RUMINAL FEED PROTEIN DEGRADATION AND MICROBIAL PROTEIN SYNTHESIS

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INTRODUCTION

There is a variety of proteins and non-protein nitrogen compounds in the composition of feeds. Proteins are large molecules that differ in size, shape, solubility and amino acid composition, and are present in the wall and cellular content of all plant and animal tissues where they perform various functions (e.g., catalytic, structural, transportation and contractile). The non-protein nitrogen compounds are smaller molecules and include peptides and amines.

Protein in feeds is, to a large extent, degraded in the rumen. Degradation is one of the most important quantitative factors determining the nutritional value of feed protein, the supply of ammonia, peptides and branched-chain fatty acids to ruminal microorganisms, and the passage of undegradable proteins to the intestine (Hvelplund and Weisbjerg, 2000).

A first order mass action model often describes the ruminal degradation of protein. An important characteristic of this model is that it considers that crude protein (CP) of feed consists of multiple fractions, which differ greatly among themselves in relation to degradation rates, and that the ruminal disappearance of protein is the result of two simultaneous activities: degradation and passage (NRC, 2001).

Several methods have been used to divide CP into rumen degradable protein (RDP) and rumen undegradable protein (RUP). These methods include *in vivo* and *in situ* evaluations, and a variety of *in vitro* methods (Schwab et al., 2003). In theory, *in vivo* methods are preferred to measure the digestibility of nutrients. However, *in vivo* techniques require large quantities of feed and a large number of repetitions to overcome the variations related to the animal and other factors. Therefore, the cost to obtain an adequate number of repetitions in addition to maintenance costs and the large number of animals may cause *in vivo* studies to be expensive and impractical. Moreover, the concept of animal welfare has contributed to a reduction in the number of *in vivo* experiments. This has led to increased interest in using *in vitro* and *in situ* techniques (Broderick and Cochran, 2000).

Ruminants with expressive pre-gastric fermentation activity evolved 14 million years ago, and their success in the evolutionary process has been attributed to the existence of a symbiotic relationship with ruminal microorganisms, where the animals provide feed and habitat, while the microorganisms provide volatile fatty acids and amino acids formed from substrates (fiber and non-protein nitrogen) that are not utilized by the host animal (Kozloski, 2002).

Most amino acids absorbed by ruminants come from microbial protein synthesized in the rumen, and the dietary requirements of metabolizable protein for ruminants are met through intestinal absorption of amino acids from undegradable dietary protein and digestible true microbial protein. Thus, the objective of ruminal nutrition has been to maximize the flow of microbial protein to the small intestine, thereby increasing production efficiency. For this reason, it is necessary to quantify the contribution of microbial protein ruminal synthesis to better understand the process of converting dietary nutrients into microbial proteins and the factors affecting this process.

The measurement techniques of synthesis and/or microbial protein flow can be divided into three main categories: direct determination by counting of microorganisms, indirect determination using the markers present in the microorganisms, such as RNA, and indirect determination by incorporation of the microorganisms from external substances, such as ^{15}N and ^{35}S.

The objectives of this chapter are to discuss some techniques used to evaluate feed proteins, as well as to address methods for identifying the microbial crude protein synthesized in the rumen, and factors affecting the ruminal microbial crude protein synthesis.

In situ METHODS

The technique used to estimate ruminal fermentation by the incubation of small samples of feed in the rumen was first used by Quin et al. in 1938, however, it was not until the introduction of mathematical tools capable of transforming the data of ruminal disappearance rates in values of effective degradability (Ørskov and McDonald, 1979) that the method became widespread (Hvelplund and Weisbjerg, 2000). Today, the *in situ* method is the most widely used in research to determine estimates of rumen protein degradability, having been adopted in several countries (Schwab et al., 2003) as well as by the NRC (2001).

The *in situ* procedure consists of placing feed samples in a nylon bag with a defined pore size (40-60 μm), and infusing them into cannulated animals (cattle, sheep or goats). The pores must be small enough to prevent the loss of particles and large enough to allow for access of microorganisms to the material. Due to the small quantity of incubated samples, they do not interfere with ruminal fermentation, and it is assumed that the conditions inside the bags are similar to those in the rumen. The samples are removed at various time intervals and the CP is quantified in the non-degraded material.

At least three fractions (A, B and C) of the CP may be determined. It is assumed that fraction A is completely degradable in the rumen and is the fraction that escapes from the pores during the process of washing with water (± 39ºC); included in this fraction are the non-protein nitrogen compounds (NPN), the rapidly soluble protein and the protein contained in the small feed particles that pass through the pores. Fraction B is potentially degradable insoluble protein associated with the larger particles. That is, the percentage of initial CP that disappears from the sample during the time of ruminal exposure. Finally, fraction C consists of matter that is not degradable in the rumen, regardless of exposure time of the sample to the ruminal environment.

