NUTRIENT DIGESTION AND NITROGEN METABOLISM OF DRIED FERMENTATION BIOMASS AND VARIOUS FRACTIONS OF RUMEN MICROBES

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INTRODUCTION

Nitrogen metabolism in the rumen consists of dietary crude protein degradation and protein synthesis by microorganisms. Nitrogen flowing from the rumen is made up of dietary, endogenous, and microbial N. Some dietary N will be degraded in the rumen (RDP, rumen degradable protein) and the rest will escape as rumen undegradable protein (RUP). Sources of RDP are dietary non-protein N (NPN) and dietary protein. Non-protein N includes DNA, RNA, ammonia (NH_3), amino acids (AA), and peptides. Protein that has been deaminated to form NH_3 can be incorporated into microbial N, and some species of bacteria can also incorporate peptides and AA for microbial protein synthesis (MPS). Microbial protein that enters the small intestine is the major form of N utilized by the ruminant, and is a high-quality AA source that is generally balanced for maintenance and moderate production. Therefore, maximizing MPS in a way that is efficient to the animal has the potential to increase milk production. Researchers have investigated how to manipulate flows of differing N fractions such as microbial N from the rumen with intent to influence growth, milk output, and overall productive efficiencies.

Overview of N metabolism in ruminants

Actual protein and AA requirements in ruminants have not been determined. Because of MPS, N recycling, and interactions between them, it is difficult to determine requirements of ruminants for individual AA, RDP, RUP, and even CP. Therefore, ruminants are commonly fed on a crude protein (CP) basis. From a practical standpoint, a meta-analysis showed maximum milk production was achieved when 22.8% of the diet is CP (Ipharraguerre and Clark, 2005). However, there is considerable variation between studies that may be explained by source of CP. Quality, amount, and source of CP in the diet all have an effect on whether a milk response is observed. Relative amounts of RDP and RUP can affect MPS and N recycling as well as what AA are available for catabolism. An animal fed more than adequate or excessive dietary CP

may not be limited by relative deficiencies of certain limiting AA, especially if the animal is at or near maintenance, but this may result in environmental concerns as excess N is excreted. Even in a fasted state, production of AA continues (Lapierre and Lobley, 2001).

The relative amount of CP from a feed or ration that is degraded has implications for the microbial population and the ruminant. Microbial protein synthesis will be limited if RDP is too low to provide adequate N substrate for MPS, so RDP levels must be high enough to support protein synthesis by the microbial population. Alternatively, adequate levels of RUP can alter the relative proportions of AA flowing to the duodenum and ideally supply higher amounts of AA that may be limiting production. In this case, it is important that RUP is not over protected rendering it undigestible in the abomasum and small intestine and causing it to be unavailable for utilization by the animal.

Protein breakdown in the rumen by microbes consists of proteolysis and deamination. Proteolysis involves the cleaving of peptide bonds in dietary protein to form peptides and individual AA. Peptides can be further broken down to AA by peptidases inside microbial cells. Microorganisms can utilize AA for MPS, or these AA can be deaminated and converted to volatile fatty acids (VFA), CO_2, or NH_3. These end-products are excreted back into the growth medium. The pathway that is utilized depends on the type of organism and the energy available in the rumen. Some microorganisms lack transporters to move excess AA into the extracellular space, so they must be excreted as ammonia. If energy is not limiting, AA are incorporated into microbial protein. However, if energy is limiting, AA are converted into VFA (Tamminga, 1978; Bach et al., 2005).

When N is absorbed as NH_3 and AA, it can then be converted to urea and recycled back to the rumen. In the goat, 70% of N ingested daily passes through the urea pool (Harmeyer and Martens, 1980), and in cattle, the proportion of dietary N converted to urea ranges from 27 to 117% in extreme situations with a typical range of 50 to 70% (Recktenwald and Van Amburgh, 2009). Due to catabolic pathways of N such as hepatic urea synthesis from AA and ammonia, N balance in ruminants would be negative without N recycling, as urea synthesis would result in greater utilization of N than intake. Once synthesized, 40 to 80% of urea is returned to the digestive tract, where it can be utilized for anabolic pathways such as MPS by conversion to NH_3

(Lapierre and Lobley, 2001). In animals at or near maintenance and growing cattle fed at low intake, most dietary N is converted to urea regardless of its form. Therefore, it was believed that urea production was mainly determined by N intake without regard for the form of N (Harmeyer and Martens, 1980). However, if growing steers are fed adequate dietary N, urea production is more closely correlated (r^2 = 0.84) to portal ammonia absorption (Lapierre and Lobley, 2001). In this case, AA can be utilized for productive purposes. Feeding a less degradable protein source may result in lower ammonia production, less absorption across the rumen wall, and thus lower urea production.

Microbial protein

Microbial biomass flowing from the rumen provides a substantial portion of protein utilized by the animal for maintenance and growth. The NRC (1989) indicated that the proportion of total protein requirement of a 600-kg dairy cow met by microbial protein was estimated to be 73, 64, and 59% at production levels of 25, 35, and 45 kg of 4% fat corrected milk (FCM) daily (Stern et al., 1994). Yields of microbial N have ranged from 14 to 49 g/kg of organic matter apparently digested (OMAD) (Chen et al., 1992).

Mechanisms of proteolysis and MPS

Nitrogen substrates utilized by rumen microorganisms are NH_3, peptides, and AA. The first step of protein breakdown is proteolysis that yields peptides and AA available to rumen microorganisms. Activity of proteolytic enzymes of rumen microorganisms is relatively moderate compared to other microbes as well as the ruminant's own digestive enzymes; however, proteins are retained within the rumen to allow fairly extensive protein breakdown.

Different peptides are broken down at different rates. The main factor determining rate of hydrolysis of peptides is the structure on the N-terminus of the chain; neutral and basic peptides tend to be hydrolyzed more rapidly than acidic peptides (Wallace et al., 1990). An N-acetyl group prevents hydrolysis of the Ala_3 sequence of AA by mixed rumen microbes, and the presence of Pro as one of the first

two N-terminal AA results in the peptide being degraded more slowly. An in vitro study with cultures of mixed rumen bacteria showed that hydrophilic peptides are preferred over hydrophobic peptides because they are catabolized twice as fast as determined by rate of NH_3 production (Chen et al., 1987). The majority of peptides in the rumen are broken down in a two-step process where they are cleaved by dipeptidyl peptidase Type I (DPP-1) into di- and tripeptides which are further catabolized by di-and tripeptidases into individual AA. Although experiments with mixed populations of rumen bacteria have shown that DPP-1 is the major enzyme of peptide hydrolysis in populations of rumen microbes, the only organism found to contain the enzyme is *Prevotella ruminicola*, a Gram-negative microorganism widespread in the rumen (McKain et al., 1992).

Diet fed to the ruminant animal has a substantial impact on the microbial population by influencing relative amounts of different microorganism such as *P. ruminicola* and can theoretically influence proteolysis by modifying which proteolytic microorganisms are active in the rumen. The Cornell Net Carbohydrate and Protein System (CNCPS) divides rumen microbes into those that ferment structural carbohydrates (SC) and those that ferment nonstructural carbohydrates (NSC). Cell walls are fermented by SC utilizing bacteria which were assumed to utilize only NH_3 as an N source while bacteria that ferment NSC utilize NH_3, as well as peptides and AA (Russell et al., 1992). However, this assumption may not be valid. Atasoglu et al. (2001) found that the cellulolytic bacteria *Fibrobacter succinogenes* were unable to grow in a cellulobiose medium without addition of peptides or AA, although growth was possible on cellulose. Hazlewood et al. (1983) were unable to culture *P. ruminicola* in 48 hours when the medium utilized leaf protein fraction 1, the major soluble protein in green leaves, as a substrate. The authors hypothesized that this was either due to the organism's slow-growth in this medium or because the cows from which rumen fluid was harvested were being fed fresh forage which may have reduced the relative proportion of *P. ruminicola*. Ling and Armstead (1995) examined the uptake and metabolism of ^{14}C-labeled peptides and AA in five species of rumen bacteria in complex, defined, and semi-defined media. They found that of the five species studied, *P. ruminicola* and *Streptococcus bovis* were the two species with highest ^{14}C-peptide uptake, which correlated to the greatest ^{14}C-peptide metabolism. *Fibrobacter succinogenes* and *S.*

bovis were shown to take up peptides and AA at similar rates, while *Selenomonas ruminantium* and *Anaerovibrio lipolytica* preferred AA. Despite these differences in highly-controlled laboratory settings, impacting proteolysis on a practical level by manipulating the microbial population has only limited potential (Wallace, 1996).

Between free AA and peptides, most studies have shown that mixed populations of rumen bacteria prefer peptides (Wallace, 1996). Prins et al. (1979) compared disappearance of free AA to peptides in vitro, and found that peptides disappeared twice as quickly as the constituent AA. Another study with incubated rumen fluid determined that peptides were used more efficiently for MPS than AA (Wright, 1967). In an in vitro study utilizing mixed cultures of rumen bacteria, peptides were taken up over two times more quickly than free AA when determined by rate of NH_3 production (Chen et al., 1987). However, a study of cellulolytic bacteria showed that AA were preferred compared with peptides (Atasoglu et al., 2001). Once proteins have been broken down into peptides, they must be further degraded into AA before they can be incorporated into microbial protein. For incorporation to be successful, there must be sufficient energy available to the microorganism, or else NH_3 will accumulate in the rumen (Wallace, 1996).

Of the N substrates exploited by rumen microbes for MPS, NH_3-N is the major N source (Cotta and Russell, 1982). Tracer studies have shown that 50 to 80% of bacterial N is derived from NH_3-N (Leng and Nolan, 1984). In fact, ruminants are unique in their ability to meet their N requirement through NPN (such as NH_3) alone if necessary. It is generally accepted that 2 to 5 mg of NH_3-N/dL of rumen fluid is adequate for maximizing microbial growth (Satter and Slyter, 1974). Above this range, increasing levels of NH_3-N had no effect on microbial growth in continuous culture, even with levels up to 80 mg/dL. Amino acids can be utilized by microbes as preformed AA directly utilized for protein synthesis, or they can provide fermentable energy, producing NH_3 (Cotta and Russell, 1982). Because NSC bacteria readily ferment AA, this population will produce NH_3 while SC bacteria are not considered to be major contributors. Bacteria that carry out deamination can be generally divided into two groups, those with high numbers but low individual deamination activity such as *S. bovis* and those with high deamination activity but are present in low numbers in the rumen such as *Clostridium aminophilium* (Wallace, 1996).

Growth and reproduction of microorganisms and the resulting yield of microbial biomass flowing from the rumen that is available physiologically to the ruminant depends on the amount of substrate available and how much substrate is utilized to meet the maintenance requirements of the microorganisms. Bacterial maintenance energy has not been clearly defined; however, three functions are commonly thought to contribute (Russell, 2007). These functions are cell motility, turnover of sensory molecules and macromolecules such as protein, and re-establishment of ion gradients across the membrane of the cell. Microbiologists once assumed that maintenance and growth rate were separate entities, but this is probably an oversimplification that does not completely account for all variation (Neijssel and Tempest, 1976). In most mechanistic models of MPS, maintenance costs are estimated to be 0.26 mmol of glucose/g of bacterial DM/h. However, this value can vary widely based on conditions in the rumen as well as the microbial species of interest. Ruminal pH can affect these maintenance costs by affecting the concentration gradient across the cellular membrane of a microorganism. Energy spilling in particular is an important factor in maintenance costs of rumen microorganisms. This concept (also known as "uncoupling," "spillage," "wastage," or "overflow metabolism") can be defined as the dissipation of excess ATP when the catabolic fate is faster than the anabolic rate in an organism (Russell and Cook, 1995). An acidic pH can increase energy spilling reactions by bacteria, resulting in inefficient growth. In order to prevent the build-up of toxic anions, *S. bovis* has the ability to decrease its intracellular pH. The relative contribution of maintenance requirements to utilization of ATP available to microorganism is higher when fermentation and growth rates are slow, limiting the amount of glucose consumed. However, growth can be limited by nutrients other than energy. The maintenance value of an organism may appear to be much greater when limited by nutrients such as ammonia, sulfate, phosphate or potassium (Russell, 2007).

Factors affecting MPS

Based on mechanisms discussed above, a number of ecological and dietary changes can affect MPS in vivo and in vitro, and many of these changes can interact with each other to further alter MPS. Throughout the following discussion, it should be considered that effects of different dietary factors on MPS have been observed under in

vitro conditions in batch or continuous culture. While these results are useful because they provide insight into potential mechanisms in the animal, conditions differ between two models, so in vivo confirmation of results observed in vitro are imperative. One of the major shortcomings of in vitro experiments is that fermentation products are not absorbed through the rumen wall (Dewhurst et al., 2000).

The ecosystem of the rumen itself can be influenced by dietary factors that can affect MPS. Microbial maintenance requirements are dynamic and can be affected by a number of factors. Typically the maintenance requirement of bacteria is determined experimentally under carbohydrate (CHO)-limiting conditions; however, a lack of other nutrients, such as protein or specific amino acids, can raise the CHO requirement of a microorganism, and this interaction between nutrients can modify the maintenance requirement (Neijssel and Tempest, 1976). Theoretical calculations show that AA synthesis has a low ATP requirement of 110.3 moles x 10^{-4} NADPH/g of cells (Stouthamer, 1973), but in spite of the inexpensive energy demand for AA synthesis, if non-carbon requirements are limited, this can result in a decrease in growth due to uncoupling (Cotta and Russell, 1982). The effect of limiting AA on CHO utilization may be due to individual effects on specific strains of microbes. There are a number of different between-species mechanisms for coping with changes to the rumen environment, and it is possible that there are differences between species for preferences of sources of CHO and N (Dijkstra et al., 1998). In fact, some species do not have the energy spilling mechanisms to cope with excess glucose and can be killed by surplus CHO, affecting the rumen ecosystem (Russell, 2007).

Interactions between bacteria and protozoa can also affect MPS. Protozoa have an impact on the microbial community because of predatory action on bacteria. Studies that analyze effects of protozoal population on protein digestion and metabolism can be difficult to design and interpret because reduction of the protozoal population and even complete defaunation are often accomplished by altering the diet, and these changes can have an intrinsic effect on studies involving protozoa (Dewhurst et al., 2000). A decrease in bacterial biomass is observed once protozoa have been reintroduced to the rumen after defaunation, thus decreasing bacterial protein available to the animal for metabolism and as a protein source. Defaunation can double the bacterial biomass flow to the duodenum, and MPS can increase from 40 to 125% (Jouany, 1996). In contrast,

it has also been hypothesized that because defaunation increases urea-N transfer into the rumen due to the decrease in NH_3 concentration, which is negatively correlated to urea-N transfer, the amount of N available for bacterial growth increases, resulting in greater bacterial biomass. However, an experiment utilizing 4 lambs in a 4 x 4 Latin square to investigate this hypothesis showed that partial defaunation of the rumen had no effect on urea-N recycling, although there was a numerically greater proportion of urea-N utilized for MPS, resulting in a higher flow of microbial N to the duodenum (Kiran and Mutsvangwa, 2010). Protozoa numbers can increase in cases of more frequent feedings at restricted intakes or decrease in response when switching to a high concentrate ration (Slyter, 1976). Growth of protozoa is stimulated by diets high in readily fermentable CHO such as starch or sugar, and their presence can result in a wide variation in turnover of the bacterial population (Recktenwald and Van Amburgh, 2009).

In addition to factors affecting environment and microbial ecology in the rumen, a number of dietary factors and their interactions have an impact on MPS in the rumen. Protein and energy in the diet, as well as the interactions between these nutrients, can play a major role in MPS. In a study to compare the effect of diet on proteolysis, sheep were fed four diets in two experiments comparing corn with timothy hay, and barley with lucerne, and the degradability of casein was observed (Siddons and Paradine, 1981). These extremes in forage and concentrate had significant impact on proteolysis of casein and the microbial population of the rumen. Feeding concentrate greatly increased proteolysis and although pH, a potential effector of protein degradability and subsequent MPS, was lower; it was still within the optimal level for proteolytic activity. Energy and protein are the two nutrients most likely to limit MPS (Clark et al., 1992). Source of N and CHO as well as N concentration can affect MPS as can relationships between N and CHO in the rumen.

Source and concentration of N play a large role in microbial protein synthesis. Limitation of N supply in the diet can decrease microbial growth due to a deficiency of substrate, but excess N can decrease EMPS, because dietary energy content will become limiting to microbial growth (Stern and Hoover, 1979). If N concentration in the diet increases, N intake will increase, even without increasing DMI. Increases in N intake have been shown to affect MPS (Hume et al., 1970; Siddons et al., 1985). This

observation may be due to an increase in ruminal degradation of dietary CP in high protein diets (Volden, 1999).

Source of protein determines relative amounts of NH_3-N and preformed AA in the diet, both of which are utilized for MPS. In a continuous culture study, replacement of an NPN source with peptide-N at four levels resulted in NH_3-N concentration decreases with increasing peptide-N (Jones et al., 1998). Microbial N responded quadratically with the highest level of microbial N at 10% peptide-N replacement of NPN. If NSC-degrading bacteria meet their requirement for AA, supplemental AA will not be degraded to NH_3. In fact, the authors concluded that these organisms may utilize peptides at the expense of NH_3 production, inhibiting microbial growth and ensuing digestion. Relative proportions of NPN and protein in the rumen can be determined by the amount of available preformed AA in the N source as well as its degradability in the rumen. Many species of rumen microbes have a requirement for certain preformed AA (Cotta and Russell, 1982), and a deficiency of preformed AA by NSC-degrading bacteria can result in a decrease in efficiency of MPS (EMPS). The peptide form of these AA is preferred over individual AA. Some species such as *Butyrivibrio fibrisolvens* have an absolute requirement for AA and are unable to grow without an AA source, while others such as *P. ruminicola* and *Megasphaera elsdenii* have been shown to adapt over time to conditions where preformed AA are limiting (Cotta and Russell, 1982). Preformed AA and peptides increase MPS, although the relationship has not been firmly established in the literature, possibly due to differences in how EMPS is expressed (Dijkstra et al., 1998). An optimum ratio of NH_3-N to AA-N for maximum growth of mixed cultures of bacteria isolated from rumen fluid was reported at 75% urea-N to 25% AA-N (Maeng et al., 1976). Using this ratio, approximately 53% of the AA supplemented was utilized for MPS, 14% was fermented into C compounds, and 33% remained unused by microbial cells. The effect of NH_3 and AA on microbial yield can be influenced by growth rate of microorganism (Van Kessel and Russell, 1996). These researchers found in an experiment with mixed rumen bacteria in vitro that when the specific growth rate of bacteria was less than 0.4, biomass yield was dependent on NH_3-N. This same study showed that AA-N only increased the yield of the predominant ruminal bacteria when CHO was in excess and when the specific growth rate allowed by NH_3 was less than the degradation rate. These results indicate that growth rate and maintenance requirement,

as determined by energy spilling reactions, influence the effect of N source on MPS. Utilization of urea by microorganisms for protein synthesis can be encouraged by feeding a readily fermentable energy source, which will promote MPS under most circumstances (Lapierre and Lobley, 2001). Other characteristics of a protein source such as degradability can influence MPS by affecting what preformed AA and peptides are available to the microorganisms. Substituting RUP for RDP at high levels has potential to decrease microbial-N flow by limiting source of AA and peptides (Dijkstra et al., 1998). The level of RUP feeding where this limitation occurs depends on level of production of the animal because high-producing animals need a greater amount of RUP to meet their AA requirement (Stern et al., 1994). Alternatively, if protein is degraded too quickly, this can result in loss of N as NH_3-N if there is not enough OM being fermented to capture it, potentially decreasing MPS with rapidly degradable protein sources. Ruminal degradation of protein is related to protein solubility as observed by Stern et al. (1978). Protein was rendered insoluble by heat treatment, and diets were arranged in a 2 x 2 factorial arrangement with two levels of solubility and urea inclusion. They found that the diet with the highest amount of soluble protein had the highest NH_3-N losses in a continuous culture experiment, and there was a corresponding decrease in MPS. These researchers noted an additive effect with urea and solubility because diets with urea were lower in microbial N. Rumen degradability of the protein source can affect what form of N is limiting to MPS. For example, under conditions when RDP is low and urea is limiting, such as in continuous culture in vitro systems or when urea recycling is limited, NH_3 may become the limiting N source for microbial growth. However, when urea is available, NH_3 can accumulate, causing peptides to be the limiting molecule (Griswold et al., 2003).

Several factors impact protein degradation in the rumen, and thus have a role in extent to which dietary protein is utilized for microbial protein synthesis. Composition of the protein itself, such as the amount of soluble protein, can affect its degradability. Specific bonds such as disulfide bonds are resistant to degradation so their relative abundance will affect degradability of the protein as a whole (Bach et al., 2005). The degree to which any feedstuff is degraded depends on both rate of degradation as well as how long it is exposed to digestive processes such as microbial fermentation in the rumen. Thus, dilution rate will affect degradability, as passage rate and degradation

have an inverse relationship (Church, 1988). Although the extent of degradation of a given protein may be lowered as dilution rate increases, microbial growth increases in spite of the potentially lower amount of dietary N available for MPS (Stern and Hoover, 1979). Typically, an increase in dilution rate will have a positive effect on EMPS (Church, 1988). In an experiment that manipulated dilution rate *in vivo* by infusing artificial saliva, both EMPS and MPS increased with increasing dilution rate (Harrison et al., 1976). Another study performed in chemostats also found an increase in microbial biomass yield with increasing dilution rates (Isaacson et al., 1975). Dilution rate itself can be affected by defaunation (Jouany, 1996) and corresponds to an increase in dry matter intake (DMI). Chen et al. (1992) showed a strong correlation with EMPS and increases in solid outflow rate (r^2 = 0.802). The effect of DMI on CP degradability has been observed (Volden, 1999). Increasing feed intake can increase passage rate, resulting in an increase in bacterial-N flow to the duodenum and a decrease in in situ degradation of CP.

