, respectively. Diets were arranged as a 3 x 4 factorial (3 levels of corn/soybean hulls and 4 levels of protein) and analyzed as a completely randomized block design. There were no statistically significant interactions seen between NFC and protein sources. Total volatile fatty acids were affected ($P<0.01$) by the NFC with 78.5, 63.2 and 71.5 mM with increasing NFC levels. The NFC level affected the acetate and butyrate whereas the protein source had an effect on the propionate. Molar ratios of acetate, propionate and butyrate averaged 60.1, 31.0 and 6.79, respectively. Varying the level of fermentable carbohydrate had a negative linear affect on ruminal pH ($P<0.01$). Extrusion did not alter pH greatly $P>0.67$). Higher extrusion temperatures altered ammonia concentrations when compared to control or low extrusion. In the low NFC diets, the medium and high extrusion increased ($P<0.10$) ammonia concentration (29.8 and 32.6 mg/dl, respectively) when compared with control and the low (18.9 and 23.4 mg/dl,

respectively). Methane concentration averaged 308 nmoles/ml and was affected by both the NFC treatment and protein source. The high and medium NFC diets increased ($P<0.01$) bacterial nitrogen percentage (9.0 and 9.5%, respectively) compared to the low NFC diet (8.7%). Data suggest that the fermentability of the structural carbohydrates in SBH was similar to the high starch corn diets. Furthermore, large differences in protein degradability did not seem to have a major impact on microbial fermentation.

Synchronization of Carbohydrate and Protein Metabolism

by Ruminal Microbes in Continuous Culture

by

Kathryn Mohney

A Thesis presented to the Graduate School
and the Animal Science Department in
partial fulfillment for the requirements
for the degree of Master of Science

Approved By:

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Dr. Vivek Fellner (Chair)

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Dr. Jack Odle (Co-chair) Dr. William Miller

Dedication

This work is dedicated to my family. My parents, Jim and Doreen Mohney for all the support they have given in all regards especially in love and encouragement to always do the best possible and never give up. I would not have been able to accomplish what I have without their unending guidance.

My sister, Robin and her husband, Don Erwin, for all the help and wisdom they provided. The unconditional understanding and love is remarkable and I feel truly blessed to have been fortunate enough to have you as my sibling.

And my future niece or nephew, may you know how much our family loves you and looks forward to helping you attempt all the dreams you set out to accomplish. Let this work be an example of how it can be done.

Biography

Kathryn Mohney was born at Fort Benning, Georgia on October 8, 1978 to the parents of Jim and Doreen Mohney. She and her older sister, Robin enjoyed attending school in different locations around the country as the family relocated often because of her father's business. She graduated from Rocky Mount Academy in Rocky Mount, North Carolina in 1996 and attended NC State University that fall. Though her exposure to any agriculture experience was essentially non-existent, it only took two weeks for Katie to decide that the Animal Science program was what she was interested in pursuing and she never thought twice about it afterwards. In May of 2000, Katie graduated Cum Laude with a Bachelor of Science in Animal Science. She spent that next summer continuing work at a research laboratory at the National Institute of Environmental Health Sciences. It was during this time that she decided to obtain her Master of Science degree from NC State University, again in the Animal Science department, with an emphasis on ruminant nutrition. Under the supervision of Dr. Vivek Fellner, Katie earned her degree in the fall of 2002. She has since moved to Manhattan, Kansas where she is currently attending Kansas State University's College of Veterinary Medicine. She will graduate as a Doctor of Veterinary Medicine in 2006.

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I would also like to thank my committee members, Dr. Jack Odle and Dr. William Miller. They have both been very helpful to me in my years here at NC State. I attribute part of my direction in life and my interests in Animal Science and the research involved to these two gentlemen. It was an honor to have them on my committee and I feel privileged that they decided to partake in those roles.

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the time spent is short and should be done with others to make the most out of life's chapters. I hope they do the same.

And finally, my family. My project would not have been completed without the time and effort spent by my family. The neverending support and love I received while earning my degree was unbelievable. They have and will always mean everything to me. I do not think I will ever be able to repay my gratitude to my sister and parents since I was fortunate to have experienced the most loving and secure environment possible. I would not be where I am today or the person I have become without the strength they provided and passed on to me.

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Introduction

The microorganisms found in the rumen consistently complicate even the simplest diet when trying to determine what to feed ruminant animals. As the agricultural industry has turned more towards profits, the ability to comprehend the rumen environment and maximize its usefulness is more important than ever. So why has it been so difficult to establish what goes on in the rumen? The different nutritional components required by the animal are met with a wide variety of possible feedstuffs. Each component of a diet is degraded in the rumen culture differently and even the individual degradation can change when included with other ingredients resulting in an interaction all due to the flora of the rumen (Van Soest, 1991). The obstacle remains due to an attempt to understand the interactions that occur with various feedstuffs while at the same time maximizing the microbial efficiency (Russell et. al., 1992). It is important to look at the main constituents that make up the ruminant diet to evaluate the digestion that occurs for each case as well as when added together. It is just as necessary to also focus on the energetics involved in the microbial populations to gain an appreciation of just how this unique complex society exists and where the possibilities exist to maximize its productiveness.

The ruminant obtains the necessary protein and energy from microbial protein and short chain fatty acids, respectively. These are produced from an environment that is energy deficient for the most part and are derived only by the ability of the microbial population to utilize the available energy and protein that comes from the diet. The energy necessary for the rumen culture is in the form of adenosine triphosphate (ATP) and is mostly produced from the fermentation of dietary carbohydrates. Without this

energy, microbial protein synthesis is not possible and dietary protein will be wasted in the form of ammonia.

In order to study the metabolism behind microbial nitrogen production, it is necessary to look at the synchrony that exists between energy and protein release in the rumen culture. Traditionally this is done in studies by altering the forage to concentrate ratio in order to maintain practicality within the diet. This is rather difficult to do however, since different forages and concentrates can have very different digestibilities. In addition, the nitrogen content of these ingredients may vary to the point of confounding the trial diets when trying to look at separate carbohydrate and protein sources. It is necessary therefore to first determine the best combination of fibrous and non-fibrous carbohydrates and the best relationship with a protein source in order to maximize microbial protein synthesis and the use of dietary nutrients within the rumen culture.

LITERATURE REVIEW

Carbohydrate

The main component of the ruminant diet is in the form of carbohydrates. This provides the majority of the energy required by the animal through the fermentation of the feed that occurs in the rumen. Most of the energy that the animal can use is derived from the microbial breakdown of simple sugars and complex carbohydrates (Van Soest, 1994).

Structural Carbohydrate

The part of the plant that makes up the fiber content is the structural carbohydrate (SC) and includes cellulose, hemicellulose and lignin (Van Soest, 1994). The ruminant is able, unlike the simple stomach animal, to breakdown the structural components primarily through the pre-gastric fermentation that occurs in the reticulo-rumen (Van Soest, 1994). The digestion of cellulose and hemicellulose is a slow process that occurs primarily by fibrolytic bacteria which break them eventually to glucose and pentoses respectively (Wolin and Miller, 1983; Leng, 1970). This results in a slow release of energy from the feed as well as faster rumen fill thereby reducing the dry matter intake of the animal (Russell et. al., 1992; Van Soest, 1994). In addition, the lower digestion rate maintains a more neutral ruminal pH and a lower passage rate from the rumen to the omasum (Allen and Mertens, 1988). Digestion of SC supports greater acetate production and is associated with greater hydrogen formation in the rumen. In order to keep the hydrogen concentration low, which is necessary for normal fermentation, a major route of hydrogen disposal is in methane production and results in lost energy to the animal (Hungate, 1966).

Non-structural Carbohydrate

The non-structural carbohydrate (NSC) in plant tissue is derived from storage carbohydrates such as starch and sugars and can be much more rapid in their fermentation (Russell et al., 1992; Sniffen et. al., 1992). This is due to greater accessibility since the NSC portion of the plant is not considered part of the cell wall and is not bound by lignin either, which impedes the rate of digestion. The rapid digestion of starch is mainly

carried out by amylolytic bacteria and has the opposite effect on the rumen environment than the slower digestion of fiber. Increased rate of digestion results in a higher rate of passage into the omasum. The higher rate of digestion has been seen to increase the total acid produced in the rumen (Van Soest, 1994). At the same time, the pH tends to decrease and while the starch fermentors can tolerate the change, the cellulolytic bacteria are negatively affected causing cellulolytic digestion to decrease since they are unable to tolerate pH values below 6.2 (Grant and Mertens, 1992; Mould and Orskov, 1983; Mould et al., 1983). The lower pH also inhibits methanogenic bacteria therefore decreasing the production of methane. The hydrogen concentration increases with the rapid fermentation and since the methanogenic bacteria are affected, the route of the hydrogen ion disposal shifts from methane to propionate production (Hungate, 1966).

Pectin

Pectin is considered a complex carbohydrate and while some include it as a component of the SC (Van Houtert, 1993), others categorize it as a NSC (Sniffen et. al., 1992). It does tend to have a more rapid fermentation rate than other SC and in many cases, feeds containing high amounts of pectin, such as soybean hulls, are used as energy sources over other more typical forages (Grigsby, 1992). It is still part of the cell wall but is more readily available than cellulose and hemicellulose. On the other hand however, many of the same species that digest the cellulose and hemicellulose of plants also break down pectin as well (Baldwin, 1965; Hungate, 1966; Leng, 1970). In addition to this, the rapid fermentation of pectin does not seem to result in the lowered pH as is seen with starch digestion (Bach et al., 1999; Van Soest et al., 1991). For this reason,

pectin containing feeds are often added to the diet to prevent problems with rumen acidosis when high concentrate diets are fed. Therefore the impact on fiber digestion should not be as severe, if any is seen at all, as compared to NSC degradation (Grigsby et al., 1992).