The effective degradability (DE) of feed is determined by the model of Ørskov and McDonald (1979), using the following equation: $DE = A + B [Kd/(Kd + Kp)]$, where the fractions A and B and the digestion rate (kd) are estimated by the potential degradability: $Dg (t) = A + B. (1 – e^{kd*t})$, where kd is the digestion rate of fraction B, kp is the rate of passage of fraction B and t is the incubation time. The RDP may be calculated as $RDP = A + B [Kd/(Kd + Kp)]$ and the $RUP = CP – RDP$ or $RUP = C + B [Kp/(Kd + Kp)]$.

Some adjustments to the original model of Ørskov and McDonald (1979) have been made. McDonald (1981) introduced a lag time value to the model, to increase precision when determining the effective degradability. The lag time is defined as the time in which the derivative of the equation of the data sets equals the true potentially degradable fraction at time zero (Mertens, 1993). Therefore, the new equations would be $Dg (t) = A + B \times [1 – e^{– kd*(t-lag)}]$ and $DE = A +[B.Kd.e^{– Kp*lag}/(Kd + Kp)]$. According to Petit et al. (1995), adding the lag time to the model has little effect on the effective

degradability. However, the values of fractions A and B and the kd are slightly different with the use or non-use of lag time in the model.

As stated above, the ruminal disappearance of CP is a function of the rates of digestion and passage. Thus, the kp will be measured or estimated using equations. The NRC (2001) proposes three equations to estimate the rates of passage, where, for wet forages, $kp = 3.054 + 0.614X_1$ (1); for dry forage $kp = 3.362 + 0.479X_1 - 0.007X_2 – 0.017X_3$ (2) and for concentrates. $Kp = 2.904+1.375X_1 – 0.020X_2$ (3), where X_1= DM intake (% of body weight); X_2 = % of concentrate in the diet (on DM basis) and X_3= % of the NDF in DM basis. As with the NRC (2001), Seo et al. (2006) proposed three equations to estimate the kp_f for forages = (2.365 + 0.0214IFpPC + 0.0734ICpPC + 0.069FDMI)/100; kp_c for concentrate = (1.169 + 0.1375IFpPC + 0.1721ICpPC)/100 and kp_l for the liquid fraction = (4.524 + 0.0223IFpPC + 0.2046ICpPC + 0.344FDMI)/100, where kp is the rate of passage (h^{-1}), IFpPC is the dry forage intake as a proportion of the body weight (g/kg), ICpPC is the ingestion of dry concentrate as a proportion of the body weight (g/kg) and FDMI is the dry forage intake (kg).

It becomes clear, from these equations, that dry matter intake (NRC, 2001) and specific diet components, such as concentrate and forage (Seo et al., 2006) are important factors affecting the rate of passage and, consequently, the content of RDP and RUP in the feeds. However, due to the complexity of modeling, some factors that exert an effect on the rate of passage (size, density and rate of particle hydration),are not yet included in the models for kp prediction.

According to Broderick and Cochran (2000), despite the broad use of the *in situ* method to determine the ruminal degradability of CP, there is still a wide variation in the results obtained in different laboratories, with the main sources of variation coming from: basal diet, type of samples and animals, replication, incubation conditions, washing technique and correction for microbial contamination. Thus, standardizing the technique is very important to allow an adequate evaluation of the feed and a comparison of obtained results. Table 1 shows conditions for evaluating ruminal degradability of the CP. These were suggested by Broderick and Cochran (2000) to standardize the evaluation conditions.

Table 1 - Recommendations for *in situ* experiments

Items	Recommendations
Basal Diet	Forage/concentrate ratio of 60:40
Feeding level	Maintenance or voluntary
Bag materials	Polyester or Nylon
Pore size	40 – 60 μm
Sample/surface area ratio	10 – 15 mg/ cm^2
Sample weight (bags measuring 10 x 15 cm)	4.5 g
Grinding (concentrate, forages)	2 mm
Animal Species	Cattle, sheep, etc.
Number of animals	2
Number of days	2 - 3
Number of bags	2-3
Bag position in the rumen	Ventral bag with free movement
Entry order/removal	Sequential entry and combined removal
Incubation times	0, 2, 4, 8, 16, 24 and 48 h (72 for forages)
Correction for microbial contamination	yes, for low-protein forages

Adapted from Broderick and Cochran (2000) and Vanzant et al. (1998).