As mentioned previously, reduction in pH can increase maintenance energy requirement of bacteria by increasing amount of energy spilling and theoretically decreasing EMPS in some cases. Although meta-analysis showed no effect of pH on EMPS, total bacterial-N flow was negatively correlated to pH (Bach et al., 2005). The pH of the rumen can affect degradability and thus MPS; however, low pH is often due to a high amount of readily fermented OM which provides more substrate for MPS, resulting in no net relationship in efficiency. Proteolysis is reduced at low pH (Bach et al., 2005). The optimal pH range for protein degradation has been estimated at 5.5 to 7.0, although it can decrease at the lower end of this range (Kopecny and Wallace, 1982). Hoover et al. (1984) showed an interaction between pH and dilution rate for protein degradation and corresponding microbial growth, with evidence of energetic uncoupling when pH was 4.5 or 7.5 and dilution rate was high (12 and 16%/h), resulting in a decrease in EMPS compared to pH 5.5 and 6.5. At extreme pH levels, dilution rate had the opposite effect than what is normally observed. One possible effect of pH on degradation and MPS is that it can have an impact on the microbial population of the rumen and activity of fibrolytic bacteria, which may affect degradability of N compounds bound to CHO (Dijkstra et al., 1998). Effects of pH may be more pronounced for feedstuffs with higher predicted degradability than lower predicted degradability (Clark

et al., 1992). In a continuous culture experiment where pH was either controlled or allowed to fluctuate, pH averaged 5.9 across 4 diets with differing ratios of SBM to lignosulfonate-treated SBM (Endres, 1995). Compared with pH controlled at 6.3 ± 0.05, the uncontrolled pH group interacted with the level of undegradable SBM and reduced degradability of SBM but not protected SBM. Loerch et al. (1983) observed an interaction between pH of the solvent and solubility of the protein source, indicating that different protein supplements may react differently to changes in pH.

In addition to N source and its degradability, CHO source will also have an effect on MPS. In general, a more rapidly degradable CHO source will result in more energy available for protein formation while N is available for incorporation (Stern and Hoover, 1979), synchronizing energy and N availability. However, high CHO can also result in a decrease in ruminal pH, limiting activity of fibrolytic bacteria and affecting protein degradation if proteins are bound to or otherwise protected by these structural compounds. The quantity of energy captured by microorganisms is critical for MPS, and this can be affected by fermentability of the CHO source. Source of CHO can also influence the microbial population, affecting MPS. For example, differences in the relative amount of NSC or SC will alter proportions of bacterial populations that degrade these compounds in the rumen. Relatively more microbial N and less non-ammonia non-microbial (NANMN), which contains both dietary and endogenous-N, pass to the small intestine when OM fermentation increases. Therefore, feeding a highly fermentable energy source can increase MPS, although interactions can occur with feed intake, the type and physical form of feed, the forage to concentrate ratio of diet, and the source and amount of N and energy in diet (Clark et al., 1992). Starch has been shown to decrease NH_3 concentration in the rumen, indicating that an increase in concentration of starch in the diet may increase the amount of NH_3-N incorporated into microbial N. Stern et al. (1978) found a decrease in MPS with decreasing starch levels in continuous culture, with a corresponding increase in NH_3 concentration with decreased starch levels. It has been suggested that feeding a high amount of readily fermentable CHO will precipitate microbes to form polysaccharides rather than stimulate microbial growth (Walker and Nader, 1970). McAllan and Smith (1977) showed a decrease in N content of microbes with an increasing amount of concentrate in the diet. Oba and Allen (2003) found that flow of microbial N increased with a high-starch diet

(32%) compared with a diet containing 21% starch. Microbial efficiency in the rumen, expressed as g of microbial-N flow to duodenum/kg true organic matter digestion (TDOM), was negatively correlated with TRDOM as well as rate of starch digestion. However, EMPS was positively correlated with passage rate for organic matter (OM) and starch, possibly due to higher passage rate decreasing microbial turnover. These results are consistent with the theory that energy spilling decreases microbial efficiency as discussed previously. A strain of ryegrass has been bred with a higher level of water-soluble carbohydrates (WSC). This ryegrass has been shown to increase microbial-N flow to the duodenum and EMPS, as well as a decrease in rumen NH_3 concentration in beef cattle (Merry et al., 2006). In a corresponding in vitro study, NH_3 concentrations decreased with higher levels of WSC, indicating greater uptake of NH_3 by rumen microorganisms. Similar to solubility of a N source affecting MPS, solubility of CHO source can also play a role. A decrease in EMPS was observed when alfalfa with high soluble protein levels was used as substrate in continuous culture (Berthiaume et al., 2010). This effect was offset by an increase in WSC and starch content of diet. They observed an increase in microbial-N flow in continuous culture, but OM digestibility also increased, so no effect was observed on efficiency of microbial protein synthesis. Furthermore, forage quality can affect MPS, as higher quality forage will increase microbial protein synthesis. In general, MPS is lower for animals fed silage compared with those fed fresh forage (Dewhurst et al., 2000).

It is difficult to determine whether effects of dietary forage to concentrate ratio on MPS and EMPS are a result of forage to concentrate ratio itself or other factors. Factors include amount and rate of OM fermentation and changes in dilution rate and pH. Rode et al. (1985) manipulated dilution rate by increasing dietary forage level, and demonstrated a strong correlation (r^2 = 0.504) of EMPS to an increase in dilution rate. A lower EMPS may be observed when diets are greater than 70% concentrate because NSC are rapidly degraded, possibly resulting in uncoupled fermentation and release of energy faster than it can be trapped and utilized by bacteria. If a greater concentration of forage is offered to the animal, energy is released more uniformly. As a result, feed intake may increase, and passage of solids and liquids may increase, increasing microbial-N flow to the small intestine. However, if a high level of forage is fed to the ruminant, available energy may become deficient, and increase maintenance costs

because of an increase in cell lysis due to slower rate of passage (Clark et al., 1992). When high grain diets were fed, Martín-Orue et al. (2000) observed an increase in microbial flow from the rumen in heifers when effective RDP was not supplemented regardless of type of concentrate (corn or barley).

Carbohydrates and N in a ruminant diet can affect MPS individually, but there are clearly interactions that occur between these two nutrients that confound their individual effects on MPS. For example, growth of mixed cultures of bacteria isolated from rumen fluid increased in response to branched-chain AA (BCAA) inclusion when cellobiose was provided as an energy source, but not when glucose provided energy (Maeng et al., 1976). This was hypothesized to be a result of cellulolytic bacteria having a requirement for branched-chain fatty acids.

"Rumen synchrony" is the hypothesis that MPS will be maximized if fermentable energy and N for MPS are available simultaneously (Dewhurst et al., 2000). Simultaneous fermentability of energy and N could be manipulated by ingredients supplied in the ration, by adjusting their relative time of feeding or a combination of both. Due to a lack of consistent experimental evidence supporting this model, rumen synchrony is widely considered not to have a profound effect on MPS because the complexity of the microbial population is believed to compensate for any asynchrony in ruminal availability of these nutrients (Bach et al., 2005). Alternatively, in certain situations, rumen synchrony may yet play some role in MPS. Cotta and Russell (1982) found that AA utilization by purified strains of rumen bacteria was never complete, which was attributed to lack of synergism between energy available for MPS and N substrate for microbial protein. These researchers observed that MPS was always highest in continuous culture when AA and glucose were supplied in equal concentration, which was the highest level that AA was supplied in this study in relation to glucose. Despite this response due to an increase in AA available, conversion of AA to microbial protein was poor for some rumen bacteria observed under these conditions. Conversion was defined as g of bacterial protein synthesized less the bacterial protein synthesized at 0 g/L divided by total AA source utilization. They determined that under these steady-state conditions, maximum microbial protein yield was generally achieved when the AA source to glucose ratio was 12.5%, although many bacterial species studied did not show a net increase in AA in the system until this ratio fell to 6.25%. An

increase in consumption of NSC can result in a decrease in NH_3 concentrations, possibly a result of an increase in MPS and subsequent uptake of NH_3 (Royes et al., 2001). A study by Valkeners et al. (2004) observed no effect on microbial protein flow to the duodenum in bulls fed the same diet in different feeding patterns in an attempt to alter rumen synchrony. They concluded that because of N recycling, there is no effect of rumen synchrony when balanced on a 48-h basis. Berthiaume et al. (2010) suggested that any potential effect of synchrony relates to the ratio of N and energy supply rather than their relative timing.

Whether or not rumen synchrony plays a part in fermentation and MPS, it is clear that N and CHO interact in various situations. Most mechanistic models assume that energy available from feed degraded in the rumen is affected by numbers of microbes available for degradative processes, which is determined by N availability (Dijkstra et al., 1998). Moreover, CHO are used as C skeletons with NH_3 for protein synthesis, so the presence of CHO can result in an improvement in N uptake (Bach et al., 2005).

Other dietary factors that affect MPS include composition of supplements, level of feeding, and frequency of feeding. In addition to protein supplementation, fat and mineral supplements can also play a role in MPS. Supplementation of high concentrations of fat to the diet can result in defaunation of the rumen, and the resulting lack of protozoa influence microbial protein synthesis. There is also a sulfur requirement for Met and Cys production by rumen microbes. Other minerals such as phosphorus are needed to synthesize nucleic acids and enzymes, so a failure to supplement adequate levels of these minerals can negatively affect MPS (Dijkstra et al., 1998). Hume and Bird (1970) showed that raising dietary sulfur level from 0.61 g/d to 1.95 g/d by including different forms of sulfur, either inorganic sulfate or Cys, in diets fed to sheep increased microbial protein production from 82 g/d to 94 g/d. However, there was no increase in microbial protein when sulfur levels increased to 3.42 g/d regardless of the form of sulfur.

In addition to composition of diet affecting MPS, level and frequency of feeding also play a role. A higher DMI will increase N intake, but does not have a positive effect on MPS, although the evidence for a positive effect of DMI on EMPS is somewhat conflicting. Clark et al. (1992) fed the same diet at 4 different levels of intake. The increase in OM intake (OMI) increased NH_3 production, resulting in more energy and N

available for MPS. The increase in OM fermentation also resulted in a decrease in pH, limiting protein degradation, although energy was not limiting to microbial growth. While evidence for high intake increasing EMPS is limited, there have been no examples of high EMPS at low intake (Dewhurst et al., 2000). Clark and others (1992) detected a poor linear (r^2 = 0.0001) and quadratic (r^2 = 0.16) relationship between OM truly digested (OMTD) and EMPS expressed as g microbial N/ unit of OMTD when summarizing results of 36 studies. They hypothesized that errors associated with measuring OMTD and microbial-N passage may fail to show an increase in EMPS with an increase in feed intake even if one does exist. Other factors may include an increase in energy spilling by rumen bacteria at high intakes, resulting in an increase in maintenance costs of bacteria (Dewhurst et al., 2000), or possibly the amount and proportion of other nutrients, synchronization, and rumen conditions (Clark et al., 1992). Alternatively, high intakes can increase dilution rate, which will also decrease maintenance costs of bacteria. Chen et al. (1992) observed the effect of DMI on excretion of purine derivatives in sheep. They found a correlation between EMPS and DMI:body weight ratio (r^2 = 0.62). In this study, degraded OM was calculated at a constant rate of 65% of DMI, which may have affected results. Volden (1999) found an increase in EMPS from 23.7 to 28.1 g bacterial N/kg OMTD when cows were fed 20 kg/d compared to 10 kg/d. However, this comparison was drawn between the same cows in two different stages of lactation and could have been confounded by a variety of factors associated with differing days in milk (DIM), such as differences in milk production. Feeding frequency can also influence level and efficiency of MPS in ruminants by increasing DMI and ruminal dilution rate. An increase in solid retention time has been shown to decrease MPS in continuous culture, possibly due to a corresponding decrease in feeding rate associated with this main effect (Crawford et al., 1980a).

Predicting MPS

Prediction of MPS has been attempted by several models of differing types. The NRC (2001) assumed 130 g of microbial-CP/kg of total digestible nutrient (TDN) intake. Yield of microbial-CP is predicted as 0.130 x TDN when RDP is 1.18 x microbial-CP yield. When RDP is less than this amount, microbial-CP is predicted as 0.85 times RDP intake.

Models used to predict MPS can be classified as empirical, mechanistic, or mixed models (Dijkstra et al., 1998). In empirical models, MPS is calculated from amount of available energy per unit of degradable OM with corrections for a possible shortage of N. Yield of microbial protein per unit of OM can be either fixed or variable. The value usually used for potential MPS is typically the lower value of MPS predicted by available OM or degradable CP. However, empirical systems do not consider interactions between microorganisms or microbial yield differences due to preformed AA or peptides, and they consider EMPS to be constant (Firkins et al., 2006). In mechanistic models, MPS is based on the amount and type of available OM, utilization of OM for growth and maintenance, and interactions between microbial populations. These models are not as common as empirical models. Mixed models, such as CNCPS, use a combination of empirical and mechanistic modeling. Ammonia requirements may be underestimated using the CNCPS model because it does not account for NH_3 absorption.

Chemical composition of rumen microbes

There is a wide range of N and OM values found in the literature for rumen microbes (Clark et al., 1992). Nitrogen and OM content of bacterial cells generally have an inverse correlation. Clark et al. (1992) summarized experiments investigating the composition of rumen bacteria and found that N values ranged from 7.35 to 13.23% of OM while OM values ranged from 60.8 to 92.2% of DM.

Chemical composition of microbial biomass in the rumen can differ between differing fractions, i.e., liquid associated bacteria (LAB), solid associated bacteria (SAB), and protozoa. A comparison of chemical content of LAB and SAB found that SAB was higher in lipid content than LAB, but that LAB was significantly higher in ash, total N, RNA, and diaminopimelic acid (DAP), which is found in the cell wall of bacteria, as well as numerically higher in total P, nucleic acid P, ^{35}S, and total carbohydrate, and had a higher ratio of RNA-N: total N and DAP-N: total N. The two fractions had a similar ratio of ^{35}S to total N (Merry and McAllan, 1983). Much work has been done to determine if the AA composition of microbial protein in the rumen varies, but general consensus in the literature is that diet does not affect AA composition of microbial protein (Hvelplund and Hesselholt, 1987), although experiments continue to be designed in attempts to find

a way to influence this because of the obvious practical benefits to the animal if this could be achieved.

The chemical composition of microbial matter can be affected by a variety of factors, including differences in methodology that can confound results between studies (Dijkstra et al., 1998). These methodological differences include diet, time of sampling after feeding, and their interactions, as well as sampling and separation techniques. In a study performed on steers fed twice daily compared to those fed 12 times daily, there was a difference in ruminal bacterial composition (Cecava et al., 1990). Ash and CP values were higher when sampled 4 hours after feeding than samples taken 0.5 hours after feeding in a study by McAllan and Smith (1977). Martin et al. (1996) did not observe any differences between AA profiles of rumen bacteria when they were sampled at 2, 5, 8, 11, and 23 h post-feeding.

The ratio of DAP:total N has been shown to change with time postfeeding when cattle are fed a high-concentrate diet, but not in trials feeding higher forage (Dufva et al., 1982). The ratio of DAP-N to total N is lower in LAB from digesta of cattle fed high roughage diets. Total DAP-N (% of total N) as well as total N was found to be higher in mixed strains of rumen microorganisms fed a high-roughage diet. This effect could be influenced by feeding a high concentrate diet that would promote growth of *S. bovis* because the same study showed that this species has been shown to lack DAP. The RNA-N: total N ratio can also vary depending on time after feeding, diet composition, environment, species, and age of the animal (Smith and McAllan, 1974). Variations in diet due to CP source, CP amount, and amount of forage can also affect purine:total N ratio (Clark et al., 1992). Stokes et al. (1991a) performed a study to evaluate the effect of CHO and protein types on ruminal digestion and microbial metabolism by feeding a NSC:RDP ratio of 2.7 across 3 diets with differing levels of RDP at 38, 31 and 24% of DM. They found that bacterial N and ash content were similar between the 2 diets with higher levels of RDP, but that the diet lowest in RDP had higher ash and lower N. Another study showed that feeding either a corn- or barely-based concentrate supplement at 75% of DMI did not affect N or purine contents of microbial fractions separated from the rumen (Martín-Orue et al., 2000). However, infusion of effective RDP as a mixture of casein and urea in increasing increments (0, 25, 50, or 75 g/kg of concentrate intake) resulted in higher levels of bacterial N and purine content without

changing purine:total N ratio. Difficulties in separating microbial fractions can lead to differences in composition. Merry and McAllan (1983) estimated that approximately 30% of bacteria associated with solids in their study may have technically been LAB. A study performed in continuous culture showed a higher standard error for most measurements in SAB compared with LAB, suggesting incomplete or contaminated recovery of SAB (Rodríguez-Prado et al., 2004).

As mentioned above, there are differences between fractions of rumen microbes, and relative abundance of these can affect composition of microbial biomass flowing from the rumen. Gram-positive bacteria are found in abundance in SAB, as they are a large proportion of the bacteria attached to cell walls of plant material (Minato et al., 1966). There are differences between composition of Gram-positive and Gram-negative bacteria, so altering relative amounts of these can theoretically alter composition of total microbial protein. Total N in pure strains of Gram-negative was found to be higher than in Gram-positive bacteria at 10.8 vs. 9.9% (Arambel et al., 1982). The DAP content of SAB was found to be lower than that of LAB, which is probably due to lack of DAP in cell walls of Gram-positive cocci (Work and Dewey, 1953). In addition, DAP:total N ratio and the purine:total N ratio were greater for SAB than for LAB (Clark et al., 1992). In contrast to observations of DAP, AA profiles of Gram-positive and Gram-negative bacteria have been shown to be similar (Arambel et al., 1982). Furthermore, differences in growth rate of bacteria can affect their composition. This has been shown in classic "shift-up and shift-down" experiments. When cells grow faster, there is an increase in ribosomal RNA ("shift-up"), and when they are grown slower ("shift-down"), ribosomal RNA decreases (Cooper, 1993).

It has been estimated that on a weight basis, approximately 25% of the bacteria in the rumen are attached to solids while the remainder are in free suspension (Minato et al., 1966). Merry and McAllan (1983) found that in steers receiving approximately equal proportions of concentrate and roughage, one half of the bacterial population was associated with solids as determined by ^{35}S. In previous studies, cattle fed a diet consisting entirely of roughage or mixed diets ranged from 9 to 49% LAB with an average of 29% (Faichney, 1980), and cattle receiving a mixed diet had about 77% SAB. Therefore differences in AA composition between bacteria and protozoa can be

reflected in AA composition of the total microbial biomass if differences exist between relative amounts of these fractions due to dietary changes.

Amino acid digestion and metabolism

Flow of AA from the rumen has physiological repercussions to the animal. However, it is difficult to control the composition of AA entering the duodenum in ruminants due to degradation of dietary protein and its incorporation into microbial protein. As milk production increases, the proportion of microbial protein contributing to the total protein requirement decreases, although it still comprises the majority of the protein required by the cow (Stern et al., 1994). Therefore, the amount of RUP required increases as milk production increases. Despite this increased RUP requirement, the effect of increased RUP on milk production is inconsistent (Santos et al., 1998). The effect can only be positive if RUP does not decrease MPS, so it is important to provide adequate RDP. Furthermore, increasing RUP when RDP is limiting microbial growth will not increase flows of essential AA (EAA), Met, or Lys to the duodenum. A poor correlation in the literature between an increase in undegraded dietary protein flowing from the rumen and an increase in milk production is probably due to a number of factors. These factors include animals that are fed higher CP than that required by the animal, thereby not limiting AA flow. Furthermore, supplemental protein may only provide a small fraction of the dietary protein leaving the rumen, so significant effects are generally only observed when supplied at 35% or more of the CP in the diet. Other limiting factors of MPS are energy, insufficient limiting AA in RUP, poor digestibility of protein in the small intestine, as well as potential interactions with mobilization and utilization of nutrients from body tissues (Clark et al., 1992).

It is extremely difficult to determine AA requirements of ruminants, although AA requirements for some species of rumen bacteria have been elucidated. The first two limiting AA for ruminants are thought to be Lys and Met, and His may potentially be limiting as well (Asplund, 1994). Infusing various combinations of AA into the abomasum indicated that Lys is either marginally first-limiting or that Lys and Met are co-limiting in cows fed diets low in protein and high in corn (Schwab et al., 1976). These authors suggested that AA limiting to milk protein and production are dependent

on dietary ingredients utilized in the ration. Requirement for some AA also appears to be partially influenced by other factors such as stage of lactation, as cows have been shown to have a more positive response in milk production in early lactation when there is a greater demand for CP, as opposed to mid or late lactation (Robinson et al., 2011).

Factors affecting AA composition of rumen microbes

Many researchers have attempted to influence the AA composition of microbial protein flowing to the small intestine. However, the generally accepted hypothesis in current literature is that AA composition of microbial protein is an intrinsic characteristic of each group of microorganisms (Martin et al., 1994), making manipulation of the AA profile of microbial protein flowing from the rumen difficult. A summary of bacterial samples from 35 experiments encompassing 61 dietary treatments expressed as g of individual AA/100 g of total AA shows large differences in AA composition (Clark et al., 1992), but much of this variation can be explained by differences in methodology (Martin et al., 1996). It is typically assumed that LAB are representative of rumen bacteria, but this is probably an invalid assumption. It has been postulated that even the SAB collected in most studies are not entirely representative of the entire SAB population (Martín-Orúe et al., 1998) because samples from this population are more likely to be contaminated by feedstuffs from the rumen (Martin et al., 1994).