Volatile Fatty Acids

Volatile fatty acids (VFAs) are by products of microbial fermentation with acetate, propionate and butyrate being the main acids produced (Stevens, 1969). The energy supplied from the production of VFAs has been estimated to make up as high as 70-80 percent of the total energy required by ruminants (Van Houtert, 1993). Pyruvate is the predominant intermediate molecule in the rumen from carbohydrate breakdown (Van Houtert, 1993). It is from pyruvate that the different VFAs are derived. Pyruvate is converted to acetate through an enzymatic pathway that results in the cleavage of pyruvate to form acetate and formate (Leng, 1970). Propionate can be derived from the conversion of pyruvate to propionate via succinate or lactate (Bergman, 1990). When the pyruvate is converted to succinate, it undergoes carboxylation first to form oxaloacetate or malate, then fumarate and finally succinate. From there, the succinate is available for other bacteria to use and convert it to propionate through decarboxylation steps (Van Houtert, 1994). When propionate is derived from lactate or acrylate, it usually occurs when concentrate is included in high levels in the diet. Providing excess rapidly fermenting carbohydrates alters the metabolic pathways of the starch fermentors, such as *Streptococcus bovis*. Although total ATP production was conventionally considered the limiting factor for microbial growth, it is now believed that ATP production per unit time

is more critical. *Strep. bovis* will normally produce acetate and propionate under normal conditions but when excess starch is included in the diet it will switch from acetate and propionate production to lactate. In order to sustain growth under excess starch production of lactate results in a greater ATP production per unit time.

For acetate and butyrate an interconversion exists within the rumen (Bergman, 1965). According to Leng and Leonard (1965) and Leng and Brett (1966), about half of the butyrate formed in the rumen is derived from acetate, the rest coming directly from fermentation of ingredients in the diet. A study by Bergman et al. (1965) observed 60% of the butyrate carbon in the rumen in equilibrium with 20% of acetate carbon.

Absorption of VFAs

The VFAs are absorbed through the rumen wall via microvilli into the rumen epithelium before going into the bloodstream (McAnally, 1944). The pH within the rumen has an inverse effect on the absorption rate. As the rumen pH declines there is formation of more free formed VFAs which absorb through the wall easier. This absorption occurs as a passive gradient since the pH of the bloodstream is more alkaline than that in the rumen (Dijkstra et al., 1993; Van Soest, 1994). When the VFAs are absorbed into the rumen epithelium, some metabolism occurs before proceeding to the circulatory system. Acetate does not undergo extensive metabolism in the epithelia tissue and will in fact pass through the rumen wall relatively intact until it reaches the peripheral tissue of the body. There is some evidence that metabolism of propionate to carbon dioxide occurs in the rumen epithelia tissue as observed in the study of Weekes and Webster (1975). Propionate can go through oxidation for tissue use to form carbon

dioxide and lactate (Bergman, 1990). Other studies observed almost no metabolism of propionate in rumen epithelium due to high butyrate concentrations inhibiting the function of proponyl-CoA synthetase and that propionate remains predominantly intact until transported and converted in the liver (Ash and Baird, 1973). It has been noted that there is a higher metabolism of propionate in rumen epithelium in sheep than in cattle (Bergman, 1990). Among the main VFA, butyrate seems to undergo extensive metabolism during absorption through the rumen epithelium. Most of the butyrate that is absorbed is transformed to the ketones acetoacetate and beta-hydroxybutyrate within the epithelium (Annison and Armstrong, 1970; Van Houtert, 1993).

Metabolism within the body

The main source of glucose to the ruminant is propionate (Van Soest, 1994). Propionate is converted to glucose by gluconeogenesis in the liver and is the only VFA to take part in this pathway and result in glucose production. Glucose produced is then available for use by peripheral tissue. Some amounts of acetate as well as any butyrate not metabolized by the rumen epithelium, are anabolized by adipose and mammary tissue to form long chain fatty acids (LCFA) and cholesterol (Bell, 1981; Bergman, 1990). The majority of acetate is utilized by peripheral tissue for energy and is the main lipogenic fatty acid (Van Soest, 1994). The muscle and adipose tissue are the main peripheral locations where acetate is consumed (Bergman, 1990; McClymont, 1952). In the ruminant, acetate is primarily oxidized by the muscle for energy purposes predominantly when at rest (Bergman, 1990). In other places such as the mammary tissue however, acetate is lipogenic, contributing to the fat content in milk.

Protein

The ruminant receives the majority of its protein from microbial protein, which is derived from the rumen environment (Cotta and Russell, 1982; Owens and Zinn, 1988). The microbial population uses nitrogen and carbon sources from the diet to synthesize the microbial protein. Therefore, ruminants are supplied with both essential and non-essential amino acids in the form of microbial protein and dietary protein that escapes rumen breakdown (Leng and Nolan, 1984).

Protein available for ruminal degradation varies among feeds and the availability of N in the rumen can be altered with treatment of the feed. Protein availability in the rumen can be reduced by complexing it to non-soluble substances such as lipids or by applying heat to the feed (Leng and Nolan, 1984). Undegraded protein from the diet is classified as bypass and is a way to preserve higher quality protein for the host animal by inhibiting its digestion by the rumen microbes. Protein sources are manipulated in order to obtain bypass ability, most commonly with heat to reduce the water-soluble form of the protein in the diet (Van Soest, 1994). The treatment of protein ingredient may not in fact render the protein as true bypass, but may decrease the digestibility of the protein in the rumen enough to result in most of it passing through in tact.

The rumen bacterial population has the ability to utilize numerous nitrogen sources from the diet. Soluble protein from feeds such as cottonseed, can provide adequate protein to the animal and are found in most dairy rations. The rumen can also utilize non-protein nitrogen which supplies the necessary ammonia nitrogen to the rumen microbes but it is not considered true protein. One of the more common feed ingredients in this category is urea. It gives the microbial population nitrogen in the form of amines

or ultimately as ammonia, once broken down. Addition of individual amino acids to the diet is another possible way to supply protein to the ruminant especially for the limiting amino acids such as methionine and lysine. These can be added separately or linked together as peptides. This supplies the bacteria with high quality amino acids that can be used to synthesize other types or be incorporated into the bacterial metabolism whole (Leng and Nolan, 1984). Microbial species differ in their preference for nitrogen sources and all populations seem to be stimulated by different sources albeit with limited ability (Allison, 1979; Cotta and Russell, 1982; Yang, 2002).

The fiber digesters in the rumen are particular as to what type of nitrogen source they are able to use. The cellulolytic bacteria require nitrogen in the form of ammonia and do not utilize amino acids or peptides as well as the non-structural bacteria (Stewart et al., 1997). This could be explained partly by the time required for the cellulolytic bacteria to digest structural carbohydrate which is greater than the time the amino acids supplied by the feed remain present intact; but the remnant of the degraded amino acids, ammonia is present for extended periods (Hungate, 1966). Cellulolytic bacteria have an absolute requirement for isoacids that are essential growth factors. However, it has been observed by Russell and Sniffen (1984) as well as others (Yang, 2002) that cellulolytic bacteria lack transport mechanisms to utilize branch chain amino acids directly. Instead, the bacteria utilize the isoacids isovalerate, isobutyrate formed during normal fermentation to re-synthesize the necessary amino acids once inside the cell (Nagaraja et al., 1997).

In a study by Yang (2002), it was shown that fiber digestion was increased when branch chain amino acids were included with the diet. The digestion of fiber was even

higher when peptides that contained the branched chain amino acids were included. The cellulolytic bacteria require the branch chain volatile fatty acids (isobutyrate, valerate and isovalerate) in order to produce microbial protein (Russell and Sniffen, 1984). The isoacids, unlike the other more common VFA which are end products of carbohydrate degradation, are derived from the breakdown of dietary protein with the exception of valerate that can be produced from either protein or carbohydrate fermentation. More specifically, isobutyrate, valerate and isovalerate are formed from the branch chain amino acids valine, leucine, and isoleucine respectively (Van Soest, 1994). Yang observed increased fiber digestion when the precursor amino acids were added compared to just adding the branch chain VFA, possibly demonstrating that the SC digesting bacteria were able to utilize the amino acids. Interestingly, the addition of leucine showed more improvement in fiber digestion than the addition of valine.

Ammonia

The ammonia that the fiber digesting bacteria use can come from a variety of sources including the breakdown of dietary peptides and amino acids by other bacteria or protozoa, from recycling of urea through saliva or direct recycling of urea through the rumen wall (Owens and Zinn, 1988).

The protein derived from the diet is broken down in the rumen by microbial proteases to numerous peptide chains which can be either incorporated into microorganisms to be converted to microbial protein, or else broken further into individual amino acids (McDonald et al., 1995). The amino acids can also be used by the bacteria to synthesize microbial protein. If amino acids are in excess of the microbial

needs and are not passed from the rumen in time, they will be degraded further into ammonia and carboxylic acids (Van Soest, 1994).

The ammonia that is not utilized in the rumen is absorbed through the rumen wall or passed onto the lower tract where it is absorbed (Church, 1988). Once in the bloodstream, it is transported to the liver where it is converted to urea, a nitrogen compound that is less toxic than ammonia (McDonald et. al, 1995). The urea is then transported to either the kidneys where it is filtered out of the bloodstream and excreted as urine waste, or recycled via saliva to act as a buffer, or diffused through the rumen wall for use as a nitrogen source when rumen concentrations of nitrogen are low (Houpt, 1969). The ruminant animal contains an ever-present nitrogen pool both in the rumen and body (Van Soest, 1994). Within the rumen it is primarily in the form of ammonia, for the body, urea is the predominant form. Changes in the concentration of nitrogen both in the rumen and the blood will alter the pool sizes and affects the amount of flux through the different pools greatly (Van Soest, 1994). If a large amount of ammonia is produced in the rumen, more will be absorbed therefore creating higher turnover of the pool. As more ammonia reaches the liver the urea production will increase concomitantly. Since the ruminant body maintains its urea concentration rigorously like all mammals, an increase in physiological urea concentration will result in higher losses as urinary output.

Carbohydrate and Protein Synchrony

Feeding a diet that is balanced for optimal release of energy and nitrogen in the rumen may provide the ruminant with valuable end products. Microbial growth and protein output is dependent on the carbohydrate and protein supplied in the diet (Hoover and Stokes, 1991). Microbial protein provides a variety of amino acids and can supply the ruminant with all of them, essential as well as non-essential. It is beneficial to maximize fermentation in the rumen in order to minimize nutrient loss. By quantifying optimal fermentation and maximal synthesis of microbial protein in the rumen, it may be possible to more accurately determine microbial nutrients requirements and the remaining nutrient requirements for the ruminant (Firkins, 1996).

There are some dietary factors to consider when trying to optimize the rumen supply of nutrients. A feed high in NSC content will be a good source of rapidly fermentable energy but it is necessary to compliment it with a rapidly degradable protein source as well. In a study by Stokes et al. (1991), maximum microbial growth occurred with the narrowest ratio of NSC and degradable intake protein (DIP) in the diet that included 50 to 55% of the total carbohydrate as NSC and 13 to 18% of the total DM as DIP. Stokes et al. (1991) also noted that based on results from other studies high NSC in the diet does not result in maximal microbial production. Sniffen and Robinson (1987) suggested that energetic uncoupling with high levels of NSC in the diet may be responsible for the decrease in microbial growth. Hoover and Stokes (1991) determined that the carbohydrate digestion and microbial efficiency were correlated with the level of DIP in the diet as well. This suggests that combining rapidly or slowly degraded

carbohydrate and protein sources in order to synchronize the degradation, leads to the greatest increase in microbial yield (Herrera-Saldana et al., 1990).