Among the main problems encountered when using the *in situ* method to evaluate the degradation of protein in forages, the main one is the high proportion of water-soluble material contained in the forages that the technique mistakenly considers degradable. Additionally, the effect of microbial contamination can be more important in forages due to its high fiber and low protein levels (Calsamiglia et al., 2000). The need for rumen fistulated animals also contributes to increased costs to determine the RDP and RUP using the *in situ* technique (Schwab et al., 2003).

Attempts have been made to consider particle loss, microbial contamination and escape of soluble protein as errors (Hvelplund and Weisbjerg, 2000). When the samples are ground, the small particles produced may escape through the pores during the incubation process, without the occurrence of any degradation and be erroneously considered as degraded. The extent of small particle loss can be estimated by the difference between the loss of particles in the bags when they are washed (P) and the solubility measured in filter paper (SOL), which is determined by weighing 0.5 g of the sample in a glass, adding 40 mL of water and leaving in solution at room temperature for one hour. Later, the material is filtered through filter paper and the N is calculated, where the value of water-soluble N is determined by the difference (Hvelplund and Weisbjerg, 2000). Assuming that the particles lost have the same rate of degradation as the remainder, the following correction may be made: $a_{cor} = a – P$; $b_{cor} = b + [b / (1 – (P + Sol))]$; $c_{cor} = c$.

The principle of the *in situ* method is that microorganisms must enter the bags that contain the samples and degrade them, similarly to the process that occurs outside the bags. Therefore, the microorganisms will colonize the samples. The washing procedure after incubation removes the degraded material and some of the microorganisms, but some remain attached to the sample and are not removed during the process. Microbial contamination has little influence on the value of dry matter degradability, but due to the high level of N in the microorganisms, the degradability of the CP may be underestimated, especially for the forages. Thus, corrections in the values of degradability obtained must be made by adding these values to a ΔDE. These corrections are based on the levels of CP and/or NDF of the samples. Therefore, $\Delta DE = 20.2 – 0.674 * CP$ (% DM) or $\Delta DE = 6.4 – 0.353 * CP$ (% DM) + $0.170 * NDF$ (% DM), (Michalet-Doreau and Ould-Bah, 1992). However, although the suggested corrections are easily applicable, as they are not based on linear equations and in the CP and NDF contents of the samples, they may not be efficient for all types of feed analyzed (Vanzant et al., 1998).

According to the equation used to estimate the effective degradability, the soluble protein is completely degradable in the rumen. Yet for feeds with a high proportion of soluble protein, such as silage, the degradability of this fraction is similar to the others. The rate of passage of the fluid phase is higher (12– 15%.h^{-1}) when compared to the rate for particles, signifying that the degradation rate for this fraction must be extremely high or there would be an escape of that fraction, resulting in overestimated values of RDP (Hvelplund and Weisbjerg, 2000). To properly estimate the RDP in feeds containing a high proportion of fraction A, it is necessary to review it in relation to its rates of passage and digestion, such as: $RDP = A [Kd_A/(Kd_A + Kp_{fluid})] + B [Kd/(Kd + Kp)]$. However, this correction is difficult due to the need for estimates of the digestion rates of fraction A.

In vitro CHEMICAL METHODS

The most widely used method to determine the nitrogen fractions in feed is the fractionation protocol used in the CNCPS (Sniffen et al., 1992; Fox et al., 2000). The CNCPS divides the CP of the feed into 5 fractions using 3 solvents and one

precipitating agent. The five fractions are: A, soluble in borate phosphate buffer (BPB), but not precipitated by trichloroacetic acid (TCA), and consisting of non-protein nitrogen compounds (NPN); B_1, true protein that is rapidly degradable in the rumen, soluble in BPB, but precipitated by TCA; B_2, true protein and large peptides that are moderately degraded in the rumen, calculated as the difference between the total CP of the feed minus the other fractions; B_3, true protein that is slowly degraded in the rumen, calculated as the difference between the content of neutral detergent-insoluble protein (NDIP) and the content of acid detergent insoluble protein (ADIP) and C, the indigestible rumen undegradable protein, similar to the ADIP.

The NDIP is obtained by determination of the CP in the insoluble residue after treatment with neutral detergent, without using sodium sulphate, and the ADIP is determined after the sequential extraction of the residue obtained after treatment with acid detergent. Fraction A is considered 100% degradable in the rumen and fraction C is 100% non-degradable.