Various fractions of rumen microbes (SAB, LAB, and protozoa) have different chemical compositions and AA compositions. Therefore, if the relative abundance of various fractions can be manipulated, so can chemical composition of microbial protein (Martin et al., 1994). For example, protozoa are high in Lys but low in Met compared with bacteria (Shabi et al., 2000). Several studies have shown that AA composition differs between LAB and SAB. An in vivo study showed that EAA contents are lower in SAB than in LAB (Martin et al., 1996). When AA profiles of LAB and SAB were compared in continuous culture, concentrations of Asp, Ala, and Arg were higher ($P < 0.05$), and concentrations of His and Tyr tended to be higher ($P < 0.1$) in SAB compared with LAB (Rodríguez-Prado et al., 2004). This study attempted to manipulate the relative amounts of SAB and LAB by utilizing differing fiber amounts and particles size (ground to 1 *vs.* 3 mm diameter) in the diets, but no differences in flow of EAA, non-essential AA (NEAA), and most individual AA from the vessels were found due to treatment.

Attempts have been made to manipulate the AA composition of microbial protein through diet. Martin and colleagues (1996) found no difference in AA composition within the LAB, SAB, and protozoal fractions as an effect of diet. Volden (1999) observed that diets with increasing levels of highly degradable protein increased duodenal levels of Arg, His, and Met compared with diets with both high and low levels of relatively undegraded protein. In a continuous culture experiment, Rodriguez-Prado et al. (2004) found significant but numerically minor differences in AA composition between outflows in fermenters in high fiber diets compared with low fiber diets depending on the bacterial population (LAB vs. SAB) considered. In LAB, Asp, Glu, Gly increased with the high fiber treatment, whereas Ile and Lys were lower. When SAB were analyzed, Ile was lower with the high fiber treatment than with the low fiber treatment. Total flow of AA from microbial origin was lower in the low fiber treatment. The proportion of EAA in LAB was 2.43% lower in the high fiber treatment, although SAB showed no effect of fiber treatment. It has been hypothesized that feed intake may also affect AA composition of microbial protein. Volden (1999) found that decreasing DMI from 20 to 10 kd/g increased Cys, Ile, and ornithine in the duodenum.

Amino acid flow from the rumen

Clark and others (1992) summarized studies totaling 152 dietary treatments and found that on average that 59% of NAN flowing from the rumen is microbial N. This value ranged from 35 to 66% with the prerequisites for study inclusion being that a large amount of a well-balanced ration was fed to cows producing 30 kg of milk/d or more. Although methods exist to separate LAB and SAB from rumen contents, determining their relative flows to the duodenum is more challenging, and it is difficult to observe their respective contributions to AA in the chyme (Martin et al., 1994; Rodríguez-Prado et al., 2004). Rodriguez-Prado et al. (2004) found greater differences in AA composition of fermenter outflows than could be accounted for in differences between LAB and SAB. This finding could be due to errors intrinsic to the analysis of microbial material. The same study found no effect of particle size on AA flow from the fermenters when particles were ground to pass through a 1-mm sieve *vs.* a 3-mm sieve, although there was an effect of fiber content. This observation was in contrast to the

hypothesis that decreasing particle size would increase surface area available to microorganisms and therefore increase the proportion of SAB in the outflow.

Manipulating AA flow from the rumen

Relative proportions of differing N fractions escaping the rumen can be influenced in a number of ways, and the protection of a portion of dietary N is one way to have an impact on AA presented to the small intestine and ideally absorbed by the animal. Because there is a low carboxypeptidase activity in the rumen, peptides can be blocked from degradation by acetic anhydrides (Wallace, 1996). In addition, many proteolytic enzymes are metal-ion dependent; chelation can protect peptides from breakdown by these enzymes.

One method of altering AA profile entering the duodenum is to feed protected AA, particularly those that would be first limiting. Theoretically, matching the profile of AA flowing from the rumen to the AA profile of milk would optimize milk production if no other factors were limiting (Robinson et al., 2011). Supplementation of a ruminally-protected Lys product in diets formulated for Lys to be the first-limiting AA provided the intestine with 9 to 10 g of intestinally absorbable Lys/d, but this resulted in milk fat depression (Swanepoel et al., 2010). Milk fat depression was probably due to an AA imbalance created at the site of absorption or stimulation of other protein processes in the body such as muscle stimulation. Piepenbrink et al. (1996) hypothesized that excessive or poorly balanced supplementation of ruminally-protected Lys and Met can decrease milk production. Alternatively, Robinson et al. (2011) provided 15 to 21 g Lys/d to early- and mid-lactation cows that elicited an increase in milk production and components compared with the early-lactation control that received no protected Lys supplement, although there was no effect with mid-lactation cows. However, plasma Lys concentration did not increase, indicating that although there was a milk response, the animals' Lys requirements still had not been met. One philosophy of adding protected AA to the diet is that it is as an opportunity to feed a lower CP diet. Socha et al. (2005) showed that cows in early lactation could produce similar amounts of milk when fed a diet with 18.5% CP compared with 16% CP, with supplementation of protected Met and Lys.

Utilization of absorbed AA in animal tissues

Essential AA are the AA that cannot be synthesized *de novo* by the animal. The essential AA have been divided into two main groups. Group 1 AA consist of His, Met, Phe + Tyr, and Trp. This group is mainly catabolized in the liver, the major site of ureagenesis, from where they are taken up into the mammary gland and secreted as milk protein at a level approximately equal to their post-liver supply. Therefore, the amount of these AA utilized to make protein is approximately equal to the amount returned from protein breakdown, resulting in no net utilization. Amino acids in Group 2 are Ile, Leu, Val, and Lys. This group undergoes minimal hepatic extraction and their post-liver supply is greater than mammary uptake, which is greater than their secretion into milk protein. Therefore, there is a net utilization of this group of AA by peripheral tissues. Because mammary uptake of these AA is greater than their secretion in milk protein (Raggio et al., 2006), it has been hypothesized that they supply N and C for NEAA synthesis and also serve as an internal energy source (Lapierre et al., 2007). In dairy cattle, when an AA is supplied at greater levels, tissues (the liver for Group 1 and peripheral tissues for Group 2) increase catabolism at a greater rate than the increase in incorporation into milk protein.

Intestinal digestion of crude protein in ruminants

The main sources of N to the duodenum from the rumen are microbial protein, RUP and endogenous protein. The NRC (1989) assumed 80% intestinal digestibility of RUP for all feedstuffs due to lack of data. However, there is a large variation among and within protein supplements in intestinal digestibility (Stern et al., 1997). Potentially, this variation can result from the source and quality of the raw material used to make the protein supplement, storage time and temperature, and drying conditions. Studies investigating the intestinal digestibility of microbial protein in the small intestine have found values between 72 to 85% (Storm and Ørskov, 1983; Wallace, 1983; Siddons et al., 1985; Larsen et al., 2000).

Nitrogen passage to the small intestine

A common weakness among studies investigating small intestinal digestibility and flow of N from the rumen is the difficulty associated with determining how much material leaves the rumen and of this material, what portion is dietary, endogenous, or microbial in nature (Dewhurst et al., 2000). Theoretically, due to N recycling, microbial-N flow to the duodenum can be greater than N intake (Dijkstra et al., 1998). Hvelplund and Hesselholt (1987) detected only small differences in AA composition of feeds incubated in the rumen for 16 h and unincubated feeds. They concluded that AA composition of undegraded protein leaving the rumen will be mostly similar to that of feed entering the rumen, although treatment of soybean meal (SBM) by formaldehyde decreased Lys and Tyr content but not digestibility in the small intestine.

Several factors affect N flow to the small intestine. Similar to the way that many of factors influence MPS by their interactions with each other, interactions can occur when discussing N flow to the small intestine. Obviously, any factor that affects MPS in the rumen will affect microbial-N passage from the rumen. The current discussion will focus on how these factors and others influence the flows of various N fractions from the rumen.

It remains unclear what effect the flow of solid and liquid fractions from the rumen have on microbial-N flow from the rumen. Obviously, changes in solid retention time and dilution rate will affect proportions of SAB and LAB, respectively, from the rumen. Furthermore, because the solid phase is carried to the rumen by the liquid phase, any change in dilution rate has the potential to affect solid retention time (Crawford et al., 1980a). Microbial-N flow has been shown to be intermediate between solid and liquid phase flow from the rumen (Faichney, 1980). It is possible that omasal sampling may provide a more accurate picture of microbial-N flow to the duodenum (Firkins et al., 2006). Rode et al. (1985) found no effect on the turnover rate of solids even though dilution rate increased with increasing forage level. Increasing dilution rate does increase MPS and EMPS, as discussed above, and has been shown to increase the flow of total AA to the duodenum (Harrison et al., 1976). An increase in feed intake can result in greater flows of N fractions from the rumen, possibly because of an increase in solid and liquid flows from the rumen (Clark et al., 1992).

Microbial ecology of the rumen can affect flow of N to the duodenum. Protozoa can have a large impact on N flow. Flow of dietary N from the rumen has been shown

to either increase or remain unaffected by defaunation. In addition, due to the lack of predation by protozoa, bacterial-N flow may almost double after defaunation. This increase in dietary and bacterial N consistently increases NAN flow to the small intestine. In addition, several studies have shown an increase in total-AA, essential AA (EAA), and non-essential AA (NEAA) flow to the duodenum upon removal of protozoa. Because of the high lysine content of protozoa, this AA increases the least (Jouany, 1996). In a meta-analysis, researchers found that defaunation increased microbial-N flow to the duodenum by 21% when expressed as a percentage of live weight (Eugene et al., 2004). In one study, two different diets were fed to wethers to encourage a large protozoal population (L) or to discourage protozoal growth (S), and sheep on both treatments were fed these diets after a defaunation period (Ushida et al., 1986). The L diet resulted in a decrease in NH_3-N concentration. Organic matter digestibilities were similar for both diets in the entire digestive tract although defaunated sheep had less OM digestion in the rumen. Flow of total N from the rumen was also greater in defaunated sheep as well as microbial N and dietary N. The latter effect on dietary N was probably due to a reduction in protozoal proteolysis. Thus, EMPS in defaunated animals was 1.5 to 2.0 times greater than in faunated animals. However, in faunated animals, the S diet promoted EMPS more than the L diet. In a study designed to test the effect of protozoa on microbial-N flow utilizing three different microbial markers (rDNA, purines, and ^{15}N), the effect of defaunation on microbial-N flow to the duodenum was dependent on marker (Belanche et al., 2011). When LAB was used as a reference, there was no difference between treatments on microbial-N flow. However, when rDNA was used as a marker, defaunated lambs had a greater microbial-N flow. Similarly, effects on EMPS were due to differences in markers rather than treatments.

Increasing feed intake can increase MPS and increase flows of NAN, NANMN, microbial N, and AA. In an experiment where cattle were fed the same diet at four levels of intake, higher intake resulted in increasing passage of OM, NAN, NANMN, and microbial N to the small intestine (Clark et al., 1992). The increase in NANMN and microbial N in turn resulted in an increase in individual and total EAA, individual and total NEAA, and total AA available for absorption in the small intestine. This effect was probably due to an increase in both solid and liquid flow to the small intestine and an increase in OMTD, possibly leading to a decrease in energy and N recycling within the

rumen. In a review by Clark et al. (1992), a summary of 39 studies showed that increasing OMI increased OMAD (r^2 = 0.53) but decreased OMAD as a percentage of OMI (r^2 = 0.39). A similar relationship between OMTD and OMI was shown in a summary of 28 experiments, with an increase in OMTD with higher OMI (r^2 = 0.56) but a decrease when expressed as a percentage of OMI (r^2 = 0.30). Summarizing results of 36 experiments showed that NAN consistently increases with greater OMI (r^2 = 0.83). The same experiments also showed that NANMN increased with increasing OMI (r^2 = 0.54), but there was considerable variation, indicating that there are other factors affecting this response. Microbial N also increases with increasing OMI also, as seen in a summary of 41 experiments (r^2 = 0.62) (Clark et al., 1992). The positive relationships between NANMN and microbial N and OMI indicate that they contribute to the increase in NAN. The increase in microbial N probably resulted from an increase in energy available from greater fermentable OM, although there are probably other factors influencing this response. Volden (1999) demonstrated an increase in total tract N digestibility at lower DMI in dairy cattle although bacterial-N flow to the duodenum increased.

In addition to interactions previously discussed between N and CHO regarding feed intake, the ratio of forage to concentrate in the ration can affect MPS and subsequent flows of different fractions of N to the duodenum. Rode et al. (1985) fed increasing levels of alfalfa hay in the diet to dairy cattle in order to modify the forage to concentrate ratio. They found that at higher levels of concentrate in the diet, bacterial-N flow to the small intestine was greater, and there was a corresponding increase in OMTD. Flow of bacterial N to the small intestine was maximized at 330 g/d when the diet consisted of 38% hay and 62% concentrate, and it was lowest at 240 g/d when the diet was 80% hay and 20% concentrate. Bacterial-N flow per unit of OMTD was greatest when hay consisted of 80% of the diet when adjusted for DMI, although no difference was observed when results were not adjusted. Bacterial N accounted for 72 to 78% of NAN in this study. Another study found that cows fed 50% forage consumed more OM than those fed 67% forage, but no difference was observed in N intake with the increase in OMI (Klusmeyer et al., 1991). As a result, there was no increase in NH_3-N concentration in the rumen. NANMN flow to small intestine was higher with lower forage level, probably due to the increase in OMI and lower degradability of corn protein

compared with alfalfa. There was a slight decrease in passage of microbial N to the small intestine at a lower level of forage feeding, although EMPS did not differ. At the higher level of forage feeding, passage of individual AA, total EAA, total NEAA, and total AA decreased numerically, but not significantly. However, effects of forage on passage of various N fractions in the literature are variable, as some studies have found no effect. Rode and Satter (1988) found that feeding a 75% forage diet to dairy cows decreased OMAD compared to 25% forage diet, but there was no effect of the proportion of forage in the diet on passage of NAN, dietary N, and microbial N to the small intestine. The combination of these effects resulted in an increase in EMPS. Tamminga (1981) concluded that varying the proportion of forage in the diet from 29 to 81% had little effect on degradation of dietary protein, EMPS, and protein supply to dairy cows.

Type of CHO in the diet, such as NSC, can also affect N flows in ruminants. A study designed to test the influence of type of CHO and protein on ruminal digestion and microbial metabolism in dairy cows with differing amounts of NSC and RDP found that the diet lowest in both NSC and RDP had the lowest OM intake, the lowest concentration of NH_3 in the rumen, and lowest digestion of OM and NSC (Stokes et al., 1991a). This diet decreased flow of microbial N to the small intestine, although NANMN flow increased, and as a result NAN and total N were comparable to other diets. There was no effect of diet on EMPS. Available N, not including the NH_3 fraction, and energy were probably limiting to MPS in the diet that had the lowest NSC and RDP. Cameron et al. (1991) designed a study to test different strategies for feeding a protein source to dairy cows with low degradability such as fish meal. They supplemented the diet with urea (0.75% of DM), starch and dextrose (9.38 and 3.12% of DM, respectively), or urea plus starch and dextrose to test the effects of these sources of CHO and protein. Results showed that feeding starch at this level decreased OM and N intake, and did not affect OMTD, but did increase ruminal starch digestion and decreased ruminal fiber digestion and NH_3 production. There was no effect of starch on flow of total N or any N fraction to the small intestine, despite the decrease in N intake. Authors hypothesized that microbial N may not have increased because of a decrease in OM intake, OM or fiber digestibilities, energetic uncoupling, or shortage of N in forms besides NH_3. Starch

inclusion in the diet decreased Arg and increased Met flow to the small intestine, but no other AA were affected.

Dietary CHO source can also affect flow of N to the small intestine. No difference was detected in OMAD between cows consuming 14 to 15 kg/d of corn- or barley-based diets at either a 60:40 or a 90:10 forage to concentrate ratio (Oldham et al., 1979). Despite the lack of difference in digestibility, passage of microbial N to the small intestine was greater in cows fed barley-based diet; therefore, EMPS was greater for cows fed the barley-based diet. However, this effect was dependent upon the microbial marker utilized. In another study (McCarthy et al., 1989), source of CHO and protein were studied in a 2 x 2 factorial arrangement with corn or barley as the CHO sources and SBM or fish meal as the protein source. Cows on the corn-based diet had higher feed intake than those on the barley-based diet at 23.8 and 20.7 kg DM/d, respectively. There was a significant increase in starch intake and a tendency for N intake to increase in corn-based diet because of an increase in DMI. Barley-based diet had higher OM and starch digestion and a decrease in NH_3 concentration in the rumen, so corn-based diets had a greater flow of OM and starch to the duodenum. The corn-based diet had higher NAN flow to small intestine because of greater NANMN. Effects of starch source on NANMN and AA flow were probably due to low degradability of corn protein and greater DMI. The amount of microbial N flowing to the small intestine and EMPS were not affected by diet. This could be due to low NH_3-N concentration in the rumen. Although SBM diets typically result in higher NH_3-N concentrations due to higher RDP, all 4 diets had NH_3 concentrations less than 4 mg/dl. However, it is likely that NH_3 production was not limiting to OM degradation because corn inclusion increased OM degradability in the rumen although barley-based diets had higher NH_3 concentrations. Corn increased flow of Thr, Met, Ile, Leu, Phe, and His, although Val, Lys, and Arg flow were not affected. However, another study found no difference in OM intake; N intake; OMAD; NH_3 concentration; microbial N, NANMN, or NAN flows to the small intestine; or EMPS when barley replaced corn as the concentrate source in the ration, although the numeric trends were similar to McCarthy et al. (Rode and Satter, 1988). Finally, Herrera-Saldana et al. (1990) fed milo or barley as concentrate and cottonseed or dried brewers grains as CP supplement. They found no differences in OM, N, and starch intake, but feeding barley increased OM and starch digestibility and decreased NH_3-N

concentration. Flow of microbial N increased, but NAN was similar between diets due to a decrease in NANMN. The best combination of ingredients for EMPS and quantity of microbial N in this study was barley and cottonseed. In the study by Martín-Orue (2000), NAN did not differ between corn-based and barley-based diets, even though dietary-N intake was greater due to increased DMI in the barley-based diet. However, microbial-N flow from the rumen was greater for the barely-based diet, although this effect was not significant when expressed as a function of organic matter apparently digested. Yanez-Ruiz et al. (2006) fed two different dietary treatments, a control grass silage and a grass silage high in water-soluble carbohydrates. Microbial-N flow from the rumen tended to be higher in animals fed silage high in water-soluble CHO, and flow of protozoal-N increased to 18.2 g/d compared with 14.2 g/d in the control (P = 0.058) as measured by 18S rDNA. The authors estimated that protozoal-N made up 21 to 25% of microbial N entering the duodenum.

Supplementation of fat in the diet can also have an impact on flow of microbial N. As mentioned previously, high levels of fat supplementation can defaunate the rumen, and the lack of protozoa may improve EMPS. In order for fat feeding strategies to successfully supply energy to the ruminant, fat cannot decrease availability of AA. Successful attempts have been made to use fat to protect AA from rumen degradation and increase flow to the duodenum without altering rumen fermentation. For example, calcium salts of long-chain fatty acids (Ca-LCFA) are inert in the rumen at recommended amounts, and are a possible way to supplement fat (Clark et al., 1992). Klusmeyer et al. (1991) showed a decrease in OM and N intake as well as OMTD when Ca-LCFA were fed, but no effect was observed on NH_3-N concentration and microbial N, NANMN, and NAN flow to the small intestine. Efficiency of MPS was numerically but not significantly greater with Ca-LCFA. Flow of Met to the small intestine decreased significantly, and passage of other EAA decreased in small amounts numerically following Ca-LCFA supplementation.

Similar to the effect of source and amount of dietary N on MPS, these factors will also affect N flow to the duodenum. In particular, the amount of CP consumed has a large impact on flow of NAN to the small intestine, mostly from undegraded protein in diets containing 11 to 25% CP. In a summary of 5 experiments encompassing 22 diets designed to investigate effects of N intake on flow of N fractions, increasing NAN flow

was correlated with increasing N in the diet (r^2 = 0.82) (Clark et al., 1992). When the regression equation included DMI, r^2 value increased (r^2 = 0.90). In the same summary, no significant effect of N intake on microbial-N flow to the small intestine was observed. Nitrogen intake only explained about 29% of the variation in microbial-N flow, but including DMI in the regression equation increased the r^2 from 0.29 to 0.65, so it is possible that there was an interaction with N intake and other dietary factors.

Dietary-N concentration becomes of particular importance when the ration is deficient in N for microbial protein synthesis. Non-protein N is of little use to the ruminant if it is not converted to NH_3 for MPS. If microbial growth is limited by either limitations in readily-fermentable energy or N, the result can be a decrease in digestion of OM and energy availability. Microbes prefer NH_3 as an N source for growth, and they are very efficient at scavenging it, so they can grow on fairly low concentrations of ruminal NH_3. At NH_3-N concentrations higher than 2 to 5 mg of NH_3-N/dl, OMTD is a better indicator of microbial-N flow, and most of the increase in AA passing from the rumen is from a decrease in ruminal protein degradation. In an experiment by Stern et al. (1983), diets were formulated to replace corn gluten meal with feeds with low CP in a grain mixture that was assumed to have equal fermentability. The increase in CP from corn gluten meal increased ruminal NH_3-N concentration from 9.6 to 14.4 mg/dl in dairy cattle without affecting OMTD or bacterial-N and bacterial-AA flow to the small intestine. The authors suggested that energy availability was more limiting than NH_3 for MPS under these circumstances. Increasing CP with corn gluten meal increased AA intake and increased the proportion of AA in NANMN. Although MPS was not affected, increasing CP from corn gluten meal increased total AA supply to small intestine. Four studies were designed to alter N intake and investigate the relationship between ruminal NH_3 and dietary N as well as the effects of ruminal NH_3 production on microbial-N flow to the small intestine (Clark et al., 1992). A summary of these studies showed that there is a relatively strong relationship between dietary CP and NH_3 (r^2 = 0.50), but a poor correlation between NH_3 production (2 to 30 mg/dL) and flow of microbial N to the small intestine (r^2 = .08).