Rate of carbohydrate degradation can affect microbial growth without affecting microbial efficiency (Hoover and Stokes, 1991). In reviewing numerous studies Hoover and Stokes (1991) observed that the synthesis of microbial protein improved when the carbohydrate level increased due to the greater availability of energy. It was noted by Stokes (1991) that greater carbohydrate in the diet led to greater fermentation overall and specifically better crude protein (CP) digestion. Likewise, it has also been noted that increased degradable protein in the diet enhances microbial efficiency and hence, microbial growth (Hoover and Stokes, 1991). Microbial protein synthesis appears to be further enhanced when peptides and amino acids are made available for direct incorporation into the bacteria rather than ammonia alone (Russell and Strobel, 1993). In order to make use of nitrogen, energy in the form of ATP is required. When peptides are utilized rather than needing to form them, less energy is necessary.

If synchrony of energy and protein release does not occur within the rumen, nutrients available will be wasted and therefore will be of no use to either the microbial population or the host animal. Asynchrony can occur when either the carbohydrate or protein source is in short supply for use by bacteria. In a study by Newbold and Rust (1992), it was observed that reducing sugar concentrations declined after 2 hours of feeding. It was proposed that this resulted in limiting the available carbohydrate supply for microbial growth when compared to the experiment where soybean meal was included. The authors suggested that the soybean meal in the second trial might have contributed additional carbohydrate for bacteria to use. Carbohydrate availability has an

influence on the end products of amino acid metabolism (Russell et al., 1992). The conversion of peptides to amino acids and ammonia is regulated by the release of energy from carbohydrates. When energy is limiting in the rumen, peptide nitrogen will be converted to ammonia and absorbed through the rumen wall (Russell et al., 1992).

Protein can also limit the ability of microbial growth if it is not adequately matched with a carbohydrate source. The same trial by Newbold and Rust (1992) looked at the affect on microbial growth when nitrogen source was in short supply. They found that when short term deficits occurred with the nitrogen available, bacterial growth was affected. However, no long term problems were reported due to the change in microbial synthesis. Whether this phenomenon would be true in vivo is not known.

Microbial Energetics

Ruminal fermentation involves the breakdown of nutrient sources for the purpose of microbial growth. The entire reason behind the complicated reactions, pathways and conversions is to gain enough energy to reproduce. The energy that is required for the bacteria to use is in the form of ATP. This molecule can be derived via a number of pathways within the rumen culture depending on what type of environment is present surrounding the microbial population.

The two types of carbohydrate, the main source of substrates from which ATP arises, are either SC or NSC (Russell et al., 1992). The fermentation of these two types will result in various end products including the amount of ATP produced. With SC, diets that are high in cellulose and hemicellulose, the fermentation results in high concentrations of acetate and methane, both indicative of high microbial ATP synthesis

(Hungate, 1966). The degradation of the carbohydrates in the diet follows the general path of being broken down to hexoses and pentoses and on to pyruvate through the Embden-Meyerhof pathway (Van Houtert, 1993). The fermentation of the SC is slow within the rumen culture. Because of this, the growth of the microbial population is not very efficient. A large majority of the energy that is produced is used for maintenance costs within the bacteria and therefore little is available to use for the benefits of growth (Russell et al., 1992).

As the NSC content in the diet increases, the microbial population becomes more efficient in the production of ATP and microbial protein (Russell and Stobel, 1993). With the increased available energy that is released from the readily fermentable storage carbohydrates, some bacteria, such as *Streptococcus bovis*, can alter their metabolic pathway to break down the NSC in order to generate smaller amounts of ATP quickly and use up the energy supply. Strep. bovis makes this switch so as to accommodate the rapid utilization of the carbohydrate which results in the production of lactic acid as opposed to pyruvate from the Embden-Meyerhof pathway (Russell, 1998). Along with this more rapid fermentation comes the increase in microbial efficiency. If there is a great deal more energy available for the bacteria to use, the same amount of ATP as always will be required to meet the maintenance costs of the microorganisms but there will also be more energy leftover to utilize for microbial growth (Russell et al., 1992; Russell, 1998).

If carbohydrates are in excess of what is directly useable, the bacteria may begin to store the energy in the form of polysaccharides (Russell, 1998). This has great potential for maintaining the level of microbial synthesis long after the rumen sources of

carbohydrate energy from the diet have been depleted. Kim et al., (1999) looked at altering the synchrony of carbohydrate and protein digestion in dairy cattle by offering maltodextrin and urea at different times during feeding. It was observed that if the maltodextrin was not incorporated into the bacteria and stored when it was not synchronized with urea content, the ATP generated by the rapid fermentation would be wasted and microbial protein synthesis would not be sustained.

If there is intracellular polysaccharide storage in the rumen culture, then the introduction of a nitrogen source to the culture could result in the assimilation of the nitrogen source and the utilization of the components for protein synthesis. A study by Matheron et al. (1999) looked at this possibility in *Fibrobacter succinogenes,* a fiber-digesting bacteria. When they studied the relationship between the storage of polysaccharides and the degradation of ammonia, they noticed that the bacteria changed its metabolism when nitrogen was added to the culture. The bacteria decreased the formation of the glycogen storage and in fact started to reverse it in the presence of ammonia. The microorganism, *F. succinogenes,* seemed able to increase microbial protein synthesis using its stored glycogen reserves when a nitrogen source was present within the culture.

Materials & Methods

Diet formulation

Diets were formulated to contain low (26%), medium (37%) or high (50%) levels of fermentable (non-fibrous) carbohydrates (NFC). Dietary ingredients used to achieve the three levels of NFC consisted of ground cracked corn and soybean hulls (Table 1).

Within each level of NFC, four levels of protein solubility were tested. Soybean meal was included either unextruded (control), or extruded at low (240°F), medium (280°F) and high (320°F) to provide high, medium or low degradable protein corresponding to the three extrusion temperatures, respectively. Diet formulation is shown in Table 1.

Extrusion of the soybean meal was performed at the University of Missouri using a twin-screw extruder with the three different exit temperatures. The samples were dried (50°C) in a forced air oven, stored in plastic containers and shipped overnight for use in the present study.

Diet Chemical Composition

Table 2 shows the chemical composition of the ingredients used for the diets. A Wiley Mill with a 1 mm sieve was used to grind the ingredients which were then analyzed for dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), crude protein (CP), ash and lipids. Dry matter was analyzed by drying a one gram sample in a forced air oven at 50°C overnight. Fiber content was determined using the Ankom® apparatus (ANKOM Technology Corp., Fairport, NY). Neutral detergent fiber and ADF detergents were prepared according to the procedure outlined by Van Soest et al (1991). Analysis for nitrogen content of the ingredients was performed on a Technicon Auto analyzer (Technicon Industrial Systems, Tarrytown, NY) using the modified version of the Association of Official Agricultural Chemists (AOAC) method (1990). Kjeltabs were used in place of mercury oxide to decrease polluted waste. Feed samples were placed overnight in a muffle furnace (500°C) to determine the ash content. Lipids were extracted from the feed samples using the procedure of Bligh and Dyer (1959).

Extracted lipids were methylated (Kramer et al., 1997) and purified using TLC with 1, 2-Dichloroethane as the solvent.

Ingredients were mixed in amounts indicated in the diet formulation (Table 1). Diets were mixed in amounts sufficient for feeding the continuous culture for a total of three weeks. All diets were kept in sealed bags within a cardboard container at room temperature.

Continuous Cultures

Rumen fluid was obtained from a dry, fistulated Holstein maintained on a predominantly forage diet. The inoculum was secured in pre-heated containers and transported to the lab. The contents were strained through double-layered cheesecloth, thoroughly mixed, and then added to the fermentation chambers (Teather & Sauer, 1988). Approximately 700 ml of the strained fluid were added into each fermentor. The water bath was switched on two hours prior to the addition of the inoculum to heat the chambers up to 39°C and remained on for the rest of the trial. Carbon dioxide was purged several hours before the addition of the fluid to create an anaerobic environment. The flow of CO_2 was maintained at 20 ml/min throughout the trial. Each fermentor was continuously stirred at a speed of 10 revolutions per minute. Artificial saliva, pH 6.8, was prepared using the method from Slyter et al. (1966) and added at a constant rate of 0.73 ml/min. An Orion (model 91-25; Fisher Scientific, Beverly, MA) combination pH electrode measured the culture pH levels in each fermentor. The fermentors were fed 13.5 g of dry matter in two equal amounts daily.

Each run consisted of an adjustment period of three days allowing the fermentors to adapt to their respective diets. After the dietary adaptation, sampling occurred for three consecutive days. Methane samples were taken from the headspace in the fermentors using a gas tight syringe before feeding, one and two hours after feeding and analyzed by gas chromatography (model CP-3800; Varian, Walnut Creek, CA). Ruminal culture pH was recorded at the same time as gas samples were obtained.

Five ml of ruminal culture were taken on the last three days for VFA and ammonia-N and refrigerated immediately at 4°C. An additional 10 ml of culture contents were taken on the last two days and immediately placed in –70°C for later determination of ruminal lipid profile. On the last day (d 6), fifty milliliters of fluid were obtained from each fermentor and stored at 0°C for later analysis of total nitrogen. The remaining 600ml from each fermentor was mixed with 300 ml of 0.9 percent sodium chloride solution to stop the fermentation. Each mixture was then aliquoted into 50 ml conical tubes and stored at 0°C overnight. Six tubes from each fermentor were used for isolation of total bacterial content. Overflow contents were stored in 50 ml sterile tubes and placed in 4°C for subsequent analysis of the fiber content.

Analysis

Volatile Fatty Acids

Samples taken for VFA and ammonia were allowed to settle out before two ml were taken and divided into two 2ml eppendorf tubes. The tubes were centrifuged using the IEC Micromax micro centrifuge for five minutes at 15,000 rpm. One ml was

removed from each tube and placed in either a glass vial for VFA analysis or in an eppendorf microtube for ammonia determination.