CNCPS also recognizes that the disappearance of the CP in the rumen is a simultaneous function of the kd and kp, and that the kp varies with intake, feed and characteristics of the diet. Thus, two equations are used to predict the kp of undegradable feeds: one for forages (kp = 0.388 + 22.0 * [DMI/$BW^{0.75}$] + 0.0002 * [% of the forage in the DM of the diet]) and another for concentrate (kp = -0.424 + [1.45 * kp for forages]). The rates of passage are adjusted for individual feed using a multiplicative adjustment factor for particle size and a physically effective neutral detergent fiber (peNDF) proposed by Mertens. Two equations are used to determine the adjustment factor (AF): one for forages (AF = 100/[peNDF + 70]) and another for concentrates (AF = 100/[peNDF + 90]).

The RDP and RUP values may be calculated directly through the association of the obtained CP fractions with the respective rates of passage and digestion. Therefore, the RDP (% CP) may be calculated as A + B1 (kdB1 / [kdB1 + kp]) + B2 (kdB2 / [kdB2 + kp]) + B3 (kdB3 / [kdB3 + kp]) and the RUP = 1 − RDP.

An interesting aspect of the approach used in the CNCPS is that the analysis (NPN, NDIP, ADIP and true soluble protein) used to determine the CP fractions is a routine procedure in the laboratory, which facilitates the adoption of this method in field conditions (Schwab et al., 2003). A negative aspect of the method, however, is that the rates of digestion of the three fractions of the true protein (B) vary significantly (B_1 = 120 to 400% $.h^{-1}$, B_2 = 3 to 16% $.h^{-1}$ and B_3= 0.06 to 0.55% $.h^{-1}$) for the main classes of feeds. Ranges of these values may be found in Sniffen et al. (1992).

In vitro ENZYMATIC METHODS

The two basic approaches to estimate *in vitro* ruminal digestion involve incubation with ruminal microorganisms (ruminal *in vitro* methods) or cell-free enzymes (non-ruminal *in vitro* methods). The first technique uses ruminal digesta usually obtained from cannulated animals, and the second is based on the use of commercially available enzymes, with the intention of obtaining a result similar to that found in the rumen fluid (Broderick and Cochran, 2000). In both cases, the protein degradation rate is measured from the rate of accumulation of amino acids and ammonia, which represent the products of protein degradation (Schwab et al., 2003).

The "ruminal *in vitro* methods" are complicated by the microbial use of liberated amino acids and ammonia, causing an underestimation of degradation. Moreover, there is a release of amino acids and ammonia from the microbial catabolism and the residual protein present in the inoculum, which leads to an overestimation of the degradation. This underestimation may be controlled by the use of a "blank". However, the use of amino acids and ammonia cannot be controlled by the same

technique (Schwab et al., 2003). To solve this problem, Broderick (1987) developed a method called "*in vitro* inhibitor (IIV)" using hydrazine and chloramphenicol as inhibitors of nitrogen metabolism of microorganisms, preventing the absorption and use of amino acids and/or ammonia. Thus, the use of the IIV system, along with the "blank", provides a better adjustment and obtained data that is more consistent with protein degradation. However, as reviewed by Broderick and Cochran (2000), the IIV system is not adequate for analyzing grass or legume silage that contains high levels of non-protein nitrogen compounds.

The use of cell-free enzymes has the advantage of eliminating the need for cannulated animals and microbial interference on the final result of the analysis. Many studies have been conducted using "non-ruminal *in vitro* methods" (Assoumani et al., 1992; Licitra et al., 1998, 1999), with the goal of identifying a protease or mixtures of proteases that produce estimates of degradation similar to those obtained with the "ruminal *in vitro* method" (Schwab et al., 2003).

According to Calsamiglia et al. (2000), due to the complexity of the interactions occurring within the ruminal environment, the proteolytic activity needed for protein degradation may require the presence of other non-proteolytic enzymes. Assoumani et al. (1992) demonstrated the interference of starch on protein degradation of cereal grains, which was increased by up to 20 percentage points by the addition of amylase to the environment. Kohn and Allen (1995) also reported an increase in protein degradation linked to NDF when cellulose was added to the environment. Thus, it seems that ruminal protein degradation requires proteolytic and non-proteolytic enzyme activities.

Aufrère et al. (1991) evaluated the degradability of 97 concentrate feeds using the *in vitro* technique with varying incubation times (1 to 24 hours) and protease from *S. griseus*, and found a high correlation with the *in situ* results (r^2 = 0.89). However, Roe et al. (1991) and Tománková and Kopency (1995) found low or moderate (r^2 = 0.21 and r^2 = 0.39, respectively) correlation between the *in vitro* and *in situ* values, suggesting that these enzymes may not be applicable for the simulation of ruminal degradation for a wide variety of feeds (Calsamiglia et al., 2000).