There are a large variety of protein supplements available for livestock feed, including byproduct feeds. Clark et al. (1992) summarized 8 experiments that replaced SBM with another CP source to investigate the effect of N source on N flow to the small

intestine of dairy cattle. Feedstuffs utilized were fish meal, blood meal, corn gluten meal, feather meal, dried distillers grains, dehydrated alfalfa and corn gluten meal, and combinations of fish meal, blood meal, and feather meal. Supplements were supplied at a range from 14 to 65% of total CP intake, and DMI ranged from 14 to 25 kg/d/cow. When NANMN flow to small intestine was set to 100%, protein supplements with lower degradability increased NANMN flow, but no effect was observed until the supplements provided 35% or greater of CP. Low degradability of feeds decreased proportion of NAN that was microbial N. They also found that passage of microbial N to the small intestine decreased when supplements with low degradability were fed, but there was a poor relationship between amount of supplement as a percentage of SBM and passage of microbial N (r^2 = 0.06). They hypothesized that this response was due to the small amount of energy provided by degradable protein to microbes. They also postulated that a lack of AA and peptides as substrate for microbial protein may contribute to the decrease in N flow. Siddons et al. (1985) compared feeding of a high-N corn silage to a low-N grass hay in wethers. Microbial-N flow to the duodenum increased in animals fed grass hay, while OM apparently digested in the rumen decreased, resulting in an increase in EMPS.

Cameron et al. (1991) observed that supplementation of fish meal with urea increased NH_3 production above the 5 mg/dl requirement of rumen microbes, and subsequently increased microbial-N passage to the small intestine by 40 g/d. In a meta-analysis, Ipharraguerre and Clark (2005) showed that treated soy products, corn by-products, animal meal, and mixtures of animal, marine, and/or plant proteins increased the proportion of NANMN flowing to the duodenum compared with SBM controls. There was a corresponding 7% depression of microbial-N flow to the small intestine with an increase in RUP supplementation. There was also an increase of about 9% in ruminal outflow of EAA when RUP was supplemented. Therefore, it is possible that the detrimental effect of depressing microbial-N outflow was compensated for by an increase in EAA flow.

In the previous summary of 8 experiments (Clark et al., 1992), a low correlation between percentage of total CP from poorly degraded supplements and flow of Met, a potentially limiting AA, to the small intestine (r^2 = 0.28) was observed. There was no

significant increase in Met flow to small intestine until supplements were fed at 35% of total CP or more. A similar relationship was detected with Lys.

There are several potential reasons why supplementing proteins with low ruminal degradability do not have a large impact on AA profile of duodenal digesta (Clark et al., 1992). First, protein supplements only provide a fraction of the AA entering the duodenum. Second, due to the effect of intake on protein degradation, degradabilities of the supplements may be overestimated for cows with high intakes, so feeds such as SBM may not be as highly degradable as estimated. Low degradability may result in other limiting factors, such as energy, NH_3, AA, peptides, or a combination of these factors.

Intestinal digestibility of protein flowing from the rumen

Digestion of bacterial protein in the small intestine is often incomplete because bacterial cell walls resist degradation, particularly for Gram-positive bacteria compared with Gram-negative. There is a trend for a decrease in digestibility in the small intestine determined using a pepsin-pancreatin in vitro assay with an increase in the ratio of Gram-positive to Gram-negative bacteria, but the effect is minor (Wallace, 1983). Siddons et al. (1985) found small intestinal digestibility of ^{15}N labeled-microbial NAN to be 72%. This value may have been slightly overestimated because small amounts of labeled endogenous proteins may also have been measured as a result of the nature of the methods utilized in the study. In rats, digestibility of protozoa was shown to be greater than bacteria (McNaught et al., 1954). Because the total flow of AA to the intestine increases in defaunated animals, AA available for absorption is still more likely to be greater when protozoa are absent (Jouany, 1996).

In contrast to the large amount of experiments that analyzed the AA composition of rumen microorganisms, experiments to determine the intestinal digestibility of these AA are limited. Apparent intestinal digestibility of AA-N of microbial origin in sheep was estimated to be 69% and true intestinal digestibility averaged 86% (Tas et al., 1981). Storm and Ørskov (1983) determined that the average true digestibility of AA-N from abomasally infused rumen microbes was approximately 84.7%. Histidine, cysteine, and proline had values significantly lower than the average (68, 73, and 76%, respectively). All other AA had values ranging from 80 to 88%. Hvelplund and Hesselholt (1987)

determined that diet did affect intestinal digestibility of microbial protein or the AA composition, possibly due to different proportions of Gram-positive and Gram-negative bacteria. There was a trend for bacteria isolated from cattle fed diets with high proportion of barley or tapioca to be more digestible in the small intestine compared with cattle fed a hay diet or mixed diet. Cys and Phe numerically had the lowest digestibilities (74 and 69%, respectively). Other AA ranged from 80 to 91%. The main differences between these two studies were in His, Phe, and Pro, while other AA showed small differences. The consensus between Tas et al. (1981) and Hvelplund and Hesselholt (1987) was that, except for Cys, the mean value for digestibility of AA of microbial protein origin is approximately 85%. Mean digestibility for Cys was 75%. Hvelplund and Hesselholt (1987) concluded that a mean value for these AA can be used in protein evaluation systems without introducing significant error.

Other sources of AA flowing to the duodenum besides that of microbial origin are either of dietary RUP or endogenous protein. Therefore, manipulating the proportion of dietary protein that remains undegraded entering the small intestine is one way to manipulate AA flow from the rumen. However, increasing RUP does not consistently have a positive effect on lactation performance. Potential reasons for this lack of response include: a decrease in MPS, poor AA profile of RUP, low intestinal digestibility of RUP, and the ability of the RUP content of controls to meet intestinal AA requirements (Bateman et al., 2005). Hvelplund and Hesselholt (1987) observed differences in AA digestibility for different protein sources infused into the abomasum of sheep. Protein sources that were investigated included SBM, formaldehyde-treated SBM, undegraded SBM, undegraded cottonseed cakes, undegraded rapeseed meal, undegraded sunflower cakes, undegraded fish meal, and undegraded coconut cakes. Undegraded sources were incubated in nylon bags in the rumen for 16 h before infusion. Treating SBM with 0.5% formaldehyde reduced digestibility of most individual AA compared with untreated SBM. The greatest reduction in intestinal digestibility was observed in Phe (0.24 units lower), and average reduction of all AA was 0.13 units. Amino acids from undegraded SBM had higher intestinal digestibility than infused SBM, ranging from 0.02 to 0.15 units higher; however, only Met was significantly different. Variation among and between undegraded feed AA was observed for digestibility in the small intestine, although Arg was consistently the highest (ranging from 0.67 to 0.90) and Cys had the lowest

digestibility in every feed tested except cottonseed cakes (ranged from 0.42 to 0.76). Some AA had values greater than 0.90. Volden (1999) found that at high intake, high protein diets with low protein degradability had higher AA flow to the duodenum than cows fed high protein with high degradability and low protein with low degradability. However, there was no effect at low intake levels.

Dried fermentation biomass

Feeding co-products of industrial processes is a common strategy for reducing feed costs without sacrificing nutrients in production settings. One such co-product is dried fermentation biomass (FB), which is a co-product of the commercial lysine production industry. Because the L-isomer of lysine is the only one available to mammals, fermentation is the preferred form of lysine production as opposed to biosynthesis (McPherson, 1966). Two species of bacteria are utilized in lysine fermentation, *Corynebacterium glutamicum* and *Escherichia coli* (Wittmann and Becker, 2007). The latter is utilized because its metabolism is well-understood and its DNA can be easily manipulated to make production more efficient. The medium used for *E. coli* in lysine production consists of inorganic salts, organic compounds, a saccharide starting material, and vitamins. Once the fermentation process has been completed, lysine must be removed from the bacterial cells that produced them (Anastassiadas, 2007). This process can be carried out in a number of ways, including rotary vacuum filtration and centrifugation, leaving a fermentation biomass or "cell paste." Unless utilized as a by-product and fed to livestock, this fermentation biomass is a waste product. The AAFCO definition of FB proposed in 2007 is "a nonviable biomass product resulting from the production of the amino acids by the fermentation of nonpathogenic, nontoxigenic, risk group 1 *Escherichia coli*. The product must contain a minimum of 75% crude protein on a dry matter basis. The product is intended as a source of protein. Non-protein nitrogen content must be guaranteed when present."

To date, limited research has been published investigating the effects of FB in livestock diets. Sulabo et al. (2011) found that the AA digestibility of FB was similar to or greater than that of fish meal, and did not differ from SBM in digestibility of CP and EAA, although the apparent total tract digestibility of energy was less than that of corn

meal or fish meal in weanling pigs. However, FB does contain more metabolizable energy than either of these feeds. There has been no published research to date on the effects of FB on ruminal metabolism. Because protein source in the diet can affect MPS as well as N flow to the intestine, more research is required to discover what influence this byproduct might have on protein metabolism in ruminants.

SUMMARY

Protein is required by all animals for maintenance, growth, and production, but the protein requirements in ruminants are difficult to determine due to alteration of dietary N by rumen microorganisms. In addition to the main functional role of rumen microbes on fermentation, they are also the main source of protein supply to the small intestine. Many factors can affect the amount of microbial protein available for digestion in the intestine, but altering their composition has proven to be difficult experimentally. More work needs to be done to determine how digestible microbial biomass is in the intestine and what factors may affect digestibility. One strategy for meeting protein requirements for ruminants cheaply is to include byproducts in the ration. Dried fermentation biomass, which is a co-product of the lysine production industry, has potential as a protein supplement for ruminants, but to date, no research has been published that has explored this possibility.

OBJECTIVES

The objectives of the following experiments are 1) to determine if FB could be utilized as a protein source in ruminant diets by mimicking conditions of the rumen in vitro with continuous culture fermentation and estimating ID in vitro, and 2) to estimate the ID of LAB, SAB, and protozoa collected from the rumen by the pepsin and pancreatin enzymatic degradation procedure of Stern and Calsamiglia (1995).

Experiment 1: EFFECT OF DRIED FERMENTATION BIOMASS ON MICROBIAL FERMENTATION IN CONTINUOUS CULTURE AND IN VITRO INTESTINAL DIGESTIBILITY

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Overview. The objective of the current experiment was to determine if fermentation biomass (FB), a dried bacterial co-product derived from lysine production (Ajinomoto Heartland, Inc.) can be used as a protein source in ruminant diets. Eight dual-flow continuous culture fermenters were inoculated with rumen fluid and used during one experimental period consisting of a 7-d adaptation period followed by 3 sampling days. Substrate for the microbes was provided by one of two isonitrogenous diets, CON or DFB. In CON, soybean meal (SBM) provided 57% of total CP, and in DFB, SBM and FB provided 12 and 45% of total CP, respectively. CON contained 3% molasses, 16% ground corn, 13% grass hay, 48% corn silage, and 20% SBM on a DM basis. DFB contained 3% molasses, 18.4% ground corn, 13% grass hay, 50% corn silage, 8.5% SBM, and 6.7% FB. Fermenters were supplied with 75 g/d of DM divided into 8 equal portions. Anaerobic conditions were maintained by infusion of N_2; pH was maintained between 5.8 and 6.8; and temperature was set at 39°C. On sampling days, liquid and solid effluent were collected, combined, and homogenized to be used for chemical analysis and in vitro estimation of intestinal digestibility (ID). Treatment did not affect (P > 0.1) average, maximum, or minimum fermenter pH. There was no effect (P > 0.1) on apparent or true OM, NDF, or ADF digestibility (%) or total VFA (mM), although branched-chain VFA (mM) was higher (P = 0.01) in CON because isobutyrate (mol/100 mol) tended (P = 0.06) to increase with CON treatment. Source of N had no effect (P > 0.1) on total, dietary, or bacterial-N flows. Addition of FB decreased (P < 0.05) NH_3-N flow from 0.4 to 0.2 g/d and tended to decrease (P =0.06) effluent NH_3-N concentration from 17.1 to 9.7 mg/100 mL. His and Met flows increased (P < 0.05) from 0.48 to 0.53 and 0.18 to 0.20 g/d, respectively, when FB partially replaced SBM in the diet, but there

were no effects ($P > 0.1$) on other AA or total AA flows. There was a trend ($P = 0.08$) in percent non-essential AA input (CON = 73.6% *vs.* DFB = 82.2%) in effluent; however, there was no effect ($P > 0.1$) on percent of essential AA or total AA input in effluent. Effluent from the DFB treatment was higher ($P < 0.05$) in ID than CON (CON = 70.4% *vs.* DFB = 79.6%). These results indicate that FB elicited a similar response in N metabolism and AA flows to SBM but had a higher estimated digestibility in the intestine, and has potential use as a protein source in ruminant diets.

Keywords: continuous culture, fermentation biomass, intestinal digestibility, protein

INTRODUCTION

Feeding co-products derived from industrial processing is a common strategy for reducing feed costs without sacrificing nutrients for animal production. Fermentation biomass (FB) is a co-product of commercial Lys production. *Escherichia coli* is utilized in Lys fermentation because its metabolism is well-understood and its DNA can be easily manipulated to make production more efficient (Wittmann and Becker, 2007). Once the fermentation process has been completed, Lys must be removed from bacterial cells by ultrafiltration or centrifugation, leaving FB (Anastassiadas, 2007).

The relative amount of protein degraded in the rumen (RDP) or passing to the small intestine (rumen undegradable protein, RUP) is important for providing amino acids (AA) for maintenance, growth, and production. Therefore, it is important when analyzing characteristics of a protein source for ruminants in vivo that RDP, RUP, and intestinal digestibility (ID) be determined. The three-step in situ/in vitro enzymatic procedure (Calsamiglia and Stern, 1995) provides a rapid and reliable way to determine degradation and ID of the protein in a feedstuff relative to use of cannulated animals.

To date, limited research has been published investigating the effects of FB in livestock diets. Sulabo et al. (2011) found that AA digestibility of FB was similar to or greater than that of fish meal, and did not differ from soybean meal (SBM) in digestibility of crude protein (CP) and essential AA (EAA) in weanling pigs. There has not been any published research on effects of FB in ruminant diets. More research is required to investigate what influence this byproduct might have on ruminal protein

metabolism and small intestinal digestion in ruminants. The objective of this study was to determine if FB could be utilized as a protein source in ruminant diets by simulating rumen conditions in vitro with continuous culture fermentation and estimating ID in vitro.

MATERIALS AND METHODS

Experimental Diets

Two diets that were formulated to be isoenergetic and isonitrogenous provided substrate for microbial metabolism in this experiment (Table 1). The control diet (CON) had no FB added while the experimental diet (DFB) included FB. High-protein SBM was utilized as the main protein source in CON, accounting for approximately 57% of the CP in the diet. Molasses, ground corn, corn silage, and alfalfa hay contributed the remaining 43% of the CP. Fermentation biomass accounted for approximately 45% of CP in the DFB diet with SBM accounting for 12% of CP. Feedstuffs were dried and ground to 2 mm particle diameter with a Wiley Mill (Thomas Scientific, Philadelphia, PA). Diets were mixed for 20 min using a Hobart mixer (Hobart Corporation, Troy, OH) and pelleted with a California pellet mill (California Pellet Mill, San Francisco, CA). Chemical and amino acid composition of experimental diets are reported in Tables 1 and 2, respectively.

Continuous Culture Fermenter Operation

Eight dual-flow continuous culture fermenters (1034 $\pm$ 40 ml) previously described by Hannah et al. (1986) were inoculated with pooled ruminal fluid strained through 4 layers of cheesecloth. Ruminal fluid was obtained from a cannulated Holstein cow fed a 60:40 forage to concentrate (DM basis) total mixed ration. Fermenters were provided with 75 g of dietary DM/d during the 10-d experimental period. Feed was given simultaneously to all fermenters with an automated feeding device (Hannah et al., 1986) that was operated using an automatic timer (DT 17, Intermatic, Spring Grove, IL), which fed 8 equal portions of the total DM every 3 h, with a 1.5-h feeding phase and a 1.5-h rest phase. Liquid dilution rate was set to 10%/h by continuous infusion of artificial saliva buffer (pH=8.25), and solid dilution rate was set to 5.5%/h by filtrate

removal. Individual fermenter pH was recorded every 10 min by an electronic data acquisition system (Daisy Lab®) and maintained between 5.8 and 6.8 by automated addition of either 5 N NaOH or 3 N HCl. Fermenter temperatures were maintained at 39°C by an electrical heater, and anaerobic conditions were maintained by constant infusion of N_2 at the rate of 40 mL/min.

Sample Collection and Analysis

The experimental period lasted for 10 d with a 7-d adaptation phase followed by 3 sampling days. During sampling, effluents were collected in vessels kept in a 1 to 2°C water bath to retard microbial growth. Solid and liquid effluents were combined and homogenized daily within fermenter, and 500 mL of homogenized effluent was subsampled each sampling day and frozen. Effluent samples from each fermenter were thawed and composited and a 500-mL subsample was collected and lyophilized (25 SL freeze drier, Virtis, Gardiner, NY) for DM, NDF, ADF, ash, purine, and AA determination. On the final sampling day, entire fermenter contents were collected to isolate bacterial cells. Contents were strained through 2 layers of cheesecloth and centrifuged at 1,000 x *g* for 10 min to remove feed particles. Supernatant was then centrifuged at 20,000 x *g* for 20 min to isolate the bacterial pellet.

Diets, effluent, and bacterial pellet DM were determined by oven drying at 105°C for 24 h. Ash was determined by weight difference after 24 h combustion at 550 °C (AOAC, 1984). Sequential detergent fiber analyses (Van Soest et al., 1991) was conducted to determine NDF and ADF concentrations of diets and effluents. Purine concentration was used to partition flow of effluent N into microbial and dietary N utilizing the method of Zinn and Owens (1986) on effluent and bacterial pellet. Total N in the effluent, bacteria, and diet was determined by the Kjeldahl method (AOAC, 1984). Ammonia-N was determined by steam distillation (Bremner and Keeney, 1965) using a Kjeltech 2300 Analyzer Unit (Tecator, Herdon, VA). Amino acids were analyzed by Ajinomoto Heartland, Inc. (Chicago, IL, USA) by total AA analysis (method 994.12; AOAC, 1995). Volatile fatty acids concentrations were determined by capillary GC analysis. Incubation fluid was solvent-extracted using ethyl acetate (3:7 ratio) during 10 min under continuous vortex. Samples were centrifuged at 5,000 x *g* for 5 min and supernatant was stored at -20 °C until analyzed. Analysis was performed using an HP

5890 GC equipped with a Stabilwax-DA capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness, Restek, CA). Chromatographic conditions were as follows: helium (1.7 mL/min); initial oven temperature, 110°C, held for 2.1 min; ramped at 25°C/min to 200°C, held for 1.5 min; injector temperature: 200°C; flame ionization detector temperature: 220°C; split injection (split ratio: 1/10); injection volume: 1 µL.

CP degradation and ID of experimental diets and fermenter effluent

A sample of each diet was mortared for in situ determination of CP degradation and subsequent determination of ID by the procedure of Calsamiglia and Stern (1995) for RUP. Approximately 0.5 g of diet was weighed into duplicate bags (5 x 10 cm, 50 µm pore size, Ankom Technology, Macedon, NY) per time point. Bags were heat sealed using an MP-8 impulse sealer (Midwest Pacific Co., Baltimore, MD) and suspended in the rumen for 2, 8, 16, 24, and 48 h of a dairy cow fed a similar diet to the cow used in the continuous culture experiment. Washout at 0 h was determined by rinsing bags in water. Bags were removed from the rumen at each time point, rinsed, and dried at 60° C for 24 h. Dry matter disappearance was determined by difference in weight, and N was measured using the Kjeldahl method (AOAC, 1984) to determine CP disappearance. Degradation of CP was calculated by the equation of Bach et al. (1998) as follows:

$$\text{Extent of CP degradation} = \text{soluble CP fraction} + \text{degradable CP fraction} \times \left[\frac{K_d}{(K_d + K_p)}\right]$$

where K_d is the rate of degradation, K_p is the rate of passage (assumed to be 0.06 h^{-1}), and degradable CP fraction = 100 – soluble CP fraction. The CP remaining in the bags after 48 h of incubation was considered to be undegradable.

In addition to bags that were used to estimate degradability, 5 Dacron polyester bags per diet were filled with 1.5 g of diet and incubated simultaneously in the rumen for 16 h as part of the three-step procedure. In addition, lyophilized effluent from each fermenter was subjected to the pepsin and pancreatin steps of the three-step procedure for estimation of ID. Intestinally absorbable dietary protein (IADP) was calculated as RUP x ID.

Statistical analysis

Results except for pH were analyzed with the TTEST procedure of SAS (version 9.3). Results for pH were averaged within hour and analyzed as repeated measures with PROC MIXED with an autoregressive order 1 structure of covariance based on minimum values of AIC (Akaike's Information Criterion). Lowest and highest hourly pH were compared for statistical analysis of maximum and minimum pH. Differences were declared at $P \leq 0.05$ and tendencies were noted at $0.1 < P < 0.05$. Due to lack of replication, statistical analysis was not completed on in situ CP degradation or ID estimation of the experimental diets.