VFA samples were analyzed with a gas liquid chromatography procedure using the Varian Gas Chromatographer (model 3380, Varian Instruments, Walnut Creek, CA) with an FID detector. A fused silica capillary column with a 30m x 0.25mm x 0.25 m film thickness (Nukol™; Supelus Inc., Bellefonte, PA) was used with He as the carrier gas in conjunction with the oven temperature program of 160 C up to 178 C at 3 C per minute and an injection temperature of 250ºC. The GC-3380 was calibrated using standard mixtures of VFA. Both the ruminal samples and standards received 200 µl of meta-phosphoric acid with ethyl butyric acid as an internal standard (MIS).

Ammonia

Ammonia was analyzed based on work by G. R. Beecher and B. K. Whitten (1970). Samples for ammonia determination were diluted (1:21) with deionized water prior to analysis. Each batch of ammonia samples was run with fresh standards made using an ammonium sulfate stock solution. Standard solution was diluted to obtain 6, 8, 10, 12 and 14 µg/ml. Each sample was run in duplicate. The sample absorbencies were determined on a Bausch & Lomb Spectronic 1001 using an endpoint assay at a wavelength of 630 nm. Calculations for concentration of ammonia-N were performed using the average absorbance readings from the sample corresponding to the standard absorbance reading from that group of samples. Ammonia-N concentration was linear for the range of standards tested and included the ammonia-N in the diluted samples.

Dry Matter and Fiber

The 50 ml aliquot from each fermentor's ruminal culture content from the overflow, was centrifuged (Sorvall RC-3) for 5 minutes at 2500 rpm. The supernatant was discarded and the pellet placed in a pre-weighed aluminum pan. Samples were placed overnight in an oven at 50°C, transferred to a dessicator for 20 minutes and weighed to obtain dry pellet weight. Approximately 0.5 g of the dry pellet was placed in Ankom fiber bags in duplicate. Bags with the sample were analyzed for NDF and ADF fiber using the Ankom apparatus for fiber determination.

Bacteria Isolation

Isolation of the bacterial pellet was performed according to the procedure outlined by Bauchart et al. (1990). Samples were centrifuged at 2500 rpm for 5 minutes and the supernatant was transferred to another tube and spun at 16500 rpm for 20 minutes. The resulting pellet was re-suspended and washed in 15 ml of 0.9 percent sodium chloride solution. The pellets from all tubes (6) were combined and re-centrifuged. Supernatant was discarded and the bacterial pellets were weighed and analyzed for DM by drying in the oven (50°C overnight), as well as N content and bacterial RNA. For DM determination, the oven-dried pellets were placed in a desicator and allowed to cool to room temperature before being weighed. For N content, dried pellets were quantitatively transferred to round-bottom centrifuge tubes and shipped to University of Missouri. Bacterial N was measured by combustion according to the procedure by Leco (Leco, 1994. FP-428 N Determinator. Leco Corp., St. Joseph, Mi.).

Total Insoluble Nitrogen

The 50 ml aliquot of the fermentor contents was centrifuged as described above for the fiber procedure. The pellets were dried at 50 C and subsequently used for insoluble N determination according to the AOAC procedure (1990).

Lipid Analysis

Lipids were extracted and analyzed according to the procedure outlined for the dietary ingredients. Fatty acid composition of ruminal cultures was determined via gas chromatograph (model 3380; Varian Instruments, Walnut Creek, CA) equipped with a flame ionization detector. A fused silica capillary column (100m x 0.25mm i.d. x 0.2µm film thickness) was used (SP-2560; Supelus Inc., Bellefonte, PA) with H_2 as the carrier gas. The temperature program began at 100°C for 5 minutes, then increased at 2°C per minute to 165°C, then increased at 0.2°C per minute to 167°C and finally increased at 1°C per minute to 225°C and held there for 15 minutes.

Bacteria RNA

The wet bacterial pellet stored at 4°C was resuspended in two milliliters of a 0.9 percent salt solution. A one milliliter aliquot was used to isolate and purify RNA using the Qiagen® Mini RNA Isolation Kit (Qiagen Inc. Valencia, CA). Initial verification of the assay using known concentrations of a standard E. coli (Sigma chemicals, St. Louis, MO) indicated good recovery and purity of the bacterial RNA. Assay verification was done twice on different days with consistent results. Duplicate samples of bacterial pellets were isolated. Each time samples were run, E. coli was included as the known

standard. The Qiagen assay is recommended for use with specific groups of bacteria, namely the gram positive (+) species. However, rumen culture consists of a mixture of several different microbial populations including gram (+) and gram (-). Therefore, based on known morphological differences among microbes and limitations of the Qiagen kit, some modifications were made to the suggested assay procedure. The incubation period for the pellets in buffer containing lysozyme (Sigma chemicals, St. Louis, MO) was extended from 10 to 20 minutes. Once isolated, RNA samples were re-suspended in 50µl autoclaved dH_2O. Part of the re-suspended sample was placed in a 1:100 dilution of dH_2O in a sterile cuvette. Samples were then read on a Beckman DU 640 spectrophotometer at a wavelength of 260 and 280 nanometers to estimate the concentration and purity of the sample.

Statistical Analysis

The experiment was performed as a completely randomized block design with a three by four factorial arrangement of treatments. There were three levels of non-fibrous carbohydrates (low, medium and high) and four sources of protein (control, extruded at 240, 280 and 320), resulting in a total of 12 diets.

Four fermentors were incubated simultaneously at any one given time which constituted a run. Each fermentor was randomly assigned one of four diets representing the four protein sources at the low NFC level. A second run was initiated immediately, the following week, and diets fermented were the medium NFC. A third run was started the third week using the high NFC diets. A total of three runs constituted a block. All diets were fermented twice resulting in two blocks (n=2). Ruminal VFA, methane and

ammonia-N were analyzed on daily samples but averaged across days before analysis. Bacterial estimates and lipids were taken on a single day. Main effects of NFC and extrusion were tested using the block*NFC interaction as the error term. The slice feature of SAS was used to determine the effect of extrusion within a NFC level. Linear and quadratic effects of NFC and extrusion were also evaluated. All diets were analyzed according to the GLM procedure of SAS (SAS, 1999).

Results

Diets

Dietary chemical composition is reported in Table 2. The dry matter (DM) percentages were typical of each ingredient. All had relatively high DM but the SBM contained the higher values on average. Both the NDF and ADF measurements also resulted in typical values for most of the diet ingredients. The corn was higher than what was expected but the fiber analysis did not include amylase digestion which could have been a factor. The soybean hulls reflected the highest fiber content, much greater than either the corn or the various soybean meal components.

As would be anticipated, the higher values for crude protein were seen with the different SBM components. Soybean hulls had a higher protein percent compared to the ground corn but values were relatively similar. The ash content was across the various SBM sources and lowest for corn.

Lipid profile of individual dietary ingredients is presented in Table 4. Linoleic acid (C18:2) was the predominant fatty acid in all ingredients ranging from 49% in SBH to an average of 61% in corn and SBM sources. Corn contained 23% Oleic acid (C18:1)

and SBH contained 22% C18:1, with the SBM sources averaging 16.5% C18:1. In all ingredients, the cis isomer of C18:1 was predominant with trace amounts of the trans C18:1. Concentration of stearic acid (18:0) was less than 5% for all ingredients except SBH that contained 8% C18:0.

VFA production

Total VFA concentration (mM) was highest ($P< 0.01$) for the low NFC diets and lowest for the medium NFC with high NFC being intermediate (Table 5). The molar percentage of acetate increased slightly as NFC was included in the high level but remained similar between the low and medium NFC. Propionate however did not change from varying NFC levels. The remaining VFA values, with the exception of valerate, all decreased with increasing NFC content. Butyrate was highest for the low NFC and similar between medium and high NFC. Isobutyrate decreased significantly across all NFC diets and isovalerate decreased for the medium and high NFC diets compared to the low. Valerate was not significant across NFC levels.

Acetate production (mol/d) was lowest for the medium NFC diets and similar between the low and high NFC (Table 6) resulting in a quadratic effect of levels of NFC. The low NFC also resulted in the highest propionate production and both medium and high NFC were similar. Propionate production was significantly different for the various protein treatments, being the highest for the medium and high extruded SBM and the lowest for the control and lowest extruded protein (Figure 3). The same trend was observed for butyrate where low NFC resulted in a higher production than either the

medium or high NFC contents. A slight numerical increase between the medium and low fiber diets did occur for both propionate and butyrate but it was not significant.

Methane, Culture pH and Ammonia

Rumen culture pH maintained an inverse relationship with increasing concentrate levels in the diet, decreasing linearly, with increasing levels of NFC (Table 7). This is consistent with earlier reports (Hungate, 1966). A decrease in pH with increasing levels of NFC was accompanied by a decrease in methane production. However, methane production was observed in the high NFC diets despite a pH as low as 4.9.

There was a linear decrease in methane output with increasing levels of NFC (Table 7). In addition, extrusion also had a significant effect on methane production (Figure 4). Methane was lowest for both the un-extruded and the SBM extruded at 280°F. Extrusion at 240°F or 320°F resulted in a higher methane output.

Feeding medium NFC resulted in the lowest ammonia-N concentration followed by the high and low NFC respectively (Table 7). There was a significant effect of protein on ammonia-N concentrations (Figure 5). Within the three levels of NFC there was an increase in the ammonia-N concentration as undegradable protein increased. The most notable increase occurred between the control SBM and the medium and high extrusion temperature samples.

Microbial values

Microbial dry matter recovery was greatest with the low NFC diets and decreased non-significantly for the medium and high NFC diets (Table 8).

The medium NFC level resulted in the highest percentage of microbial nitrogen compared to either the low or high NFC diets. Microbial N percent was lowest for the low NFC diets and intermediate for the high NFC diet.

Microbial nitrogen production (g/d) reflected the opposite trend of the percent microbial nitrogen. The low NFC diets resulted in highest production of microbial nitrogen compared to either the medium or high NFC diets. This was perhaps due to the higher microbial dry matter in fermentors receiving the low NFC diets.

Microbial DM (g) per kilogram of organic matter digested and microbial nitrogen (g) per kilogram of organic matter digested resulted in a similar pattern as the microbial nitrogen production. The medium NFC level had the highest amount of DM and the low NFC the intermediate value. Neither of these microbial efficiency parameters however, were significantly affected by levels of NFC or extrusion temperature.

Digestibility

The amount of substrate used by the microbes for VFA production varied significantly for all three levels of NFC (Table 9). The medium NFC had the lowest rate of conversion with the low NFC resulting in the highest rate of substrate conversion into VFA. Looking at the substrates used for VFA plus gas production the low NFC level utilized the most substrate and the medium was lowest. The high NFC was similar to both the low and medium NFC.

Apparent fermentability, derived from the amount of substrate fermented to VFA was highest with the low NFC and lowest with the medium NFC; high NFC resulted in digestibility higher than medium but lower than the low NFC level.

Total fermentability of the diets was derived from the amount of substrate fermented to VFA plus gas (CO_2 and CH_4) (Table 9). The low NFC diet resulted in the highest fermentability when accounting for both VFA and gas and the medium NFC resulted in the lowest. However, the high NFC diet was not different from either the low or medium NFC diets.

Lipids

The lipid profile of ruminal culture fed the different diets is reported in Table 10. Non-fibrous carbohydrates or extruded soybean meal did not seem to have any affect on the major fatty acids measured in ruminal fluid. The major fatty acid across all treatments was C18:1 which comprised more than 40% of the total fatty acids. More than 70% of the total C18:1 consisted of the trans isomer. Stearic acid ranged between 22.4% and 26.9% of the total fatty acis. Linoleic acid was also not affected by either NFC level or extruded SBM and ranged between 7.8% and 10.5% of total fatty acids in the culture. The conjugated cis/trans isomer were significantly reduced with an increase in NFC level from low to medium.

RNA

The initial testing of the isolation kit with the *E. coli* standard was performed with increasing concentrations that resulted in good quality recovery of sample (Figure 1). A

second test was performed to ensure that the results could be duplicated (Figure 2). The bacteria isolated from the rumen mixed culture were assayed with the same methods, along with E. coli samples as standards. Isolation of the bacteria samples, including the standard concentrations done with each set, ended up not as consistent as the standard bacteria alone.

Discussion

Total volatile fatty acid production was contrary to our expectations; with increasing NFC content. Total VFA production has been shown to increase with increasing NFC levels (Van Soest, 1994). Hoover and Stokes (1991) reported an increase in total VFA concentrations with an increase in NFC content of the diets. The discrepancy between our results and those reported earlier may be related to the nature of dietary ingredients. In the present trial, soyhulls were used as a source of fibrous carbohydrates. However, we know that soyhull fiber is rapidly and extensively degraded in the rumen and including it in the high NFC diet seemed to have supported vigorous fermentation.

Similarly, production of individual VFA was also unexpected with the increase in the non-fibrous content of the diet. Acetate remained significantly high even with the high NFC level while the propionate did not change. Acetate is considered to be related to fiber digestion in rumen fermentation since production of it typically increases with high fiber diets (Hungate, 1966). Propionate increases with diets high in NFC particularly when corn is included as the NFC source (Van Kessel and Russell, 1996). High NFC levels have been reported to increase propionate production while either not

changing or decreasing ruminal acetate (Hungate, 1966), therefore decreasing the acetate to propionate ratio. The high pectin content in the soyhulls may have contributed to altering the fermentation of the culture. Pectin has been shown to ferment differently than other non-structural carbohydrates because it is degraded to pentoses and trioses rather than hexoses (Prins, 1977). It has also been shown to have as an end product, acetate, which could explain the concentration of acetate remaining similar across all NFC levels even when soyhulls were included at only 20 percent of diet dry matter. Grigsby et al. (1992) suggested that soyhulls, although readily fermentable, do not result in the formation of the detrimental lactic acid and are perhaps better suited for maintaining fiber digestion than other typical fibrous feeds.

The isoacids were significantly affected by increasing NFC content in the diets. In particular, concentrations of isobutyrate were undetectable in the high NFC levels. Isoacids are formed from the breakdown of protein rather than the carbohydrate in the diet (Yang, 2002). They are essential growth factors for the cellulolytic bacteria because these bacteria lack the transport mechanisms to absorb the pre-formed amino acids from which they are derived (Russell and Sniffen, 1984). Once absorbed by the bacteria, they are then converted back to the amino acids to be used within the cell. The low concentrations of these acids with the higher NFC level therefore negatively affected the cellulolytic bacteria in this study. Another important VFA to observe is valerate. This is a unique VFA because it can be produced from the degradation of either carbohydrate or protein (Van Soest, 1994). The concentration of valerate remained consistent throughout the NFC levels as well as the protein sources. This could potentially be due to a shift in the source of valerate production from carbohydrate and protein degradation.

Increasing NFC content resulted in a typical decrease in pH and methane production in the rumen cultures. The pH level is directly associated with the dietary content of NFC and their rapid rate of fermentation. Normal rumen function with a high roughage, low concentrate diet will maintain a pH between 6.5 and 6.8. As the concentrate level in the diet increases, the pH in the rumen culture decreases (Anderson et al., 1988) due to the increase in rapidly degradable starch and sugars in the diet and subsequent increase in lactic acid. When readily fermentable carbohydrate is in excess, the pH will rapidly drop potentially causing a problem of metabolic acidosis for the animal (Van Soest, 1991). This occurs primarily due to a shift in bacterial metabolism of excess carbohydrate from a propionate production to a predominantly lactic acid production. This shift towards lactic acid formation is related to bacterial growth. Production of lactic acid from excess, rapidly fermentable carbohydrates results in a higher ATP yield per unit time (Van Houtert, 1994. The bacteria that cause the pH to drop alleviate the competition of substrate by eliminating other bacteria that cannot tolerate a low pH. In addition however, pH below 6.0 is considered to be detrimental to methanogenic bacteria and if it drops below 5.0 methane production is inhibited (Van Kessel and Russell, 1996). The production of methane is a major route for the rumen culture to dispose of hydrogen ions that are produced during fermentation. Methane output did decrease linearly with a drop in pH. Interestingly however, the pH remained consistently low across all diets but methane production was maintained, albeit in smaller quantities, even at a pH of 4.9. A pH of 4.9 is lower than the minimum pH essential for methane production (Van Kessel and Russell, 1996; Bach et al., 1999). Although we did not measure lactic acid production, it seems that soyhulls, while being rapidly

fermentable, maintained high levels of acetate production resulting in a ready supply of hydrogen equivalents as substrate for methanogenic bacteria. It is also possible that soyhulls altered microbial populations resulting in altered metabolic pathways and end product formation.

There was a general trend for ammonia concentration to increase with decreasing protein availability. This increase occurred predominantly with the low NFC diet. This increase in ammonia was contrary to expectations since ammonia should decrease with increasing extrusion temperatures. Ammonia is derived from the degradation of dietary protein by microbes in the rumen culture (Leng and Nolan, 1984). As the protein is degraded to ammonia and amino acids, they are used by microorganisms to form microbial protein. Therefore an increase in the concentration, such as what was observed with the low NFC diet, suggests an increase in the production of ammonia, i.e. higher protein degradation. Higher extrusion temperature is meant to render the protein more unavailable to microbes. It is unusual therefore to have ammonia concentration actually increase with increasing extrusion temperature.

It is possible that pH played a role in the concentration of ammonia within the rumen culture. The lower pH associated with the high NFC diet may have had a detrimental effect on the ammonia producing bacteria hence the lower concentration of ammonia with this NFC level. The higher pH with the high soyhull content may have alleviated the effect and thus resulted in higher ammonia production.

The high concentration could also be an indication of a decrease in the utilization of the ammonia which can occur if amino acids are the preferred nitrogen substrate for the bacteria. The idea of amino acids as a preferred source of nitrogen has been reported

in numerous studies where microbial protein synthesis was increased when amino acids were included in a diet already containing urea (Maeng et al., 1976; Cotta and Russell, 1982). Oldham (1980) proposed that slow protein degradability in the rumen could potentially increase microbial protein synthesis due to the slow release of the amino acids. This implies that the bacteria within the culture are either unable or prefer not to utilize the ammonia that becomes available over time. It has also been shown that simply increasing the supply of N as ammonia will not always increase microbial protein output and, that the source of carbohydrate affects the optimal concentration of ammonia for maximal microbial protein synthesis (Satter and Slyter, 1974).

It is well documented that soybean hulls are rapidly fermented in rumen culture (Van Soest, 1991) as was evidenced in the low NFC diet in the present study. It is likely, therefore that the high soyhull diet (low NFC) was rapidly degraded and the release of energy to the bacterial population was not synchronized by the protein that was more slowly degraded. Odle and Schaefer (1987) noted that changing the chemical or structural components of the carbohydrate substrate without altering the protein source will change the optimal concentration of ammonia necessary to maximize microbial protein synthesis. There could however, be a point where higher ammonia concentrations are no more useful to the bacteria then what was already supplied therefore allowing for higher It seems that the medium NFC level provided the best balance of readily available carbohydrates to ensure optimum use of the proteinThis optimal level of NFC in the medium diet may also explain the low ammonia concentration irrespective of protein sources.

The average microbial dry matter (DM), percent was 4.27, and tended to be higher than values published earlier (Stokes et al., 1991). The DM of the rumen culture was verified on two separate occasions to confirm the higher bacterial DM numbers. Medium NFC level resulted in the highest microbial nitrogen percent, and although not significant, it resulted in numerically higher microbial production both with dry matter and nitrogen produced per gram of organic matter digested. It appears that the combination of readily fermentable soyhulls with the corn in the medium NFC diet resulted in the most favorable release of energy for the microbial population to utilize for growth. In the study by Grigsby et al. (1992) increasing soyhull content in the diet resulted in higher microbial nitrogen flow to the duodenum of cattle.

Based on stoichiometric estimates, more substrate was used for VFA production in the low NFC diet and less in the medium NFC diet. However, when the substrate used for gas was included with the VFA production, apparently more gas was produced with the medium level of NFC since total substrate utilized was not significantly different between the high NFC and medium NFC. The low NFC diet used the most substrate for VFA as well as gas production. Fermentability of various diets was calculated from the amount of substrate used for VFA (apparent) and VFA and gas (total) and followed the same trend as substrate use discussed above.

There was no effect of either the NFC levels or protein sources on fatty acid content. Stearic acid, C18:0, increased in the rumen culture as a whole when compared to its concentration in the diet. As expected, the unsaturated linoleic acid (18:2) followed the opposite trend and declined within the culture. Oleic acid, C18:1 was the predominant fatty acid in the culture and consisted mainly of the trans isomer. Although

dietary treatments did not affect biohydrogenation activity, the nature and profile of biohydrogenation end-products was not similar to results reported earlier using a typical forage to concentrate diet (Fellner et al., 1995). In the present study, C18:0 comprised less than 30% of the total fatty acids compared to almost 50% in earlier reports (Fellner et al., 1995). It also seems that a very large proportion of fatty acids consisted of the trans C18:1. Irrespective of dietary treatment, biohydrogenation occurred in ruminal cultures however formation of C18:0 was inhibited and production of C18:1 trans was enhanced. A greater proportion of Linoleic acid remained intact when compared to earlier reports. However, previous studies that reported fatty acid profiles and biohydrogenation activity used diets comprising of alfalfa hay as the predominant forage source.