RESULTS AND DISCUSSION

In situ CP disappearance

Although not statistically analyzed, numerical values for CP disappearance of the treatments in the rumen are useful for understanding the fermentation and degradation of the diets in continuous culture. Table 3 shows that both diets appeared comparable in RDP (approximately 84%). The soluble fraction of the diets was lower than the mean solubility (67.4% of CP) reported for silages by Hoffman et al. (1999). Alternatively, Colombini et al. (2010) found a solubility of 33.1% of CP for corn silage, and an RDP value of 79.4% of CP, which approaches the RDP value for diets in the current study. Bach et al. (2008) reported a solubility of 30% of CP when SBM was the source of protein in a 60:40 forage to concentrate ration.

Nutrient digestion

Results for digestion are presented in Table 4. Apparent ($P = 0.56$) and true ($P = 0.89$) OM digestion were not affected by treatment, and NDF ($P = 0.71$) and ADF ($P = 0.12$) digestion were also not influenced by treatment. Acid detergent fiber digestion in the current experiment (65.3% and 63.3% for CON and DFB, respectively) was similar to that of Chester-Jones et al. (1990) in a diet provided in continuous culture that contained extruded SBM as the main protein source. However, this level of ADF digestibility is elevated compared to numerous other studies in continuous culture (Hussein et al., 1991; Griswold et al., 2003; Berthiaume et al., 2010). The high level of ADF digestion could be a result of high CP content of the experimental diets (Table 1).

Windschitl and Stern (1988) reported greater ($P < 0.05$) ADF digestion when urea inclusion increased N content of the experimental diets. This effect is presumably due to an increase in activity of cellulolytic bacteria, which would also elevate NDF digestion. Köster et al. (1996) showed that an increase in RDP levels in diets fed to steers resulted in a cubic response in NDF digestion ($P = 0.01$). When RDP levels increased in their experimental diets, NDF digestibility increased initially, but at the highest levels of RDP, NDF digestibility decreased. The response in their study was assumed to be due to the higher passage rate associated with increased feed intake, which does not occur in continuous culture. Digestion of NDF in the current experiment was higher than that reported by Cerrato-Sánchez et al. (2008) when a high protein diet (19.2%) was provided to continuous culture fermenters.

pH

Because there was not a difference in OM and fiber digestion, or total VFA concentration (Table 6), there was no effect ($P > 0.1$) of treatment on pH (Table 5). However, there was an effect ($P < 0.01$) of hourly pH measurements. Average and minimum hourly pH showed an interaction ($P < 0.05$) between treatment and hour, while maximum hourly pH tended ($P = 0.08$) to show an interaction between treatment and hour. Figure 1 shows hourly values for average, maximum, and minimum pH during the 72-h sampling period. There was an increase in average hourly pH in DFB to above 6.00 between 3 and 7-h after the onset of the sampling period. However, there was no subsequent increase at corresponding daily times on the 2 remaining sampling days. Similarly, CON average hourly pH exceeded 6.00 at 25, 26, and 72-h after the onset of the sampling period with no visible pattern in pH fluctuations. Hourly maximum and minimum pH rose at similar times. Because of the controlled feeding schedule throughout the day, it is unlikely that this effect on pH is due to patterns in feeding. The lack of any discernible pattern in pH fluctuations due to time indicates that this effect has limited physiological repercussions.

The average pH for CON was 5.89 and 5.91 for DFB. A pH of 5.9 has been shown to decrease digestion of OM and fiber (Hoover et al., 1989). In the current experiment, pH did not appear to influence nutrient digestion based on high values for OM, NDF, and ADF digestion as well as total VFA concentration.

VFA concentration

VFA concentrations are reported in Table 6. Consistent with nutrient digestion and pH results, there was no difference (P = 0.10) in total VFA concentration. One of the main differences between in vitro and in vivo conditions is the inability in vitro to absorb VFA, resulting in potentially elevated total VFA concentrations. Mansfield et al. (1995) found greater concentrations of total VFA in continuous culture compared with lactating dairy cows fed the same diet (94.9 *vs.* 99.9 m*M* for rumen and in vitro, respectively). In continuous culture experiments, total VFA concentrations ranged from well below 100 m*M* to concentrations greater than 150 m*M* in several experiments. Much of this difference between studies can probably be accounted for by other sources of variation, such as inconsistencies between analytical techniques between laboratories, rather than diet fermentability alone. Crawford, Jr. et al. (1980b) found no differences in total VFA production with differing dilution rates and solid retention times except at a solid retention time of 14.3 h. Values for total VFA concentration were not reported in their experiment. Because there were no differences in dilution rate between treatments in the current experiment, there was not expected to be any differences in VFA concentration. Mean total VFA concentrations in the current experiment were 227.7 and 201.3 m*M* for CON and DFB, respectively. Chester-Jones et al. (1990) observed concentrations of total VFA ranging from 167.1 to 179.9 m*M* in continuous culture when high-concentrate diets (70 to 85% ground corn) provided substrate for microbes in continuous culture. Dahlberg et al. (1988) observed total VFA concentrations as high as 181.2 m*M* when pellets composed entirely of four different forage sources (alfalfa, cicer milkvetch, birdsfoot trefoil, or sanfoin) were supplied to microbes in continuous culture. In a second experiment, Dahlberg et al. (1988) reported total VFA concentrations ranging from 194.6 to 211.5 m*M* using these same forages to supply 85% of total dietary N in diets containing approximately 20% non-structural carbohydrates. Considering the extreme differences in carbohydrate composition in these three experiments, elevated levels of total VFA concentration can partially be accounted for by variations inherent to laboratory analysis. Another factor that can increase total VFA concentration is high concentration of CP in the diet (Table 1). Brooks et al. (2012) showed an increase (P < 0.01) in total VFA concentration in continuous culture when

urea was added to a diet to increase dietary CP to 13.0% compared with the same diet without urea (8.8% CP).

Mean acetate to propionate ratio (A:P) in effluents of both diets were similar (*P* = 0.63). Acetate concentrations were 39.0 and 37.9 mmol/100 mol, and propionate concentrations were 35.7 and 38.5 mmol/100 mol for CON and DFB, respectively. This resulted in A:P of approximately one for both diets, consistent with previous research in high concentrate diets. Stokes et al. (1991b) observed an increase in propionate production with no change in molar concentration of acetate when increasing levels of non-structural carbohydrates were fed in continuous culture. Chester-Jones et al. (1991) found a similar ratio as the current experiment in continuous culture when using a high concentrate diet with differing protein sources.

There was a difference (*P* = 0.01) in total branched-chain VFA concentration resulting from a tendency (*P* = 0.06) for isobutyrate to be higher as a percentage of total VFA in CON. Branched-chain VFA are required by several species of rumen bacteria (Allison et al., 1962), and are derived from branched-chain AA. Specifically, Val is converted to form isobutyrate, which can be resynthesized to Val. Although Val flow from the fermenters was similar (Table 6), CON had more Val delivered to the fermenters daily (0.70 *vs.* 0.56 g/d for CON and DFB, respectively). Although Val flow was not different (*P* = 0.15) between treatments, DFB had a numerically greater flow of Val from fermenters daily than CON (Table 7), possibly indicating that bacteria in these fermenters resynthesized a higher concentration of Val. The combination of these 2 factors may have increased the proportion of isobutyrate in CON effluent.

Nitrogen metabolism

Results for N flows and other variables of N metabolism are presented in Table 7. Total (*P* = 0.29), dietary (*P* = 0.99), and bacterial N (*P* = 0.96) flow from the fermenters was not different between treatments, but NH_3-N flow was lower (*P* = 0.05) for DFB treatment. Similarly, NH_3-N concentration had a tendency (*P* = 0.08) to be higher in CON than DFB. Although NH_3-N flow and concentration differed or tended to differ between treatments, bacterial-N flow (*P* = 0.96) or efficiency of bacterial protein synthesis (EBPS; *P* = 0.99) were not affected by treatment expressed on a DM or OM basis. Ammonia-N concentrations were above the range of 2 to 5 mg/100 mL required

for optimal bacterial growth (Satter and Slyter, 1974). Because NH_3-N cannot be absorbed from fermenter flasks, there are two possible reasons for differences in NH_3-N concentrations between treatments. Either the inclusion of FB in the diet resulted in a decrease in NH_3-N production or there was an increase in utilization of NH_3-N for microbial protein synthesis. However, there was no difference in CP degradation, so the former explanation is not valid. There was a large standard error (0.45) for CON in bacterial N, possibly due to an error associated with estimation of bacterial-N flow by purine analysis. Calsamiglia et al. (1996) showed that bacterial flow calculated by purine N had a higher variation than ^{15}N as a marker in continuous culture, most likely a result of low precision with the assay. Because dietary N flow is determined mathematically by difference between effluent non-ammonia N (NAN) and bacterial N, this value also contains variation from purine analysis. It is possible that differences between treatments for dietary N or bacterial N or both dietary and bacterial N were masked by variation associated with the assay. Because results of the in situ degradation procedure indicate that rate and extent of degradation of dietary CP were similar between diets, it is more likely that bacterial N flow differed between treatments rather than dietary N flow. If this is the case, then the observed decrease ($P < 0.05$) in NH_3-N flow for DFB is likely due to an increase in uptake by microorganisms for microbial protein synthesis, although this cannot be substantiated.

No effect ($P = 0.99$) was observed on percent CP degradation (Table 7) which is consistent with results of the in situ degradation procedure (Table 3). The average pH in this study (Table 4) was within the range of 5.5-7.0 for optimal proteolysis by rumen microorganisms (Kopecny and Wallace, 1982); however, proteolysis may still be reduced at the lower end of this range (Bach et al., 2005). Although pH was below 6.0 in the present experiment, there was no deleterious effect on CP degradation based on comparisons to previous research in continuous culture. Crude protein degradation was higher in the current experiment than it was in a study performed by Cerrato-Sánchez et al. (2008) where CP degradation ranged from 35.5% at pH 5.1 to 44.3 and 50.0% at pH 5.6 and 6.4, respectively, with a 19.2% CP diet. Alternatively, Bach et al. (2008) observed values similar to the current experiment for CP degradation ranging from 52.6 to 71.3% when continuous culture fermenters were infused with differing sources of

soluble protein to bring CP content of the basal ration to 15.3%. Minimum pH was set to 6.0 in the study by Bach et al. (2008).

Amino acid flows

Amino acid flows are presented in Table 8. Dietary N source had no effect on total ($P = 0.35$), essential ($P = 0.23$), non-essential ($P = 0.55$), or most individual AA ($P \geq 0.11$) flows from fermenters. Although numerical differences were small, flows of His ($P = 0.02$) and Met ($P = 0.05$) were higher for the DFB treatment. The level of FB in the experimental diet was high enough to alter the composition of AA flow from the fermenters if it had been sufficiently undegradable (Clark et al., 1992). Determination of AA requirements in ruminants is difficult; however, Met is considered to be first or second limiting AA after Lys, and His is also believed to be potentially limiting in some cases (Asplund, 1994). However, despite being statistically different, the potential impact of these AA in vivo is probably minimal because of minute differences.

Percent input of AA in effluent was used to determine the change in AA profile in the fermenters by dividing g of AA in the effluent by g of AA fed daily to the each fermenter (Table 9). There was a tendency ($P = 0.08$) in percent of non-essential AA (NEAA) input to have a treatment effect; however, percent of EAA ($P = 0.17$) and total AA ($P = 0.85$) input in effluent were not affected by treatment. When SBM was the N source, EAA in the effluent had a greater similarity to dietary EAA than FB. When FB was the primary N source, there was a numerically greater similarity between NEAA in the effluent and the dietary treatment. This pattern resulted in no difference in percent of input of total AA in the effluent.

Estimation of ID of diets and outflows

Samples of dietary treatments were exposed to rumen microbes in situ for 16 h and lyophilized effluent samples from each fermenter from the continuous culture experiment were only subjected to the in vitro pepsin and pancreatin steps of the 3-step procedure. Statistical analysis could only be performed on the effluent because of the lack of replication with dietary samples (Table 10). The diet containing FB had a numerically higher ID than CON as well as a higher IADP. Estimates of ID of effluents were consistent with these results. Because effluents from DFB were higher ($P < 0.01$)

in ID than CON, results may indicate potential for DFB to provide more AA to the ruminant for production purposes.

Outflow from fermenters contains dietary N as well as NH_3-N and bacterial N. Bacterial N is highly digestible in the small intestine with estimations as high as 88% for some bacterial AA (Storm and Ørskov, 1983), and is present in fermenter outflow. This is likely the reason for numerically higher estimations of ID for effluents compared to diets. Calsamiglia and Stern (1995) estimated an average ID value of 89.8% for SBM using their 3-step procedure. Maiga et al. (1996) found a similar ID value of 85% also utilizing the same procedure. Boruckl Castro et al. (2007) found an ID value of 87.5% for SBM when the 3-step procedure was used to estimate ID, but a value of 98% when the in situ mobile bag technique (Hvelplund and Weisbjerg, 2000) was applied, possibly due to material escaping from the bags or hindgut digestion by bacteria. Unpublished data from the University of Minnesota estimated the ID of various FB samples to be 80.5% with the 3-step technique, with a range from 74.5 to 87.7%. However, determination of CP degradation in the rumen is difficult with this product because of an extremely small particle size. To prevent material from escaping from the 50 µm pore size bags, 25 µm pore size material was used to make bags with comparable surface area for incubation in the rumen. Depending on the bacterial population and the relative abundance of large microorganisms such as protozoa, this may have resulted in underestimation of CP degradation by limiting exposure of the product to rumen microorganisms. Pore size of bag material in the current literature for this procedure has generally ranged from 20 to 60 µm, with the majority of studies utilizing bags between 40 to 60 µm (Vanzant et al., 1998). Thus, incubated material exposed to enzymatic digestion in vitro may not have been truly representative of the material after 16 h in the rumen. Because diets from the current continuous culture experiment were similar in chemical composition and ingredients other than SBM and FB (Table 1), it can be assumed that the increase in ID with DFB is due to inclusion of FB in the diet.

IMPLICATIONS

Results of this study indicate that dried FB may be utilized in ruminant feeding systems and may even increase the amount of His and Met, two potentially limiting AA,

to the small intestine. In addition, ID of material flowing from the fermenter was estimated to be 11.6% higher for DFB, indicating that this supplement has potential to increase the amount of protein available to the ruminant for maintenance and production. More research with live animals is necessary to validate these results, but these experiments show that this co-product has potential as a feedstuff in ruminant production systems.

Table 1. Ingredient and chemical composition of experimental diets.

	Diet	
Ingredient, % of DM	CON	DFB
Soybean meal	20.0	8.5
Fermentation biomass	-	6.7
Corn silage	48.0	50.3
Corn, ground	16.0	18.4
Alfalfa hay	13.0	13.1
Molasses, liquid	3.0	3.0
Chemical composition	-------% of DM-------	
Crude protein	18.6	18.6
Neutral detergent fiber	28.0	28.3
Acid detergent fiber	17.3	16.7
Non-fiber carbohydrate	43.7	43.1
Ether extract	2.9	3.4
Ash	6.8	6.6
Total digestible nutrients	73	74
NEl (Mcal/kg)	1.7	1.7

Table 2. Amino acid composition of experimental diets.

	Nitrogen Source	
Amino acid (wt/wt)	SBM	FB
Alanine	0.88	1.03
Arginine	2.01	1.23
Aspartic acid	1.57	1.44
Cysteine	0.23	0.17
Glutamic acid	2.62	2.22
Glycine	0.67	0.66
Histidine	0.37	0.30
Isoleucine	0.64	0.63
Leucine	1.28	1.28
Lysine	0.83	0.97
Methionine	0.23	0.29
Phenylalanine	0.82	0.91
Proline	0.91	0.74
Serine	0.81	0.78
Threonine	0.67	0.73
Tryptophan	0.17	0.18
Tyrosine	0.32	0.22
Valine	0.70	0.87

Table 3. Effect of dietary N source on in situ CP degradation.

	Nitrogen Source	
Item	SBM	FB
Soluble protein, % of CP	38.0	36.5
Rumen degradable protein, % of CP	84.0	83.7
K_d[a], h^{-1}	0.08	0.12

[a]Rate of CP degradation

Table 4. Effect of dietary N source on digestion.

	Nitrogen Source			
Digestion	SBM	FB	SEM[a]	*P*-value
Organic matter, apparent	46.7	47.5	0.69	0.56
Organic matter, true[b]	62.7	63.8	3.57	0.89
Neutral detergent fiber	60.4	60.4	0.54	0.71
Acid detergent fiber	65.3	63.3	0.65	0.12

[a]Standard error of the mean.

[b]Corrected for bacterial contribution to OM.

Table 5. Effect of dietary N source on pH in continuous culture fermenters.

	Nitrogen Source			*P*-value		
pH	SBM	FB	SEM[a]	Treatment	Hour	Treatment*Hour
Average	5.89	5.91	0.041	0.54	< 0.01	0.03
Maximum[b]	5.96	5.99	0.050	0.53	< 0.01	0.08
Minimum[c]	5.84	5.85	0.037	0.60	< 0.01	0.04

[a]Standard error of the mean.

[b]Mean of hourly maximum pH over 3-d sampling period.

[c]Mean of hourly minimum pH over 3-d sampling period.

Table 6. Effect of dietary N source on total VFA concentration and VFA molar proportions.

Item	Nitrogen source		SEM[a]	*P*-value
	SBM	FB		
Total VFA (m*M*)	227.7	201.3	8.05	0.10
Individual VFA (mol/100 mol)				
Acetate	39.0	37.9	1.33	0.16
Propionate	35.7	38.5	1.86	0.48
Butyrate	1.0	0.8	0.06	0.14
Isobutyrate	15.1	13.3	0.51	0.06
Isovalerate + 2-methylbutyrate	3.4	3.9	0.37	0.75
Valerate	5.1	4.8	0.17	0.44
Caproate	0.7	0.7	0.09	0.89
Branched-chain VFA (m*M*)	42.0	34.5	0.65	0.01
A:P Ratio	1.1	1.0	0.09	0.63

[a]Standard error of the mean.

Table 7. Effect of dietary N source on N metabolism in continuous culture fermenters.

Item	Nitrogen Source		SEM[a]	*P*-value
	SBM	FB		
Ammonia, mg/100mL	17.1	9.7	2.01	0.06
N flow (g/d)				
Total	2.2	2.1	0.07	0.29
Ammonia	0.4	0.2	0.05	0.05
Dietary	0.6	0.6	0.25	0.99
Bacterial	1.2	1.2	0.22	0.18
CP degradation, %	73.6	73.8	10.28	0.99
EBPS (OM)[b]	33.8	33.9	5.71	0.99

[a]Standard error of the mean.

[b]Efficiency of bacterial protein synthesis (g of bacterial N/kg of OM truly digested).

Table 8. Effect of dietary N source on amino acid flow.

	Nitrogen source			
Amino acid flow (g/d)	SBM	FB	SEM[a]	*P*-value
Total flow	7.87	8.28	0.200	0.35
Essential	4.44	4.74	0.115	0.23
Nonessential	3.42	3.54	0.088	0.55
Individual flow				
Alanine	0.66	0.71	0.015	0.11
Arginine	0.46	0.50	0.017	0.29
Asparagine	0.97	1.02	0.026	0.43
Cysteine	0.13	0.12	0.004	0.31
Glutamine	1.26	1.25	0.032	0.85
Glycine	0.49	0.51	0.011	0.31
Histidine	0.48	0.53	0.013	0.02
Isoleucine	0.76	0.83	0.023	0.14
Leucine	0.54	0.56	0.013	0.32
Lysine	0.31	0.32	0.008	0.48
Methionine	0.18	0.20	0.005	0.05
Phenylalanine	0.51	0.51	0.011	0.87
Proline	0.54	0.54	0.014	0.96
Serine	0.45	0.45	0.012	0.93
Threonine	0.50	0.52	0.011	0.30
Tryptophan	0.17	0.18	0.008	0.83
Tyrosine	0.18	0.20	0.013	0.68
Valine	0.54	0.58	0.015	0.15

[a]Standard error of the mean.

Table 9. Effect of dietary N source on percent change of input of AA.

	Nitrogen Source			
Amino Acid % change of input[a]	SBM	DBC	SEM[b]	*P*-value
Total AA	71.3	72.0	1.69	0.85
Essential	68.9	63.7	1.82	0.17
Nonessential	73.6	82.2	2.47	0.08

[a]100 x (g of AA in effluent/g of AA in diet DM).

[b]Standard error of the mean.

Table 10. Effect of dietary N source on in vitro intestinal protein digestion.