The RNA isolation assay was accurate when used with the pure *E. coli* standards (Figures 1 and 2). The protocol assumes that pure cultured bacteria, either gram negative or gram positive will be processed with the assay. It does make a change in the protocol to extend the incubation of the lysozyme for the gram negative bacteria. It was with this in mind that the time was extended longer for the ruminal mixed culture. It was apparent however, that the mixed culture resulted in inconsistencies most likely due to the variation of species within the innoculum.

It is possible that the soyhulls altered the fermentation process in the rumen culture having a positive effect on the bacteria even with the high NFC diet. Grigsby et al. (1992) surmised that when soyhulls were included in a ration, all the microorganisms within the culture had been supported or perhaps that the fiber digesting bacteria had been able to continue fermenting more efficiently. This possible explanation is further supported by the fact that the soyhulls despite providing vigorous fermentation did not

decrease the pH as a starch feed would and as noted in numerous studies (Anderson et al., 1988; Van Soest, 1991; Grigsby, 1992).

The readily fermentable soyhulls could have allowed the energy release from the diet to be uncoupled from the protein in the low NFC diet. The high concentration of ammonia with the high extrusion soybean meal shows the potential of limited ability to utilize the ammonia for microbial protein synthesis. This possibility was seen in the study by Haig et al (2002) using soluble intake protein at various amounts and was also predicted using the Cornell net carbohydrate and protein system within the same study.

Conclusion

The chemical composition of the dietary ingredients did not differ greatly and were similar to values reported in the NRC. The soybean hulls reflected the expected higher fiber content when compared to the ground corn although both seemed to degrade similarly in the culture. There is evidence to suggest that perhaps soybean hulls were in fact fermented more rapidly and resulted in a unique fermentation where the acetate production was supported even at the high NFC levels. Including the soybean hulls at the low level of 20% seemed to alleviate the negative effects of high starch.

Soybean hulls have been used in various trials as an energy supplement despite the high NDF content (Bach et al., 1999). Pectin that is found in large quantity within soybean hulls could be considered in the category of structural carbohydrate. It is easily accessible however which is what allows for the rapid fermentation of soybean hulls to occur in the rumen. The digestion of pectin differs from what is usually thought of with energy supplement feeds (Van Houtert, 1994). The pH does not tend to decrease with

higher amounts of pectin in the diet unlike with supplements such as corn added to the diet (Van Soest, 1994). This therefore allows for the cellulolytic bacteria, which are some of the most sensitive organisms with regard to pH, to continue digesting the fiber within the diet. Indeed, Grigsby et al. (1992) noted that the highest NDF digestion occurred when soybean hulls were added to bromegrass hay diets.

The use of soyhulls and corn as our carbohydrate sources eliminated the confounding effects of altered protein content when forage and concentrate levels are changed to achieve different NFC levels. Altering the NFC levels in practical diets is very difficult due to the confounding effects of changing the protein content. Our diets are by no means practical, however in order to fully understand synchrony of carbohydrate and protein in the rumen our diets provided least variation in other parameters. Extrusion did affect nitrogen degradability at higher temperatures. However, differences in nitrogen degradability and extrusion did not have an impact on ruminal fermentation. The resulting differences in the study were not obvious however, and led to the conclusion that there was not a large variation among protein sources.

Varying NFC levels had a greater impact on ruminal fermentation compared to protein levels. Providing NFC at the medium level (37%) resulted in the greatest response. The substrate digested, microbial production, and ammonia concentration indicate that the most protein synthesis occurred at the medium NFC level. By synchronizing the release of energy with a protein source, the diet can potentially minimize the effect that the rumen fermentation has on the waste products of the ruminant by ensuring that the optimal utilization of nutrients occurs within the culture. It would be beneficial to synchronize diets for many reasons including maximizing the

nutrients going towards animal use, minimizing waste, and alleviating waste problems on farms.

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Table 1. Composition of experimental diets.

	Non-Fibrous Carbohydrate (NFC)											
	Low (26%)[1]				Medium (37%)[1]				High (49%)[1]			
	Protein				Protein				Protein			
	C	H	M	L	C	H	M	L	C	H	M	L
Soybean Hulls, %	60	60	60	60	40	40	40	40	20	20	20	20
Ground Corn, %	20	20	20	20	40	40	40	40	60	60	60	60
Soybean Meal, %												
Un-extruded (C)	20	-	-	-	20	-	-	-	20	-	-	-
Extruded 240°F, % (H)	-	20	-	-	-	20	-	-	-	20	-	-
Extruded 280°F, % (M)	-	-	20	-	-	-	20	-	-	-	20	-
Extruded320°F, % (L)	-	-	-	20	-	-	-	20	-	-	-	20

[1]Based on NRC values (2001).

Table 2. Chemical composition of dietary ingredients (n=2).

	Dry Matter (%)	NDF (%)	ADF (%)	CP (%)	Ash (%)	Total Lipid (%)	NFC[1] (%)
Corn	89.03	24.37	4.90	10.31	1.31	5.18	58.83
Soybean Hulls	89.53	67.84	47.49	12.66	4.13	1.02	14.35
Soybean Meal							
Unextruded	90.51	11.99	4.19	53.93	6.89	4.47	22.72
Extruded SBM 240°F	88.45	14.14	4.28	55.86	6.71	3.04	20.25
Extruded SBM 280°F	91.22	12.63	4.26	54.12	6.62	5.6	21.03
Extruded SBM 320°F	93.18	13.52	4.32	53.46	6.79	4.39	21.84

[1] NFC = 100 – (% NDF + % CP + % Ash + % Total Lipid)

Table 3. Nitrogen and non-fibrous carbohydrate fractions of soybean hulls and corn[1].

	SBH	Corn
N Fraction, %		
A	23	24
B	72	72
C	5	4
Kd of B, %/hr	6	5
NFC Fraction, %		
Sugars	19	20
Starch	19	80
Pectin	62	0

[1]NRC, 2001.

Table 4. Lipid profile of dietary ingredients (n=2).

	Fatty Acid (FA)				
	C 16	C 18	C 18:1t	C18:1c	C18:2
			% of Total FA[1] g/100 g of Total FA		
Corn	11.36	4.42	0.46	22.61	60.96
SBH	20.72	7.84	0.33	21.4	48.91
Unext SBM	19.17	4.75	0.18	15.79	60.0
Ext 240	17.88	4.82	0.10	16.57	61.04
Ext 280	17.34	4.77	0	16.72	61.04
Ext 320	17.35	4.68	0.11	16.4	61.3

[1]Total FA consisted of C14 to C20.

Table 5. Concentrations of volatile fatty acids (VFA) in continuous culture fed diets differing in NFC content and protein solubility.

	NFC level				Extrusion temperature, °F					[1]P<	
	Low	Med	High	SE	Unext	240	280	320	SE	NFC	Protein
Total VFA, mM	78. 5[a]	63.2[b]	71.5[c]	2.2	69.1	71.7	71.5	71.9	2.54	< 0.01	0.85
Individual VFA, mol %											
Acetate	57.2[a]	59.4[a]	63.8[c]	1.53	62.3	61.7	58.6	57.9	1.76	0.04	0.26
Propionate	30.6	32.3	30	1.32	30.0[ab]	27.7[a]	33.4[b]	32.9[b]	1.52	0.49	0.08
Butyrate	8.3[a]	6.4[b]	5.6[b]	0.51	6.1	8.0	6.3	6.9	0.59	0.01	0.17
Valerate	0.88	0.86	0.69	0.191	0.7	1.1	0.6	0.8	0.22	0.76	0.34
Isobutyrate	0.42[a]	0.16[b]	0[c]	0.04	0	0.33	0.21	0.29	0.062	0.01	0.27
Isovalerate	2.02[a]	0.62[b]	0.59[b]	0.219	0.7	1.3	1.1	1.2	0.25	< 0.01	0.35

1 No interaction observed.

a, b, c Means with different superscripts differ. P values are tabulated.

Table 6. Production of volatile fatty acids (VFA) in continuous culture fed diets differing in NFC content and protein solubility.

	NFC level				Extrusion temperature, °F					[1]P<	
	Low	Med	High	SE	Unext	240	280	320	SE	NFC	Protein
VFA mmol/d											
Total VFA	78.5	63.2	71.5	2.2	69.1	71.7	71.5	71.9	2.54	<0.01	0.85
Acetate	47.4[a]	39.7[b]	47.6[ac]	1.55	45.6	46.4	44.1	43.6	1.79	0.01	0.68
Propionate	25.3[a]	21.3[b]	22.5[bc]	1.04	21.6[a]	21.0[a]	25.0[b]	24.7[b]	1.20	0.06	0.09
Butyrate	6.7[a]	4.3[b]	4.6[bc]	0.48	4.3	6.1	4.8	5.6	0.56	0.01	0.17
Valerate	0.72	0.53	0.49	0.129	0.54	0.79	0.42	0.57	0.149	0.43	0.41
Isobutyrate	0.32[a]	0.10[b]	0[c]	0.048	0	0.25	0.16	0.23	0.057	0.026	0.36
Isovalerate	1.6	0.4[a]	0.5[a]	0.18	0.49	1.03	0.85	0.95	0.211	<0.01	0.34

1 No interaction observed

a, b, c Means with different superscripts differ. P values are tabulated.

Table 7. Methane, pH, ammonia, nitrogen, acid detergent and neutral detergent fiber in continuous culture fed diets differing in NFC content and protein solubility.

	NFC level				Extrusion temperature, °F					[1]P<	
	Low	Med	High	SE	Unext	240	280	320	SE	NFC	Protein
DMI, g/d	13.5	13.5	13.5	--	13.5	13.5	13.5	13.5	--	--	--
Ruminal pH	5.7[a]	5.3[b]	4.9[c]	0.12	5.4	5.2	5.3	5.4	0.14	0.01	0.67
Ammonia-N, mg/dl	26.2[a]	15.8[b]	20.5[c]	1.62	16.4[a]	20.5[ab]	21.5[b]	25.0[b]	1.87	< 0.01	0.06
Methane, mmol/d	11.0[a]	8.7[b]	6.9[c]	0.45	7.3[a]	9.8[b]	8.2[a]	10.1[b]	0.52	< 0.01	0.02
Insoluble Nitrogen, %	1.8	1.5	1.5	0.4	1.7	1.8	1.5	1.5	0.13	0.15	0.29
NDF, %	62.5[a]	49.4[b]	53.6[b]	1.65	54.4	55.6	55.9	54.6	1.91	< 0.01	0.93
ADF, %	38.7[a]	28.7[b]	29.6[b]	1.89	32.1	33.6	31.3	32.2	2.18	< 0.01	0.90

1 No interaction observed

a, b, c Means with different superscripts differ. P values are tabulated.

Table 8. Microbial yield and efficiencies of bacterial synthesis in continuous culture fed diets differing in NFC content and protein solubility.

	NFC level				Extrusion temperature, °F					[1]P<	
	Low	Med	High	SE	Unext	240	280	320	SE	NFC	Protein
Microbial DM, g/d	37.3	36.2	24.4	5.43	28.8	26.1	35.7	39.8	6.26	0.23	0.43
Microbes, % N	8.7[a]	9.5[b]	9.0[c]	0.08	9.13[a]	8.77	9.24[ab]	9.15[ab]	0.098	<0.01	0.03
Microbial N, g/d	3.2	3.5	2.2	0.52	2.6	2.3	3.3	3.7	0.60	0.25	0.37
Microbial DM, g/ OM digested, kg	613.0	889.5	411.3	123.79	646.1	429.7	798.5	677.4	142.94	0.19	0.60
Microbial N, g/ OM digested, kg	52.3	84.2	37.5	11.61	57.6	37.8	74.0	62.5	13.41	0.16	0.56

1 No interaction observed

a, b, c Means with different superscripts differ. P values are tabulated.

Table 9. Fermentability of diets fed to continuous culture fed diets differing in NFC content and protein solubility.

	NFC level				Extrusion temperature, °F					[1]P<	
	Low	Med	High	SE	Unext	240	280	320	SE	NFC	Low
Substrate used, g/d											
VFA	5.3[a]	4.3[b]	4.9[c]	0.14	4.71	4.87	4.92	4.94	0.164	<0.01	0.76
VFA + Gas	7.6[a]	6.4[b]	7.2[ab]	0.32	7.0	7.6	6.7	7.0	0.38	0.07	0.62
Digestibility, %											
Apparent (OM)	39.4[a]	32.2[b]	36.5[c]	1.05	34.9	36.1	36.4	36.6	1.21	<0.01	0.76
True (NDF)	56.2[a]	47.0[b]	53.7[ab]	2.41	51.6	55.7	49.8	52.1	2.78	0.07	0.62

1 No interaction observed

a, b, c Means with different superscripts differ. P values are tabulated.

Table 10. Ruminal lipid profile of continuous culture fed diets differing in NFC content and protein solubility.

	NFC level				Extrusion temperature, °F					[1]P<	
	Low	Med	High	SE	Unext	240	280	320	SE	NFC	Protein
C 16:0	14.7	15.5	16.0	0.57	14.6	16.0	16.0	15.0	0.47	0.41	0.13
C 18:0	26.8	22.4	30.3	3.80	25.0	26.7	26.9	27.3	1.69	0.48	0.78
C 18:1 trans	35.1	35.8	31.3	1.80	35.5	32.5	33.9	34.4	1.82	0.35	0.72
C 18:1 cis	12.2	13.5	12.6	0.87	12.7	12.9	12.8	12.8	0.28	0.64	0.95
C 18:1 cis/trans	3.2	2.4[a]	2.0[a]	0.14	3.5	2.8	1.9	2.0	0.76	0.05	0.45
C 18:2	8.0	10.5	7.8	1.81	8.8	9.1	8.5	8.64	0.51	0.60	0.87

1 No interaction observed

a, b, c Means with different superscripts differ. P values are tabulated.

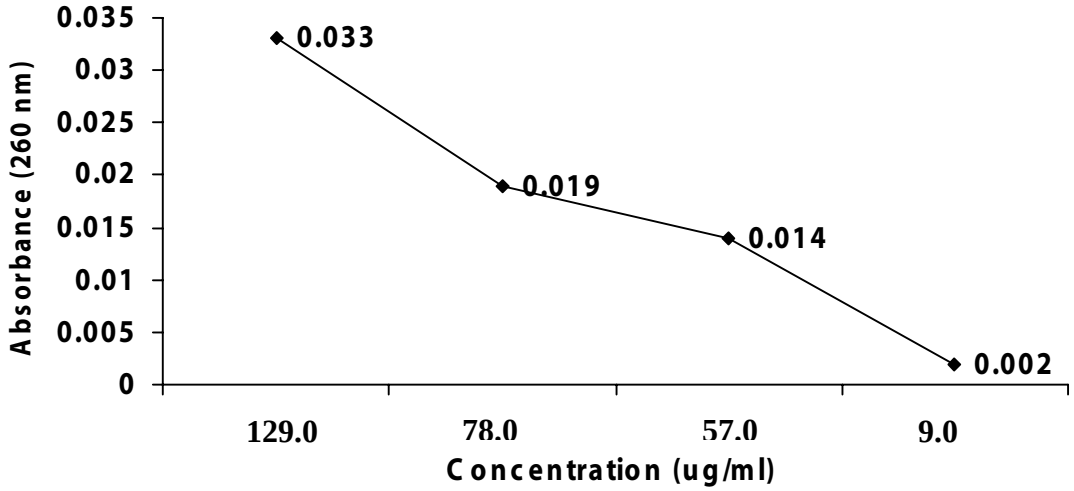

Figure 1. RNA concentration at 260 nm of *E. coli* standard.

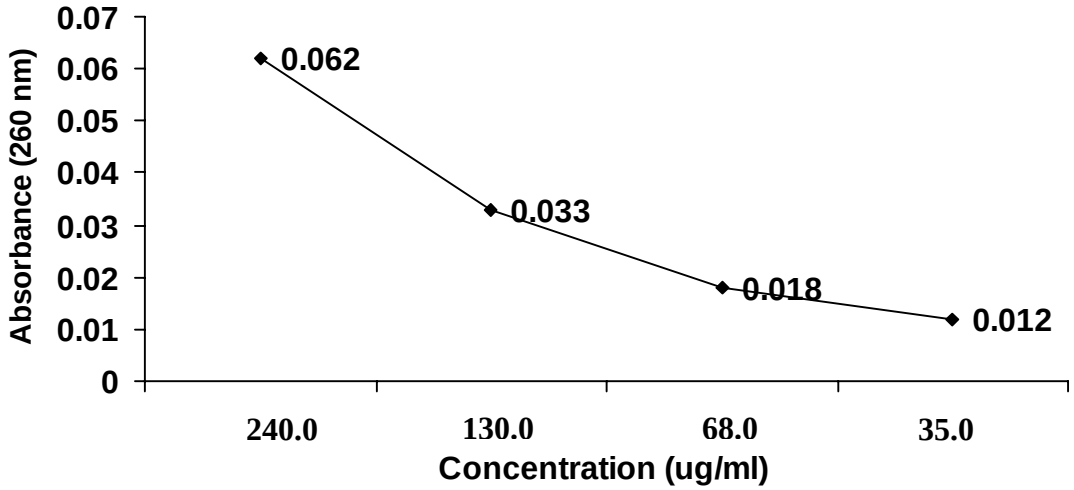

Figure 2. RNA concentration at 260 nm of *E. coli* standard, second set.

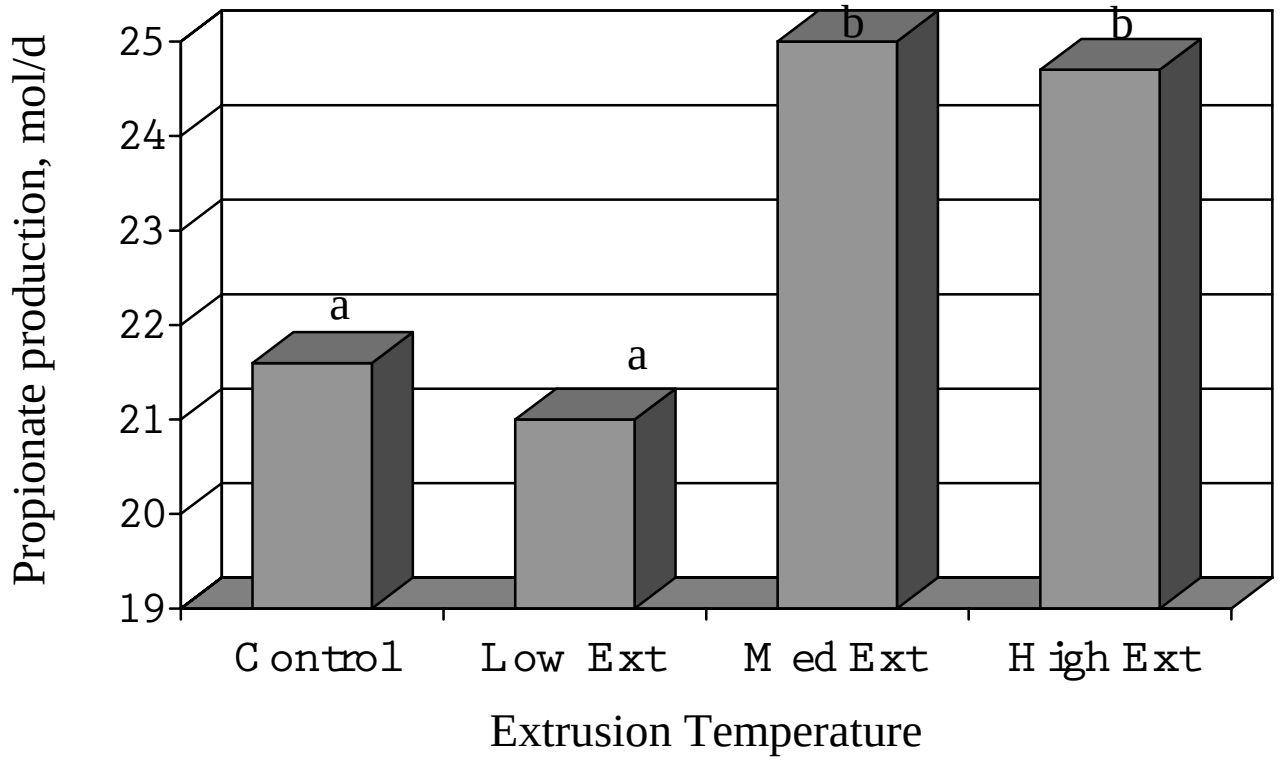

Figure 3. Effect of extrusion temperature on propionate production by continuous culture fed diets differing in NFC content and protein solubility.