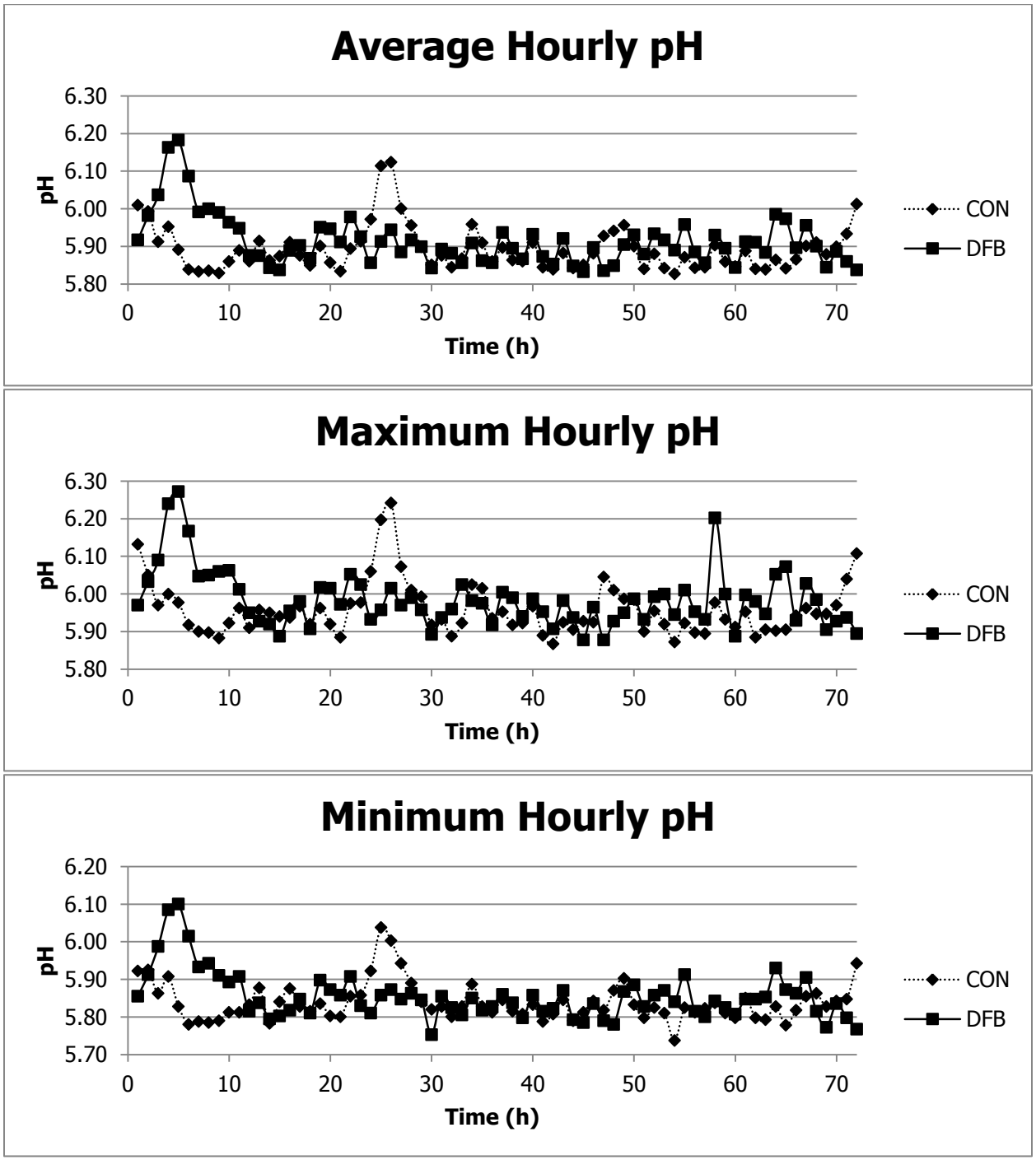
	Nitrogen source			
Item	SBM	FB	SEM[a]	*P*-value
Intestinal digestibility (%), diet[b]	61.5	65.1	NA	NA
IADP[c] (%), diet[b]	9.8	10.6	NA	NA
Intestinal digestibility (%), effluent	70.4	79.6	2.05	0.01

[a]Standard error of the mean.

[b]Statistical analysis was not performed on diets.

[c]Intestinally absorbable dietary protein (RUP x ID).

Figure 1. Effect of dietary treatment on average, maximum, and minimum hourly pH in continuous culture fermenters over time.



Experiment 2: CHEMICAL COMPOSITION AND IN VITRO INTESTINAL DIGESTIBILITY OF ISOLATED RUMEN MICROBIAL FRACTIONS

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Overview. Rumen fluid was collected from 4 steers fed the same diet, and 3 populations of rumen microorganisms (liquid-associated bacteria (LAB), solid-associated bacteria (SAB), and liquid-associated protozoa (LAP)) were isolated by differential centrifugation. Intestinal digestibility of N (ID) was determined using the in vitro (pepsin and pancreatin) steps of the 3-step procedure of Calsamiglia and Stern (1995). Total-N content of each fraction was different ($P < 0.05$) with LAB having the highest N content (8.1 *vs.* 6.1 *vs.* 5.6% of DM for LAB, SAB, and LAP, respectively). Purine concentration was greatest ($P < 0.05$) in LAB compared with SAB and LAP (3.1 *vs.* 1.0 *vs.* 0.9 mg/g bacterial DM, respectively). Similarly, LAB had a higher ($P < 0.05$) purine:N ratio compared with SAB and LAP (37.4 *vs.* 12.3 *vs.* 11.1 mg/g of bacterial N, respectively). Total amino acids (AA) (wt/wt) varied ($P < 0.05$) with each treatment (35.9 *vs.* 29.8 *vs.* 27.72% for LAB, SAB, and LAP, respectively). Most individual AA (g/100 g of total AA and wt/wt) exhibited differences ($P < 0.05$) between the 3 populations. Essential AA content (g/100 g of total AA) was highest ($P < 0.05$) in LAB and lowest in LAP (47.15 *vs.* 46.02, respectively). Non-essential AA content was greatest ($P < 0.05$) in LAP followed by SAB and LAB (53.3 *vs.* 52.3 *vs.* 51.8 g/100 g total AA, respectively). LAB was highest ($P < 0.05$) in ID followed by LAP and SAB (71.2% *vs.* 68.2% *vs.* 57.5% for LAB, LAP, and SAB, respectively). These results demonstrate that microbial fractions have differing digestibilities in the small intestine and suggest that ID of mixed rumen microbes can be influenced by the relative amount of each microbial population.

Keywords: amino acids, intestinal protein digestibility, liquid-associated bacteria, protozoa, solid-associated bacteria, rumen

INTRODUCTION

In addition to the functional role of rumen microorganisms in fermentation and nutrient utilization by ruminant animals, microbial biomass is the greatest source of protein to the small intestine of the ruminant. Clark et al. (1992) estimated that on average, 59% of non-ammonia N flowing from the rumen is microbial N, with values ranging from 35 to 66% in cows producing at least 30 kg of milk/d. Populations of rumen microorganisms can be broadly classified as liquid-associated bacteria (LAB), solid-associated bacteria (SAB), and liquid-associated protozoa (LAP). Each of these fractions has unique characteristics and chemical composition.

The quality and quantity of protein flowing from the rumen that is digested in the intestine provides amino acids (AA) for maintenance, growth, and production. Therefore, it is essential when analyzing the behavior of a protein source in vivo to determine its availability in the rumen as well as the small intestine. The 3-step in vitro enzymatic procedure (Calsamiglia and Stern, 1995) provides a rapid and reliable way to determine intestinal digestibility of N (ID) in a feedstuff.

Few studies have investigated the availability of microbial protein to the animal by digestion and absorption in the intestine. The NRC (2001) assumes that 80% of microbial CP is digested in the small intestine regardless of fraction. Studies have been performed to investigate the ID of N and AA in mixed rumen bacteria (Tas et al., 1981; Storm et al., 1983; Wallace, 1983; Siddons et al., 1985; Hvelplund and Hesselholt, 1987; Larsen et al., 2000), but information regarding differences in ID between the different isolations of microbes within the rumen is lacking.

Because there is a lack of research comparing differences in ID between LAB, SAB, and LAP, the objective of this study was to analyze the chemical composition of each fraction and estimate the ID of each fraction using a modification of the 3-step procedure of Calsamiglia and Stern (1995).

MATERIALS AND METHODS

Animals and Diets

Rumen fluid was collected approximately 2.5 h post-feeding from 4 ruminally-cannulated Holstein steers housed at the University of Minnesota Rosemount Research and Outreach Center (Rosemount, MN). Steers were all fed the same TMR balanced for 8.4% degradable intake protein (DIP). Diets are presented in Table 1.

Sample Collection and Isolation of Microbial Fractions

Microbial fractions were isolated according to the procedure of Martin et al. (1994) with modifications (Figure 1). Approximately 2 L of whole rumen contents were collected from each steer, strained through 4 layers of cheesecloth, mixed with pre-warmed (41°C) Coleman buffer (Coleman, 1978) at the collection site, and transported back to the laboratory in pre-warmed thermoses. All solids remaining after straining were cooled on ice until frozen at -20 °C prior to isolation of SAB. Upon arrival at the laboratory, liquid samples were allowed to flocculate in the thermoses for approximately 30 min, and the top layer of debris containing feed particles was removed by aspiration. The particle-free rumen fluid was then centrifuged at 800 x *g* for 5 min at 20°C to isolate protozoa in the pellet. Supernatant was centrifuged at 15,000 x *g* for 20 min at 4°C to isolate LAB in the pellet.

Rumen solids (450 to 500 g) were warmed to room temperature and mixed with pre-warmed (39°C) Coleman buffer (approximately 4 mL/g of solids) for isolation of solid-associated bacteria. The mixture of rumen solids and buffer was gently agitated for 5 min in a shaking water bath and strained through a 100 µm filter. Filtrate was centrifuged at 1,000 x *g* for 10 minutes at 20°C. Particles in the filter and the pellet from centrifugation were combined within steer, mixed with cooled (4°C) buffer (4 mL/g), and homogenized in a Waring blender (Waring, New Hartford, CT) for 3 min. Homogenate was filtered through a 100 µm filter and rinsed with cooled buffer (4 mL/g). Filtrates were pooled within steer and centrifuged at 1,000 x *g* for 10 min at 4 °C. The supernatant was centrifuged at 18,600 x *g* for 30 min at 4 °C to isolate SAB in the pellet. Use of chemical treatments was avoided to prevent modification of the chemical composition of microbial cells.

Sample analysis

After isolation, fractions were lyophilized (25 SL freeze drier, Virtis, Gardiner, NY) for N, AA, and purine analysis and in vitro ID determination. Total-N of each microbial fraction was determined by the Kjeldahl method (AOAC, 1984). Amino acids were determined at the University of Missouri Experiment Station Chemical Laboratories (Columbia, MO) by hydrolysis and performic acid oxidation analysis according to the method of AOAC (1984) with modifications. Only 2 mg of protein were used due to limited sample amounts for both hydrolysis and oxidized methods. The protein was hydrolyzed using 2 mL of 6N HCl. Purines were measured by the procedure of Zinn and Owens (1986) with modifications by Obsipo and Dehority (1999) for small sample amounts. Adenine and guanine were used in a 1:1 ratio to create the standard curve, and centrifugation at 25,000 x *g* for 25 min was replaced with centrifugation at 18,600 x *g* for 25 min. Purines were considered to comprise 22% of RNA (Obispo and Dehority, 1999). Samples of microbial fractions were subjected to pepsin and pancreatin enzymatic degradation by the procedure of Calsamiglia and Stern (1995) to estimate ID.

Statistical analysis

Data were analyzed using the GLM procedure of SAS (version 9.3) with means separated by LSD. Significance was declared at $P \leq 0.05$, and tendencies were noted at $0.1 < P < 0.05$.

RESULTS AND DISCUSSION

N content of microbial isolates

Composition of each microbial fraction is presented in Table 2. Total-N content of each fraction was different ($P < 0.01$), with LAB having the highest N concentration (8.1% of DM), followed by SAB (6.1% of DM), and LAP having the lowest (5.6% of DM).

Clark et al. (1992) compiled data on the composition of mixed rumen bacteria from 22 studies and found a wide range in results. The mean N content was 7.7% of DM and ranged from 4.8% to 10.6%, consistent with the values observed in the present study. Variation in previous research can be accounted for by a number of factors such as feed intake, diet composition, and time of sampling. The amount of N in both LAB and SAB was shown to increase in wethers fed an experimental diet at 80 g of DM/kg

of body weight compared with 40 g of DM/kg of body weight (Rodriguez et al., 2000). Additionally, SAB are more likely to be contaminated by feed than LAB or protozoa because of their strong adhesion to particles.

Using similar protocol to the current study, Vincente et al. (2004) also found significant differences in N content between LAP, LAB, and SAB (7.91, 8.69, and 9.58% of OM, respectively). Martin et al. (1994) observed a similar pattern. The authors noted an effect of diet, with a higher percent of N in microorganisms isolated from cows fed hay and barley compared with cows fed hay alone. They also showed an effect of time after feeding on N% of each population as well as an interaction between time and population. In general, Martin et al. (1994) found that N content of isolates increased with greater time post-feeding.

Previous research has shown that usually, LAB will have a higher amount of N than SAB, as observed in the present study. The observed variation between studies is probably due to inconsistencies in diet and sampling time between studies, both of which have been shown to affect the amount of N in rumen bacteria. Rodríguez et al. (2000) detected no difference in N content between LAB and SAB (70 and 72% of DM, respectively). Merry and McAllan (1983) detected a lower amount of N in SAB (7.01% of DM) compared with LAB (8.03% of DM). Craig et al. (1987) only observed a significant difference in N content between LAB and SAB at 1 h post-feeding, at which time SAB had lower N content than LAB (6.4 *vs.* 7.5% of OM, respectively). A similar relationship with time after feeding was observed by Cecava et al. (1990). They observed a quadratic effect of time after feeding with LAB and a linear relationship with time in SAB. In this experiment, SAB (9.35% of OM) were lower in N than LAB (10.13% of OM) when a high forage diet was fed, but no difference was detected between fractions when steers were fed a low forage diet (97.8 and 93.6% of OM for LAB and SAB, respectively). Alternatively, González et al. (2011) found LAB (7.44% of DM) to be lower in N content than SAB (7.94% of DM), but only when wethers were fed sunflower meal as concentrate. In a different experiment reported in the same paper, LAB and SAB were similar when concentrate was provided by soybean meal (8.30 and 8.69% of DM, respectively).

Purine-N content of microbial isolates

Purine concentration and purine:N ratio for each fraction are presented in Table 2. Although N content of the microbial isolates was consistent with previous literature, purine content was noticeably lower in the present study than in previous experiments (Clark et al., 1992; Illg and Stern, 1994), resulting in a depressed purine:N ratio (mg of purine/g of microbial N). Purine concentration as well as purine:N ratio was higher ($P < 0.05$) in LAB than in both SAB and LAP (Table 2). This is consistent with the findings of a review by Clark et al. (1992), who observed a higher purine:N ratio in LAB than SAB across experiments. However, Craig et al. (1987) and Firkins et al. (1987) found no difference between LAB and SAB in purine:N ratio. Rodríguez-Prado et al. (2004) detected higher concentrations of both N and purines in LAB compared with SAB but observed no difference in their ratio. Purine concentration and purine:N ratio of LAP was not different ($P > 0.1$) from SAB in the current experiment. This is in disagreement with the findings of Firkins et al. (1987), who found that the purine:N ratio was similar ($P < 0.05$) between LAB and SAB, but was lower in protozoa (0.75 to 0.80, 0.75 to 0.84, and 0.42 to 0.45 g of purine/g of microbial N, respectively).

Taking into consideration the already low purine concentration across all fractions in the present study, the values for purine:N in SAB and LAP were exceptionally low. This could possibly be due to contamination by feed particles. Obispo and Dehority (1999) reported that the purine:protein ratio was approximately 3 times greater in pure strains of rumen bacteria compared with mixed cultures isolated from the rumen. They attributed this increase to contamination by other sources of N such as feed particles. Sylvester et al. (2005) attributed the higher variation between studies in protozoal N:RNA ratios compared with bacterial N:RNA ratios to differences in separation techniques and resulting contamination by feed particles.

Mixed rumen bacteria collected in previous experiments often include predominantly LAB because of difficulties extracting SAB from particulate material (Dewhurst et al., 2000) and the lack of a standardized procedure for isolation of SAB from other rumen microorganisms. Thus, estimates of purine:N ratios of mixed bacteria may be biased by an increase in the proportion of LAB. Even at low purine concentrations, the purine:N ratio in LAB was elevated compared with SAB and LAP in this experiment, which is consistent with previous studies. This higher purine:N ratio

may result in overestimation of bacterial-N flow when LAB are overrepresented in samples of rumen microorganisms.

AA profile of microbial isolates

Total AA (wt/wt) was highest ($P < 0.05$) in LAB compared with both SAB and LAP (Table 3). As a result, LAB were highest ($P < 0.05$) of the three fractions in most of the individual AA on a weight basis. There was no difference ($P > 0.1$) between fractions in Ser, Pro, Leu, and His. However, LAB were lower ($P < 0.05$) than LAP but not SAB in Cys, and higher ($P < 0.05$) than SAB but not LAP in Glu. Solid-associated bacteria were higher ($P < 0.05$) on a weight basis than LAP in Asp, Thr, Gly, Val, Met, and Arg. There was no difference ($P > 0.1$) on a weight basis between SAB and LAP in Glu, Ala, Cys, Ile, Tyr, Phe, or Lys.

When expressed as g of individual AA/g of total AA (Table 4), there was no difference ($P > 0.1$) between fractions in Ile, but all other AA analyzed differed ($P < 0.05$) between fractions. Liquid-associated bacteria were highest ($P < 0.05$) of the three fractions in Asp, Thr, Tyr, and Lys when measured as a percentage of total-AA; SAB were highest ($P < 0.05$) of the three fractions in Ser, Met, and Arg; and LAP were highest ($P < 0.05$) in Glu, Pro, Cys, Leu, and His. Essential AA were highest ($P < 0.05$) in LAB and lowest in LAP; conversely, LAP was higher ($P < 0.05$) than both LAB and SAB in non-essential AA. Values for all individual AA in LAB and SAB were within the range for rumen bacteria reported by Clark et al. (1992). Martin et al. (1996) found differences between LAB and SAB in means (g/100 g of total AA) of Thr, Leu, Phe, His, Arg, Cys, Met, Asp, Gly, and Ala from rumen fluid harvested from dry cows at various times. González et al. (2011) summarized the composition of LAB and SAB collected from 3 wethers over the course of 4 independent experiments for *in situ* feed evaluation. Only Ala was different ($P < 0.05$) between LAB and SAB across all 4 experiments. In continuous culture, Rodríguez-Prado et al. (2004) found less variation between bacterial fractions ($P < 0.05$) in g of individual AA/100 g of total AA. In this study, differences ($P < 0.05$) were only observed in Lys, Arg, Asp, and Glu. When comparing protozoa to bacterial isolates collected from cows, Martin et al. (1996) found differences between Thr, Val, Ile, Phe, Lys, His, Arg, Cys, Met, Asp, Ser, Gln, Gly, and Ala.

Clark et al. (1992) compared the results of AA composition of rumen bacteria across 35 experiments and 61 dietary treatments and found a wide variation. Much of this variation can be accounted for by differences in separation and analytical techniques, but within-study variation of AA composition was limited. In addition, Martin et al. (1996) investigated the effect of diet and time of sampling on the AA composition of LAP, LAB, and SAB. They discovered a time effect on the amount of Arg, Glu, and Ala in total AA and an effect of diet on Leu and Glu, although as a whole, AA composition was not greatly affected by diet and time of sampling.

Although protozoa are typically higher in Lys than bacteria (Storm and Ørskov, 1983; Martin et al., 1996), in this study, LAP were lower in Lys than LAB and SAB. This result may be indicative of contamination of LAP samples by feed particles due to incomplete removal of feed particles from rumen fluid by suction before centrifugation for LAP removal. It is possible that the process of transporting rumen fluid from the farm to the laboratory (approximately 45 km) resulted in insufficient flocculation before suction of the top layer of particles. In previous unpublished research performed in our lab (Nelsen, 2002), the Lys content of protozoa was determined to be 11.0 g/100 g of total AA compared with 8.3 and 7.7 g/100 g of total AA in LAB and SAB, respectively. This is likely due to differences in separation techniques utilized. Nelsen separated protozoa by gravimetric sedimentation in separatory funnels. We chose to separate LAP by centrifugation at slow speed in the current study in an attempt to decrease contamination by plant material (Sylvester et al., 2005).

Intestinal N digestibility of microbial isolates

Results for ID are presented in Table 2. There was no difference ($P > 0.1$) between LAB and LAP in ID, although the protozoal fraction was numerically lower. Solid-associated bacteria were lower ($P < 0.05$) in ID than both other fractions. Average ID across populations was 65.6%, although this figure ignores the relative proportions of each fraction flowing to the small intestine. This estimation of mean ID is lower than that found in previous studies. Unpublished research from our lab (Nelsen, 2002) determined an ID of 96.6, 92.2, and 91.6% for protozoa, SAB, and LAB, respectively using the 3-step procedure. Siddons et al. (1985) estimated digestibility of ^{15}N-labelled microbial NAN at 72%, although this value may have been slightly

overestimated due to potential contamination by labeled endogenous N. Storm et al. (1983) determined the ID of individual AA of microbial origin by infusing mixed rumen microbes into the abomasum of sheep. They found an average ID of 84.7%, and most AA were in the range of 80 to 88% digestible in the small intestine with the exceptions of His (68%), Cys (73%), and Pro (76%). Tas et al. (1981) assessed the ID of rumen microbial AA by infusing mixed rumen microbes into the duodenum of sheep. Apparent ID was 69% and true ID was 86%, similar to the finding of Hvelplund and Hesselholt (1987), who found an average ID of rumen microbial AA to be approximately 85% with the exception of Cys that had a mean digestibility of 75%. Similarly, Larsen et al. (2000) found that Cys had the lowest ID of the microbial AA (65.5%) in cattle while Lys had the highest ID (79.9%). Mean ID for all microbial AA-N was 75.1% in this study. Using an enzymatic pepsin-pancreatin procedure similar to the 3-step procedure, Wallace (1983) estimated the ID of isolated strains of rumen bacteria and found that Gram-negative bacteria had a higher ID than Gram-positive bacteria. All Gram-negative bacteria had estimated apparent ID of greater than 80%, while Gram-positive bacteria only averaged 39% ID.

The lower mean value for ID in the present study compared with results from previous research could be due to differences in methodology or contamination of SAB and LAP by feed particles. Solid-associated bacteria may not have completely detached from feed particles. Except for Wallace (1983) and Nelsen (2002), all cited digestibility studies were performed in vivo. Although the 3-step technique was utilized to determine ID in both the present study and by Nelsen (2002), differences in separation methods and sample storage may have affected results. Furthermore, SAB was both higher in Cys than LAB, which may have contributed to a decrease in ID. However, it is unlikely that this small amount of Cys (1.03, 1.37, and 1.50 g/100 g of total AA in LAB, SAB, and LAP, respectively) would have a significant influence on ID, especially considering that LAP was higher in Cys than either other fraction but was similar to LAB in ID.

Alternatively, the lower ID of SAB is consistent with findings that an increase in proportion of Gram-positive bacteria resulted in a decrease in ID (Wallace, 1983) because SAB are higher than LAB in Gram-positive bacteria (Lantham, 1980). Wallace found that Gram-negative bacteria had estimated apparent ID greater than 80% and

usually more than 90%, while Gram-positive bacteria averaged 39% digestibility in the species tested. In the same study, a trend was observed between increased concentration of Gram-positive bacteria isolated from rumen fluid and decreased ID. The relative amounts of Gram-positive and Gram-negative bacteria in the rumen are influenced by the diet. High concentrate diets have been shown to result in a higher proportion of Gram-positive bacteria (Hungate, 1966; Nagaraja et al., 1978). It is possible that the concentrate level in the current study (Table 1) was sufficient to promote greater Gram-positive populations in the rumen.

IMPLICATIONS

All microbial fractions collected from the rumen had greater than 50% ID. These results are lower than estimations in previous research, and may reflect differences in sampling and sample handling methodology. Further research is required to define the ID of microbial fractions in vivo and to determine if any factors may influence the digestibility of these fractions in the small intestine.

Table 1. Ingredient and chemical composition of diets fed to ruminally-cannulated steers.

Diet Composition	
Ingredient	*% of DM*
Corn silage	43.0
Corn grain earlage	20.0
Distiller's grains	19.5
Bromegrass hay	6.0
Rumensin	4.5
Soybean straw	2.0
Item	
Dry matter	38.5
Crude protein	16.1
Neutral detergent fiber	35.5
Acid detergent fiber	21.5
Non-forage carbohydrates	33.8
Crude fat	4.6
Ash	10.2
Total digestible nutrients	66
NEg (Mcal/lb)	0.4

Table 2. Characteristics of isolated rumen microbial fractions.

	Microbial Fraction			
Item	LAB	SAB	LAP	SEM
Nitrogen, % of DM	8.1[a]	6.1[b]	5.6[c]	0.33
Purine bases, mg/g of bacterial DM	3.1[a]	1.0[b]	0.9[b]	0.31
Purine bases, mg/g of bacterial N	37.4[a]	12.3[b]	11.1[b]	3.77
Intestinal digestibility, % of CP	71.2[a]	57.5[b]	68.2[a]	1.88

[a,b,c]Means with uncommon superscripts within row differ ($P < 0.05$).

[d]LAB = liquid-associated bacteria, SAB = solid-associated bacteria, LAP = liquid-associated protozoa.

[e]Standard error of the mean.

Table 3. Amino acid composition of isolated rumen microbial fractions (wt/wt).

	Microbial Fraction[d]			
Amino acid (g/100 g of bacterial DM)	LAB	SAB	LAP	SEM[e]
Total AA	35.91[a]	29.83[b]	27.72[b]	1.139
Alanine	2.90[a]	2.30[b]	1.98[b]	0.122
Arginine	1.69[a]	1.45[b]	1.27[c]	0.057
Aspartic acid	4.45[a]	3.39[b]	2.96[c]	0.194
Cysteine	0.37[b]	0.41[ab]	0.42[a]	0.009
Glutamic acid	4.38[a]	3.79[b]	4.15[ab]	0.096
Glycine	1.99[a]	1.68[b]	1.38[c]	0.078
Histidine	0.59[a]	0.55[a]	0.55[a]	0.012
Isoleucine	1.96[a]	1.58[b]	1.43[b]	0.074
Leucine	2.83[a]	2.63[a]	2.72[a]	0.062
Lysine	2.86[a]	1.98[b]	1.78[b]	0.147
Methionine	0.94[a]	0.81[b]	0.69[c]	0.033
Phenylalanine	1.89[a]	1.64[b]	1.50[b]	0.056
Proline	1.34[a]	1.38[a]	1.48[a]	0.036
Serine	1.47[a]	1.29[a]	1.39[a]	0.041
Threonine	2.12[a]	1.68[b]	1.42[c]	0.092
Tyrosine	1.71[a]	1.26[b]	1.12[b]	0.081
Valine	2.07[a]	1.68[b]	1.42[c]	0.090

[a,b,c]Means with uncommon superscripts within row differ ($P < 0.05$).

[d]LAB = liquid-associated bacteria, SAB = solid-associated bacteria, LAP = liquid-associated protozoa.

[e]Standard error of the mean.

Table 4. Amino acid composition of isolated rumen microbial fractions (g/100 g of total AA).

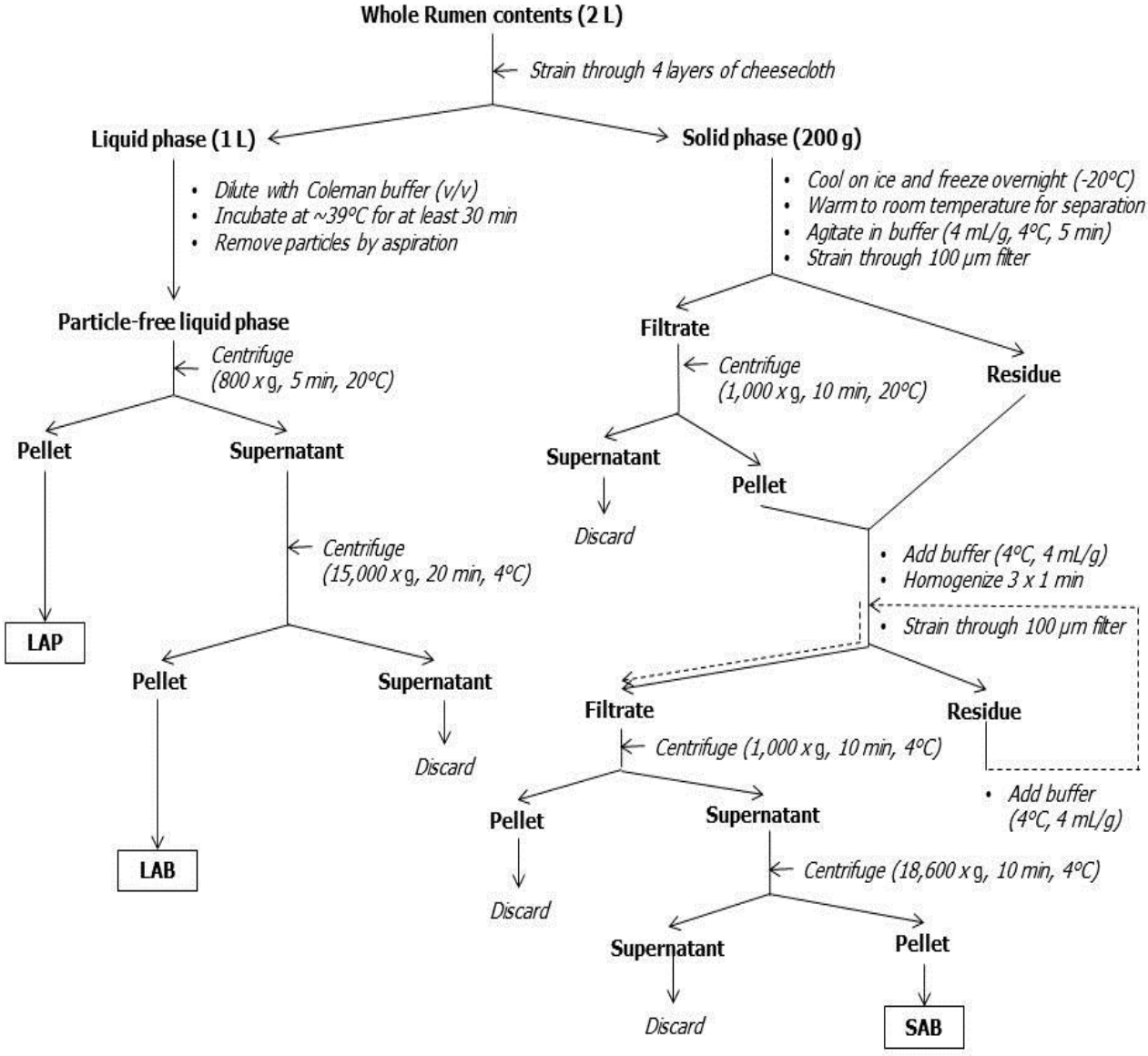
	Microbial Fraction[d]			
Amino acid (g/100 g of total AA)	LAB	SAB	LAP	SEM[e]
Alanine	8.09[a]	7.71[a]	7.13[b]	0.136
Arginine	4.70[b]	4.86[a]	4.56[c]	0.042
Aspartic acid	12.39[a]	11.38[b]	10.70[b]	0.236
Cysteine	1.03[c]	1.37[b]	1.50[a]	0.061
Glutamic acid	12.21[b]	12.69[b]	14.96[a]	0.371
Glycine	5.55[a]	5.64[a]	4.98[b]	0.089
Histidine	1.64[c]	1.84[b]	1.97[a]	0.043
Isoleucine	5.46[a]	5.29[a]	5.15[a]	0.069
Leucine	7.88[c]	8.80[b]	9.80[a]	0.257
Lysine	7.96[a]	6.63[b]	6.44[b]	0.238
Methionine	2.61[b]	2.71[a]	2.48[c]	0.029
Phenylalanine	5.25[b]	5.48[a]	5.40[ab]	0.042
Proline	3.74[c]	4.64[b]	5.32[a]	0.205
Serine	4.09[b]	4.64[a]	4.67[a]	0.109
Threonine	5.91[a]	5.12[b]	5.63[c]	0.104
Tyrosine	4.75[a]	4.26[b]	4.03[b]	0.101
Valine	5.75[a]	5.64[a]	5.10[b]	0.108
Essential AA	47.15[a]	46.88[ab]	46.02[b]	0.215
Non-essential AA	51.84[b]	52.31[b]	53.27[a]	0.238

[a,b,c]Means with uncommon superscripts within row differ ($P < 0.05$).

[d]LAB = liquid-associated bacteria, SAB = solid-associated bacteria, LAP = liquid-associated protozoa.

[e]Standard error of the mean.

Figure 1. Procedure used to isolate LAB, SAB, and LAP from whole rumen contents.



LITERATURE CITED

Allison, M.J., Bryant, M.P., Doetsch, R.N., 1962. Studies on the metabolic function of branched-chain volatile fatty acids, growth factors for ruminococci. I. Incorporation of isovalerate into leucine. J. Bacteriol. 83, 523-532.

Anastassiadas, S., 2007. L-lysine fermentation. Recent Patents on Biotechnology 1, 11-24.

AOAC, 1984. Official Methods of Analysis (14th ed.). Assoc. Offic. Anal. Chem. Washington, D.C.

AOAC, 1995. Official Methods of Analysis (16th ed.). Assoc. Offic. Anal. Chem. Washington, D.C.

Arambel, M.J., Bartley, E.E., Dufva, G.S., Nagaraja, T.G., Dayton, A.D., 1982. Effect of diet on amino and nucleic acids of rumen bacteria and protozoa. J. Dairy Sci. 65, 2095-2101.

Asplund, J.M.e., 1994. Principles of Protein Nutrition of Ruminants. CRC Press, Inc., Boca Raton, FL.

Atasoglu, C., Newbold, C.J., Wallace, R.J., 2001. Incorporation of [15N] ammonia by the cellulolytic ruminal bacteria Fibrobacter succinogenes BL2, Ruminococcus albus SY3, and Ruminococcus flavefaciens 17. Appl. Environ. Microbiol. 67, 2819-2822.

Bach, A., Calsamiglia, S., Stern, M.D., 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88: (E. Suppl.), E9-E21.

Bach, A., Ruiz Moreno, M., Thrune, M., Stern, M.D., 2008. Evaluation of the fermentation dynamics of soluble crude protein from three protein sources in continuous culture fermenters. J. Anim. Sci. 86, 1364-1371.

Bach, A., Stern, M.D., Merchen, N.R., Drackley, J.K., 1998. Evaluation of selected mathematical approaches to the kinetics of protein degradation in situ. J. Anim. Sci. 76, 2885-2893.

Bateman, H.G., Clark, J.H., Murphy, M.R., 2005. Development of a system to predict feed protein flow to the small intestine of cattle. J. Dairy Sci. 88, 282-295.

Belanche, A., Abecia, L., Holtrop, G., Guada, J.A., Castrillo, C., de la Fuente, G., Balcells, J., 2011. Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chyme. J. Anim. Sci. 89, 4163-4174.

Berthiaume, R., Benchaar, C., Chaves, A.V., Tremblay, G.F., Castonguay, Y., Bertrand, A., Bélanger, G., Michaud, R., Lafrenière, C., McAllister, T.A., Brito, A.F., 2010. Effects of nonstructural carbohydrate concentration in alfalfa on fermentation and microbial protein synthesis in continuous culture. J. Dairy Sci. 93, 693-700.

Borucki Castro, S.I., Phillip, L.E., Lapierre, H., Jardon, P.W., Berthiaume, R., 2007. Ruminal degradability and intestinal digestibility of protein and amino acids in treated soybean meal products. J. Dairy Sci. 90, 810-822.

Bremner, J.M., Keeney, D.R., 1965. Steam distillation methods for determination of ammonium, nitrate and nitrite. Analytica Chimica Acta 32, 485-495.

Brooks, M.A., Harvey, R.M., Johnson, N.F., Kerley, M.S., 2012. Rumen degradable protein supply affects microbial efficiency in continuous culture and growth in steers. J. Anim. Sci.

Calsamiglia, S., Stern, M.D., 1995. A three-step in vitro procedure for estimating intestinal digestion of protein in ruminants. J. Anim. Sci. 73, 1459-1465.

Calsamiglia, S., Stern, M.D., Firkins, J.L., 1996. Comparison of nitrogen-15 and purines as microbial markers in continuous culture. J. Anim. Sci. 74, 1375-1381.

Cameron, M.R., Klusmeyer, T.H., Lynch, G.L., Clark, J.H., Nelson, D.R., 1991. Effects of urea and starch on rumen fermentation, nutrient passage to the duodenum, and performance of cows. J. Dairy Sci. 74, 1321-1336.

Cecava, M.J., Merchen, N.R., Gay, L.C., Berger, L.L., 1990. Composition of ruminal bacteria harvested from steers as influenced by dietary energy level, feeding frequency, and isolation techniques. J. Dairy Sci. 73, 2480-2488.

Cerrato-Sánchez, M., Calsamiglia, S., Ferret, A., 2008. Effect of the magnitude of the decrease of rumen pH on rumen fermentation in a dual-flow continuous culture system. J. Anim. Sci. 86, 378-383.

Chen, G., Strobel, H.J., Russell, J.B., Sniffen, C.J., 1987. Effect of hydrophobicity of utilization of peptides by ruminal bacteria in vitro. Appl. Environ. Microbiol. 53, 2021-2025.

Chen, X.B., Chen, Y.K., Franklin, M.F., Ørskov, E.R., Shand, W.J., 1992. The effect of feed intake and body weight on purine derivative excretion and microbial protein supply in sheep. J. Anim. Sci. 70, 1534-1542.

Chester-Jones, H., Stern, M.D., Metwally, H.M., Linn, J.G., Ziegler, D.M., 1991. Effects of dietary protein-energy interrelationships on Holstein steer performance and ruminal bacterial fermentation in continuous culture. J. Anim. Sci. 69, 4956-4966.

Chester-Jones, H., Stern, M.D., Su, A., Donker, J.D., Ziegler, D.M., Miller, K.P., 1990. Evaluation of various nitrogen supplements in starter diets for growing Holstein steers and their effects on ruminal bacterial fermentation in continuous culture. J. Anim. Sci. 68, 2954-2964.

Church, D.C.e., 1988. The Ruminant Animal: Digestive Physiology and Nutrition. Prentice-Hall, Inc., Englewood Cliffs, NJ.

Clark, J.H., Klusmeyer, T.H., Cameron, M.R., 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy Sci. 75, 2304-2323.

Coleman, G.S., 1978. Rumen entodiniomorphid protozoa, In: Baker, J.R., Taylor, A.E.R. (Eds.), Methods of Cultivating Parasites In Vitro, Academic Press, London, UK, pp. 39-54.

Colombini, S., Rapetti, L., Colombo, D., Galassi, G., Matteo Crovetto, G., 2010. Brown midrib forage sorghum silage for the dairy cow: nutritive value and comparison with corn silage in the diet. Ital. J. Anim. Sci. 9, 273-277.

Cooper, S., 1993. The origins and meaning of the Schaeter-Maaloe-Kjeldgaard experiments. J. Gen. Microbiol. 139, 1117-1124.

Cotta, M.A., Russell, J.B., 1982. Effect of peptides and amino acids on efficiency of rumen bacterial protein synthesis in continuous culture. J. Dairy Sci. 65, 226-234.

Council, N.R., 1989. Nutrient Requirements of Dairy Cattle. 6th rev. ed., Natl. Acad. Sci., Washington, D. C.

Craig, W.M., Brown, D.R., Broderick, G.A., Ricker, D.B., 1987. Post-prandial compositional changes of fluid- and particle-associated ruminal microorganisms. J. Anim. Sci. 65, 1042-1048.

Crawford, R.J., Hoover, W.H., Junkins, L.L., 1980a. Effects of solids and liquid flows on fermentation in continuous cultures. II. Nitrogen partition and efficiency of microbial synthesis. J. Anim. Sci. 51, 986-995.

Crawford, R.J., Hoover, W.H., Knowlton, P.H., 1980b. Effects of solids and liquid flows on fermentation in continuous cultures. I. Dry matter and fiber digestion, VFA production and protozoa numbers. J. Anim. Sci. 51, 975-985.

Dahlberg, E.M., Stern, M.D., Ehle, F.R., 1988. Effects of forage source on ruminal microbial nitrogen metabolism and carbohydrate digestion in continuous culture. J. Anim. Sci. 66, 2071-2083.

Dewhurst, R.J., Davies, D.R., Merry, R.J., 2000. Microbial protein supply from the rumen. Anim. Feed Sci. Technol. 85, 1-21.

Dijkstra, J., France, J., Davies, D.R., 1998. Different mathematical approaches to estimating microbial protein supply in ruminants. J. Dairy Sci. 81, 3370-3384.

Dufva, G.S., Bartley, E.E., Arambel, M.J., Nagaraja, T.G., Dennis, S.M., Galitzer, S.J., Dayton, A.D., 1982. Diaminopimelic acid content of feeds and rumen bacteria and its usefulness as a rumen bacterial marker. J. Dairy Sci. 65, 1754-1759.

Endres, M., 1995. Evaluation of Factors Affecting Ruminal Protein Metabolism in Continuous Culture, Department of Animal Science, University of Minnesota, Minneapolis, MN.

Eugene, M., Archimede, H., Sauvant, D., 2004. Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. Livest. Prod. Sci. 85, 81-97.

Faichney, G., 1980. Measurement in sheep of the quantity and composition of rumen digesta and of the fractional outflow rates of digesta constituents. Aus. J. Agric. Res. 31, 1129-1137.

Firkins, J.L., Berger, L.L., Merchen, N.R., Fahey, G.C., Mulvaney, R.L., 1987. Ruminal nitrogen metabolism in steers as affected by feed intake and dietary urea concentration. J. Dairy Sci. 70, 2302-2311.

Firkins, J.L., Hristov, A.N., Hall, M.B., Varga, G.A., St-Pierre, N.R., 2006. Integration of ruminal metabolism in dairy cattle. J. Dairy Sci. 89, E31-E51.

Gonzalez, J., Arroyo, J.M., Ouarti, M., Guevara-Gonzalez, J., Rodriguez, C.A., Alvir, M.R., Moya, V.J., Piquer, O., 2011. Composition of free and adherent ruminal bacteria: inaccuracy of the microbial nutrient supply estimates obtained using free bacteria as reference samples and ^{15}N as the marker. Animal 6, 468-475.

Griswold, K.E., Apgar, G.A., Bouton, J., Firkins, J.L., 2003. Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. J. Anim. Sci. 81, 329-336.

Hannah, S.M., Stern, M.D., Ehle, F.R., 1986. Evaluation of a dual flow continuous culture system for estimating bacterial fermentation in vivo of mixed diets containing various soya bean products. Anim. Feed Sci. Technol. 16, 51-62.

Harmeyer, J., Martens, H., 1980. Aspects of urea metabolism in ruminants with reference to the goat. J. Dairy Sci. 63, 1707-1728.

Harrison, D.G., Beever, D.E., Thomson, D.J., Osbourn, D.F., 1976. Manipulation of fermentation in the rumen. J. Sci. Fd Agric. 27, 617-620.

Hazlewood, G.P., Orpin, C.G., Greenwood, Y., Black, M.E., 1983. Isolation of proteolytic rumen bacteria by use of selective medium containing leaf fraction 1 protein (ribulosebisphosphate carboxylase). App. Environ. Microbiol. 45, 1780-1784.