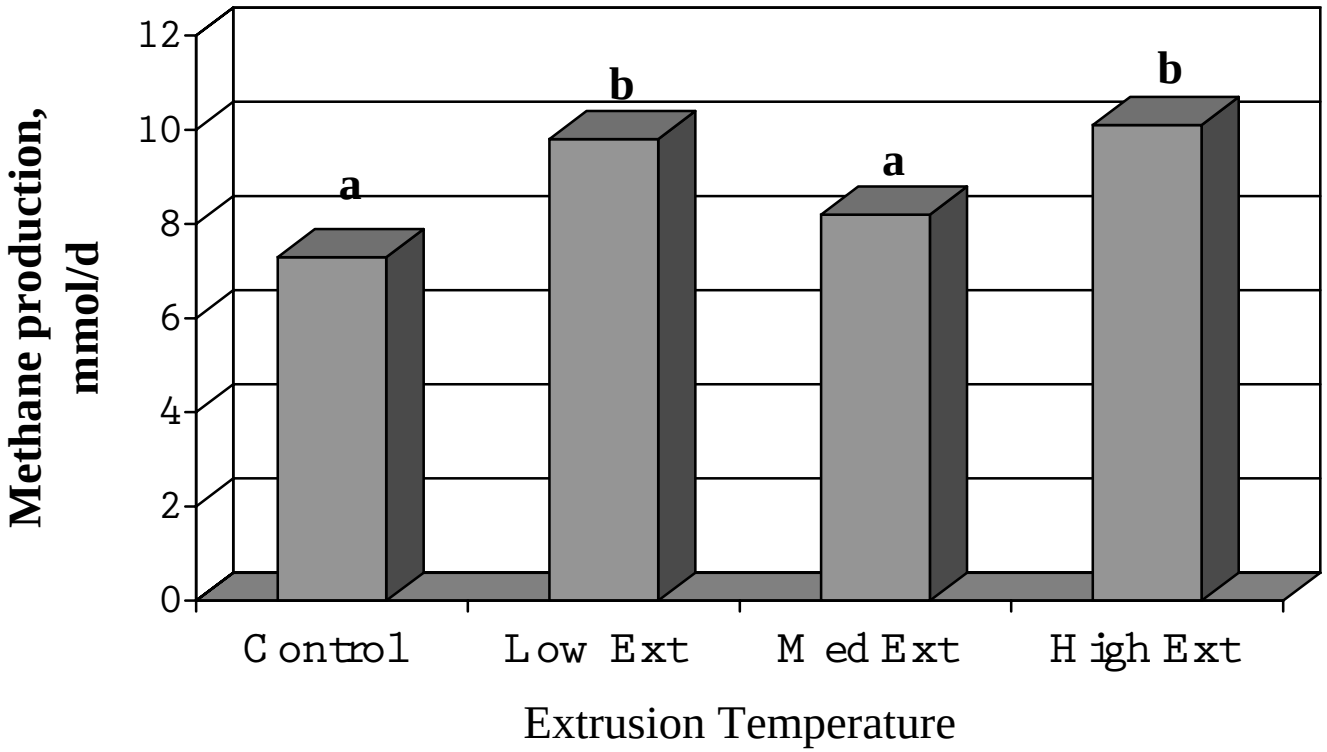

Figure 4. Effect of extrusion temperature on methane production by continuous culture fed diets differing in NFC content and protein solubility.

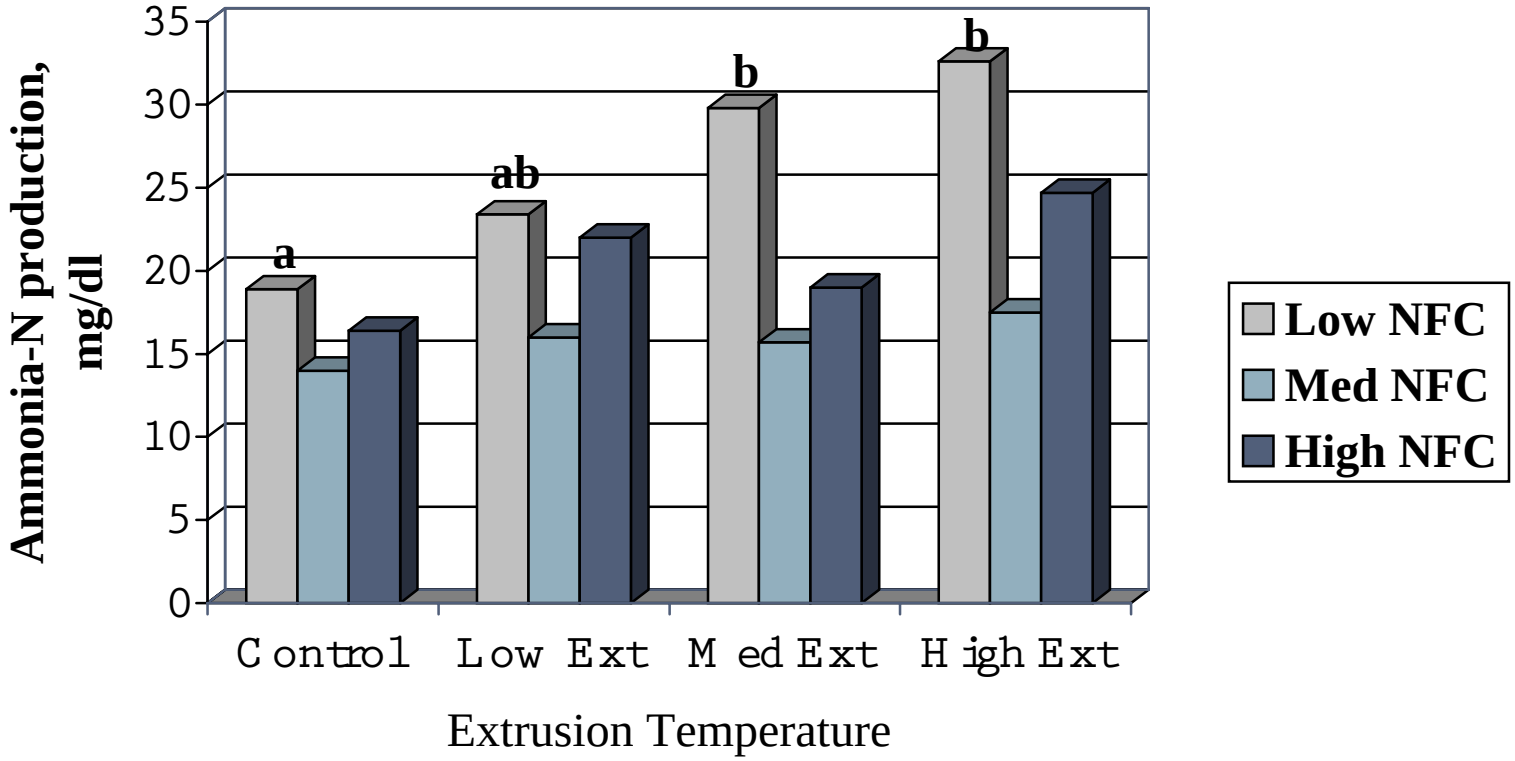

Figure 5. Effect of extrusion temperature on ammonia-N production by continuous culture fed diets differing in NFC content and protein solubility.

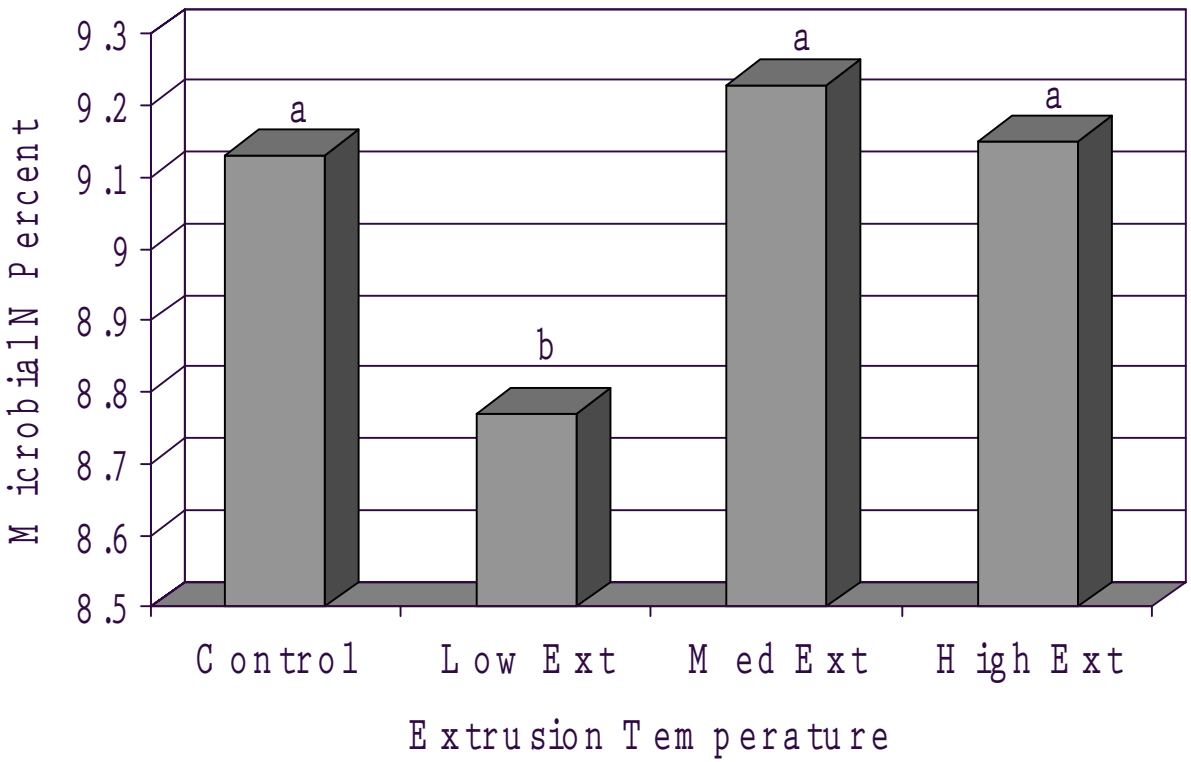

Figure 6. Effect of extrusion temperature on microbial nitrogen percent by continuous culture fed diets differing in NFC content and protein solubility.