Herrera-Saldana, R., Gomez-Alarcon, R., Torabi, M., Huber, J.T., 1990. Influence of synchronizing protein and starch degradation in the rumen on nutrient utilization and microbial protein synthesis. J. Dairy Sci. 73, 142-148.

Hoffman, P.C., Brehm, N.M., Bauman, L.M., Peters, J.B., Undersander, D.J., 1999. Prediction of laboratory and in situ protein fractions in legume and grass silages using near-infrared reflectance spectroscopy. J. Dairy Sci. 82, 764-770.

Hoover, W.H., Kincaid, C.R., Varga, G.A., Thayne, W.V., Junkins, L.L., 1984. Effects of solids and liquid flows on fermentation in continuous cultures. IV. pH and dilution rate. J. Anim. Sci. 58, 692-699.

Hoover, W.H., Miller, T.K., Stokes, S.R., Thayne, W.V., 1989. Effects of fish meals on rumen bacterial fermentation in continuous culture. J. Dairy Sci. 72, 2991-2998.

Hume, I., Bird, P., 1970. Synthesis of microbial protein in the rumen. IV. The influence of the level and form of dietary sulphur. Aus. J. Agric. Res. 21, 315-322.

Hume, I., Moir, R., Somers, M., 1970. Synthesis of microbial protein in the rumen. I. Influence of the level of nitrogen intake. Aus. J. Agric. Res. 21, 283-296.

Hungate, R.E., 1966. The Rumen and Its Microbes. Academic Press, New York, NY.

Hussein, H.S., Stern, M.D., Jordan, R.M., 1991. Influence of dietary protein and carbohydrate sources on nitrogen metabolism and carbohydrate fermentation by ruminal microbes in continuous culture. J. Anim. Sci. 69, 2123-2133.

Hvelplund, T., Hesselholt, M., 1987. Digestibility of individual amino acids in rumen microbial protein and undegraded dietary protein in the small intestine of sheep. Acta Agric. Scand. 37, 469-477.

Illg, D.J., Stern, M.D., 1994. In vitro and in vivo comparisons of diaminopimelic acid and purines for estimating protein synthesis in the rumen. Anim. Feed. Sci. Technol. 48, 49-55.

Ipharraguerre, I.R., Clark, J.H., 2005. Impacts of the source and amount of crude protein on the intestinal supply of nitrogen fractions and performance of dairy cows. J. Dairy Sci. 88, E22-E37.

Isaacson, H.R., Hinds, F.C., Bryant, M.P., Owens, F.N., 1975. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. J. Dairy Sci. 58, 1645-1659.

Jones, D.F., Hoover, W.H., Miller Webster, T.K., 1998. Effects of concentrations of peptides on microbial metabolism in continuous culture. J. Anim. Sci. 76, 611-616.

Jouany, J.-P., 1996. Effect of rumen protozoa on nitrogen utilization by ruminants. J. Nutr. 126, 1335S-1346S.

Kiran, D., Mutsvangwa, T., 2010. Effects of partial ruminal defaunation on urea-nitrogen recycling, nitrogen metabolism, and microbial nitrogen supply in growing lambs fed low or high dietary crude protein concentrations. J. Anim. Sci. 88, 1034-1047.

Klusmeyer, T.H., Lynch, G.L., Clark, J.H., Nelson, D.R., 1991. Effects of calcium salts of fatty acids and proportion of forage in diet on ruminal fermentation and nutrient flow to duodenum of cows. J. Dairy Sci. 74, 2220-2232.

Kopecny, J., Wallace, R.J., 1982. Cellular location and some properties of proteolytic enzymes of rumen bacteria. Appl. Environ. Microbiol. 43, 1026-1033.

Köster, H.H., Cochran, R.C., Titgemeyer, E.C., Vanzant, E.S., Abdelgadir, I., St-Jean, G., 1996. Effect of increasing degradable intake protein on intake and digestion of low-quality, tallgrass-prairie forage by beef cows. J. Anim. Sci. 74, 2473-2481.

Lantham, M.J., 1980. Adhesion of rumen bacteria to plant cell walls, In: Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R., Vincent, B. (Eds.), Microbial Adhesion to Surfaces, Ellis Horwood, Chinchester, UK, pp. 339-350.

Lapierre, H., Lobley, G.E., 2001. Nitrogen Recycling in the Ruminant: A Review. J. Dairy Sci. 84 (E. Suppl.), E223-E236.

Lapierre, H., Lobley, G.E., Ouellet, D.R., Doepel, L., Pacheco, D., 2007. Amino acid requirements for lactating dairy cows: reconciling predictive models and biology, Cornell Nutrition Conference for Feed Manufacturers, Ithaca, NY.

Larsen, M., Madsen, T.G., Weisbjerg, M.R., Hvelplund, T., Madsen, J., 2000. Small intestinal digestibility of microbial and endogenous amino acids in dairy cows.

Leng, R.A., Nolan, J.V., 1984. Nitrogen metabolism in the rumen. J. Dairy Sci. 67, 1072-1089.

Ling, J.R., Armstead, I.P., 1995. The in vitro uptake and metabolism of amino acids by five species of rumen bacteria. J. Appl. Bacteriol. 78, 116-124.

Loerch, S.C., Berger, L.L., Gianola, D., Fahey, G.C., 1983. Effects of dietary protein source and energy level on in situ nitrogen disappearance of various protein sources. J. Anim. Sci. 56, 206-216.

Maeng, W.J., Van Nevel, C.J., Baldwin, R.L., Morris, J.G., 1976. Rumen microbial growth rates and yields: effect of amino acids and protein. J. Dairy Sci. 59, 68-79.

Maiga, H.A., Schingoethe, D.J., Henson, J.E., 1996. Ruminal degradation, amino acid composition, and intestinal digestibility of the residual components of five protein supplements. J. Dairy Sci. 79, 1647-1653.

Mansfield, H.R., Endres, M.I., Stern, M.D., 1995. Comparison of microbial fermentation in the rumen of dairy cows and dual flow continuous culture. Anim. Feed Sci. Technol. 55, 47-66.

Martin, C., Bernard, L., Michalet-Doreau, B., 1996. Influence of sampling time and diet on amino acid composition of protozoal and bacterial fractions from bovine ruminal contents. J. Anim. Sci. 74, 1157-1163.

Martin, C., Williams, A.G., Michalet-Doreau, B., 1994. Isolation and characteristics of the protozoal and bacterial fractions from bovine ruminal contents. J. Anim. Sci. 72, 2962-2968.

Martín-Orue, S.M., Balcells, J., Vicente, F., Castrillo, C., 2000. Influence of dietary rumen-degradable protein supply on rumen characteristics and carbohydrate

fermentation in beef cattle offered high-grain diets. Anim. Feed Sci. Technol. 88, 59-77.

Martín-Orúe, S.M., Balcells, J., Zakraoui, F., Castrillo, C., 1998. Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: effect of detachment procedure. Anim. Feed Sci. Technol. 71, 269-282.

McAllan, A.B., Smith, R.H., 1977. Some effects of variation in carbohydrate and nitrogen intakes on the chemical composition of mixed rumen bacteria from young steers. Br. J. Nutr. 37, 55-65.

McCarthy, R.D., Klusmeyer, T.H., Vicini, J.L., Clark, J.H., Nelson, D.R., 1989. Effects of source of protein and carbohydrate on ruminal fermentation and passage of nutrients to the small intestine of lactating cows. J. Dairy Sci. 72, 2002-2016.

McKain, N., Wallace, R.J., Watt, N.D., 1992. Selective isolation of bacteria with dipeptidyl aminopeptidase type I activity from the sheep rumen. FEMS Microbiol. Lett. 95, 169-174.

McNaught, M.L., Owen, E.C., Henry, K.M., Kon, S.K., 1954. The utilization of nonprotein nitrogen in the bovine rumen 8. The nutritive value of the proteins of preparation of dried rumen bacteria, rumen protozoa, and brewer's yeast for rats. Biochem. J. 56, 151-156.

McPherson, A.T., 1966. Production of Lysine and Methionine, World Protein Resources, American Chemical Society, Washington, DC, pp. 65-74.

Merry, R.J., Lee, M.R.F., Davies, D.R., Dewhurst, R.J., Moorby, J.M., Scollan, N.D., Theodorou, M.K., 2006. Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 1. In vitro and in vivo studies of nitrogen utilization. J. Anim. Sci. 84, 3049-3060.

Merry, R.J., McAllan, A.B., 1983. A comparison of the chemical composition of mixed bacteria harvested from the liquid and solid fractions of rumen digesta. Br. J. Nutr. 50, 701-709.

Minato, H., Endo, A., Higuchi, M., Ootomo, Y., Uemura, T., 1966. Ecological treatise on the rumen fermentation I. The fractionation of bacteria attached to the rumen digesta solids. J. Gen. Appl. Microbiol. 12, 39-52.

Nagaraja, T.G., Bartley, E.E., Fina, L.R., Anthony, H.D., 1978. Relationship of rumen Gram-negative bacteria and free endotoxin to lactic acidosis in cattle. J. Anim. Sci. 47, 1329-1337.

Neijssel, O.M., Tempest, D.W., 1976. Bioenergetic aspects of aerobic growth of *Klebsiella aerogenes* NCTC 418 in carbon-limited and carbon-sufficient chemostat culture. Arch. Microbiol. 107, 215-221.

Nelsen, K., 2002. Chemical Composition and Small Intestinal Protein Digestibility of Various Rumen Microbial Fractions, Science in Agriculture Program, College of Agricultural, Food, and Environmental Sciences, University of Minnesota, St. Paul, MN.

NRC, 1989. Nutrient Requirements of Dairy Cattle. 6th rev. ed. Natl. Acad. Sci., Washington, D. C.

NRC, 2001. Nutrient Requirements of Dairy Cattle, 7th rev. ed. Natl. Acad. Press, Washington, D. C.

Oba, M., Allen, M.S., 2003. Effects of diet fermentability on efficiency of microbial nitrogen production in lactating dairy cows. J. Dairy Sci. 86, 195-207.

Obispo, N.E., Dehority, B.A., 1999. Feasibility of Using Total Purines as a Marker for Ruminal Bacteria. J. Anim. Sci. 77, 3084-3095.

Oldham, J.D., Sutton, J.D., McAllan, A.B., 1979. Protein digestion and utilization by dairy cows. Ann. Rech. Vet. 10, 290-293.

Piepenbrink, M.S., Overton, T.R., Clark, J.H., 1996. Response of cows fed a low crude protein diet to ruminally protected methionine and lysine. J. Dairy Sci. 79, 1638-1646.

Prins, R.A., Van Gestel, J.C., Counotte, G.H.M., 1979. Degradation of amino acids and peptides by mixed rumen microorganisms. Z. Tierphysiol. Tierenahr. Futtermittelk. 42, 1566-1574.

Raggio, G., Lemosquet, S., Lobley, G.E., Rulquin, H., Lapierre, H., 2006. Effect of casein and propionate supply on mammary protein metabolism in lactating dairy cows. J. Dairy Sci. 89, 4340-4351.

Recktenwald, E.B., Van Amburgh, M.E., 2009. Refining nitrogen feeding using current knowledge of recycled urea and microbial nitrogen uptake, Cornell Nutrition Conference for Feed Manufacturers, Ithaca, NY.

Robinson, P.H., Swanepoel, N., Shinzato, I., Juchem, S.O., 2011. Productive responses of lactating dairy cattle to supplementing high levels of ruminally protected lysine using a rumen protection technology. An. Feed Sci. Tech. 168, 30-41.

Rode, L.M., Satter, L.D., 1988. Effect of amount and length of alfalfa hay in diets containing barley or corn on site of digestion and rumen microbial protein synthesis in dairy cows. Can. J. Anim. Sci. 68, 445-454.

Rode, L.M., Weakley, D.C., Satter, L.D., 1985. Effect of forage amount and particle size in diets of lactating dairy cows on site of digestion and microbial protein synthesis. Can. J. Anim. Sci. 65, 101-111.

Rodriguez, C.A., Gonzalez, J., Alvir, M.R., Repetto, J.L., Centeno, C., Lamrani, F., 2000. Composition of bacteria harvested from the liquid and solid fractions of the rumen of sheep as influenced by feed intake. Br. J. Nutr. 84, 369-376.

Rodríguez-Prado, M., Calsamiglia, S., Ferret, A., 2004. Effects of fiber content and particle size of forage on the flow of microbial amino acids from continuous culture fermenters. J. Dairy Sci. 87, 1413-1424.

Royes, J.B., Brown, W.F., Martin, F.G., Bates, D.B., 2001. Source and level of energy supplementation for yearling cattle fed ammoniated hay. J. Anim. Sci. 79, 1313-1321.

Russell, J.B., 2007. The energy spilling reactions of bacteria and other organisms. J. Mol. Microbiol. Biotechnol. 13, 1-11.

Russell, J.B., Cook, G.M., 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiol. Rev. 59, 48-62.

Russell, J.B., O'Connor, J.D., Fox, D.G., Van Soest, P.J., Sniffen, C.J., 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. J. Anim. Sci. 70, 3551-3561.

Santos, F.A.P., Santos, J.E.P., Theurer, C.B., Huber, J.T., 1998. Effects of rumen-undegradable protein on dairy cow performance: a 12-year literature review. J. Dairy Sci. 81, 3182-3213.

Satter, L.D., Slyter, L.L., 1974. Effect of ammonia concentration on rumen microbial protein production in vitro. Br. J. Nutr. 32, 199-208.

Schwab, C.G., Satter, L.D., Clay, A.B., 1976. Response of lactating dairy cows to abomasal infusion of amino acids. J. Dairy Sci. 59, 1254-1270.

Shabi, Z., Tagari, H., Murphy, M.R., Bruckental, I., Mabjeesh, S.J., Zamwel, S., Celik, K., Arieli, A., 2000. Partitioning of amino acids flowing to the abomasum into feed, bacterial, protozoal, and endogenous fractions. J. Dairy Sci. 83, 2326-2334.

Siddons, R.C., Nolan, J.V., Beever, D.E., Macrae, J.C., 1985. Nitrogen digestion and metabolism in sheep consuming diets containing contrasting forms and levels of N. Br. J. Nutr. 54, 175-187.

Siddons, R.C., Paradine, J., 1981. Effect of diet on protein degrading activity in the sheep rumen. J. Sci. Food. Agric. 32, 973-981.

Slyter, L.L., 1976. Influence of acidosis on rumen function. J. Anim. Sci. 43, 910-929.

Smith, R.H., McAllan, A.B., 1974. Some factors influencing the chemical composition of mixed rumen bacteria. Br. J. Nutr. 31, 27-34.

Socha, M.T., Putnam, D.E., Garthwaite, B.D., Whitehouse, N.L., Kierstead, N.A., Schwab, C.G., Ducharme, G.A., Robert, J.C., 2005. Improving intestinal amino acid supply

of pre- and postpartum dairy cows with rumen-protected methionine and lysine. J. Dairy Sci. 88, 1113-1126.

Stern, M.D., Bach, A., Calsamiglia, S., 1997. Alternative techniques for measuring nutrient digestion in ruminants. J. Anim. Sci. 75, 2256-2276.

Stern, M.D., Hoover, H., Sniffen, C.J., Crooker, B.A., Knowlton, P.H., 1978. Effects of nonstructural carbohydrate, urea and soluble protein levels on microbial protein synthesis in continuous culture of rumen contents. J. Anim. Sci. 47, 944-956.

Stern, M.D., Hoover, W.H., 1979. Methods for determining and factors affecting rumen microbial protein synthesis: a review. J. Anim. Sci. 49, 1590-1603.

Stern, M.D., Rode, L.M., Prange, R.W., Stauffacher, R.H., Satter, L.D., 1983. Ruminal protein degradation of corn gluten meal in lactating dairy cattle fitted with duodenal T-type cannulae. J. Anim. Sci. 56, 194-205.

Stern, M.D., Varga, G.A., Clark, J.H., Firkins, J.L., Huber, J.T., Palmquist, D.L., 1994. Evaluation of chemical and physical properties of feeds that affect protein metabolism in the rumen. J. Dairy Sci. 77, 2762-2786.

Stokes, S.R., Hoover, W.H., Miller, T.K., Blauweikel, R., 1991a. Ruminal digestion and microbial utilization of diets varying in type of carbohydrate and protein. J. Dairy Sci. 74, 871-881.

Stokes, S.R., Hoover, W.H., Miller, T.K., Manski, R.P., 1991b. Impact of carbohydrate and protein levels on bacterial metabolism in continuous culture. J. Dairy Sci. 74, 860-870.

Storm, E., Brown, D.S., Ørskov, E.R., 1983. The nutritive value of rumen micro-organisms in ruminants. 3. The digestion of microbial amino and nucleic acids in, and losses of endogenous nitrogen from, the small intestine of sheep. Br. J. Nutr. 50, 479-485.

Storm, E., Ørskov, E.R., 1983. The nutrivitive value of rumen micro-organisms in ruminants. 1. Large-scale isolation and chemical composition of rumen micro-organisms. Br. J. Nutr. 50, 463-470.

Stouthamer, A.H., 1973. A theoretical study on the amount of ATP required for synthesis of microbial cell material. Antonie van Leeuwenhoek 39, 545-565.

Sulabo, R.C., Mathai, J.K., Usry, J.L., Ratliff, B.W., McKilligan, D.M., Stein, H.H., 2011. Amino acid digestibility and energy content in Dried Fermentation Biomass, Peptone 50, and P.E.P. Two Plus fed to weanling pigs (abstract), ADSA-ASAS Joint Annual Meeting, New Orleans, LA.

Swanepoel, N., Robinson, P.H., Erasmus, L.J., 2010. Amino acid needs of lactating dairy cows: impact of feeding lysine in a ruminally protected form on productivity of lactating dairy cows. Anim. Feed Sci. Technol. 157, 79-94.

Sylvester, J.T., Karnati, S.K.R., Yu, Z., Newbold, C.J., Firkins, J.L., 2005. Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. J. Dairy Sci. 88, 2083-2095.

Tamminga, S., 1978. Protein degradation in the forestomachs of ruminants. J. Anim. Sci. 49, 1615-1630.

Tamminga, S., 1981. Effect of the roughage/concentrate ratio on nitrogen entering the small intestine of dairy cows. Neth. J. Agric. Sci. 29, 273-283.

Tas, M.V., Evans, R.A., Axford, R.F.E., 1981. The digestibility of amino acids in the small intestine of the sheep. Br. J. Nutr. 45, 167-174.

Ushida, K., Jouany, J.P., Thivend, P., 1986. Role of rumen protozoa in nitrogen digestion in sheep given two isonitrogenous diets. Br. J. Nutr. 56, 407-419.

Valkeners, D., Théwis, A., Piron, F., Beckers, Y., 2004. Effect of imbalance between energy and nitrogen supplies on microbial protein synthesis and nitrogen metabolism in growing double-muscled Belgian Blue bulls. J. Anim. Sci. 82, 1818-1825.

Van Kessel, J.S., Russell, J.B., 1996. The effect of amino nitrogen on the energetics of ruminal bacteria and its impact on energy spilling. J. Dairy Sci. 79, 1237-1243.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy. Sci. 74, 3583-3597.

Vanzant, E.S., Cochran, R.C., Titgemeyer, E.C., 1998. Standardization of in situ techniques for ruminant feedstuff evaluation. J. Anim. Sci. 76, 2717-2729.

Vincente, F., Guada, J.A., Surra, J., Balcells, J., Castrillo, C., 2004. Microbial contribution to duodenal purine flow in fattening cattle given concentrate diets, estimated by purine N labelling (^{15}N) of different microbial fractions. Anim. Sci. 78, 159-167.

Volden, H., 1999. Effects of level of feeding and ruminally undegraded protein on ruminal bacterial protein synthesis, escape of dietary protein, intestinal amino acid profile, and performance of dairy cows. J. Anim. Sci. 77, 1905-1918.

Walker, D., Nader, C., 1970. Rumen microbial protein synthesis in relation to energy supply: diurnal variation with once-daily feeding. Aus. J. Agric. Res. 21, 747-754.

Wallace, R.J., 1983. Digestion of rumen bacteria in vitro. Br. J. Nutr. 49, 101-108.

Wallace, R.J., 1996. Ruminal microbial metabolism of peptides and amino acids. J. Nutr. 126, 1326S-1334S.

Wallace, R.J., Newbold, C.J., McKain, N., 1990. Patterns of peptide metabolism by rumen microorganisms, In: Hoshino, S., Onodera, R., Minato, H., Itabashi, H. (Eds.), The Rumen Ecosystem: The Microbial Metabolism and Its Regulation,

Japan Scientific Societies Press and Springer-Verlag, New York City, NY, pp. 43-50.

Windschitl, P.M., Stern, M.D., 1988. Effects of urea supplementation of diets containing lignosulfonate-treated soybean meal on bacterial fermentation in continuous culture of ruminal contents. J. Anim. Sci. 66, 2948-2958.

Wittmann, C., Becker, J., 2007. The L-lysine story: From metabolic pathways to industrial production. Microbiol. Monogr. 5, 39-70.

Work, E., Dewey, D.L., 1953. The Distribution of α, ε-Diaminopimelic Acid among various micro-organisms. J. Gen. Microbiol. 9, 394-409.

Wright, D.E., 1967. Metabolism of peptides by rumen microorganisms. Appl. Microbiol. 15, 547-550.

Yanez-Ruiz, D.R., Scollan, N.D., Merry, R.J., Newbold, C.J., 2006. Contribution of rumen protozoa to duodenal flow of nitrogen, conjugated linoleic acid and vaccenic acid in steers fed silages differing in their water-soluble carbohydrate content. Br. J. Nutr. 96, 861-869.

Zinn, R.A., Owens, F.N., 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. Can. J. Anim. Sci. 66, 157-166.