The use of *in vitro* enzymatic methods to predict the rate of ruminal protein degradation offers the practicality of a laboratory and higher analytical precision. However, no single method has been scientifically accepted for all types of feed and challenges associated with the interference of some compounds (fiber, starch, NPN) were linked to the appropriate identification of the enzyme: remaining substrate ratio (Schwab et al., 2003).

MICROBIAL PROTEIN SYNTHESIS

Ruminants with expressive pre-gastric fermentation activity evolved 14 million years ago, and their success in the evolutionary process has been attributed to the existence of a symbiotic relationship with the ruminal microorganisms, where the animals provide the feed and the habitat, while the microorganisms supply volatile fatty acids and amino acids formed from substrates that are not utilized (fiber and non-protein nitrogen) by the host animal (Kozloski, 2002).

Qualitative observations of the presence of microorganisms and volatile fatty acids in the rumen have been reported throughout the 19th century; however, only in the early 1940s did researchers at Cambridge University conducte the first quantitative studies on the production of volatile fatty acids. After their importance to the host was identified, studies related to ruminal microorganisms began (Hobson and Stewart, 1997).

Most of the amino acids absorbed by ruminants come from microbial proteins synthesized in the rumen. The dietary requirements of the metabolizable protein for ruminants are met by the absorption of the digestible true microbial protein and the digestible rumen undegradable protein in the small intestine. The microbial protein may supply from 50 to 100% of the metabolizable protein required by beef cattle and it is considered to be of good quality due to its intestinal digestibility (around 80%) and its amino acid profile (NRC, 2000).

The amino acid composition of the microbial protein is similar to that of the protein in the animal tissue, as well as the protein found in milk. When compared to the protein composition of vegetable protein concentrates, microbial protein contain a higher proportion of methionine and lysine, and after the prohibition of the use of animal-based feed in diets for ruminants in Brazil, the best source to supply the amino acid requirements of the animal has become microbial protein (Verbic, 2002).

Considering these qualities, the goal of ruminant nutrition has been to maximize the microbial protein flow to the small intestine, increasing the production efficiency. Therefore, it is necessary to quantify the contribution of the ruminal microbial protein synthesis to better understand the conversion process of dietary components into microbial protein and the factors affecting it. However, measurement of the production of microbial protein is difficulted by the fact that it involves three distinct populations (bacteria, protozoa and fungi) that are constantly exposed to selection pressure in their habitat, which is frequently altered.

Due to the importance of microbial protein for the protein metabolism of ruminants, the quantification of its flow under different dietary and physiological conditions is essential to meet the requirements in absorbed amino acids. With this purpose, several microbial markers have been used, each with its own advantages and limitations. Broderick and Merchen (1992) affirmed that no individual marker is entirely appropriate for all situations, so the estimates obtained are relative and not absolute. Many techniques require fistulated animals and the estimate of abomasal flow. Currently, there is an increased interest in replacing the surgical implantation of fistulas in different parts of the gastrointestinal tract using non-invasive techniques.

The techniques used to measure synthesis and/or flow of microbial protein may be divided into three main categories: direct determination by counting of microorganisms, indirect determination using the markers present in the microorganisms, such as RNA and some other substances found in these microorganisms, and indirect determination through incorporation of microorganisms from external substances, such as the elements ^{15}N and ^{35}S.

MICROORGANISM COUNTING

This technique consists of direct counting of bacteria, protozoa and fungi in samples of ruminal digesta after successive dilutions necessary to identify the number of microorganisms in a given volume, observed with the aid of a microscope. Counting may be conducted to determine the quantity of viable individuals or simply the amount of fixed individuals. The bacteria and protozoa can be quantified in an appropriate counting chamber. The concentration of bacteria can be obtained using the most probable number procedure, described by Dehority et al. (1989). Fungi can be determined from the number of zoospores in the ruminal fluid, although this technique is highly questionable.

Some difficulties of this technique include the incapacity to distinguish between small feed particles and bacteria, the incapacity to identify the microorganisms that adhere to particles, the superposition of bacteria, the distribution of individuals in the

counting chamber, the viscosity of the diluents and the sampling error of ruminal content.

The identification of microorganism concentration in the ruminal fluid collected before and at various times after feeding, indicates the rate and extent of microbial growth in the rumen and may be useful in comparing diets. However, the concentration of microorganisms is a result of the balance between growth, lysis and passage of microorganisms to a determined ruminal volume, and, therefore, has little use in determining microbial protein as a source of absorbable amino acids. The biomass of microorganisms varies greatly per cell, thus, microorganism counting must be converted into microbial biomass for nutritional evaluation.

DAP

The discovery of a unique amino acid, diaminopimelic acid (DAP), was reported in 1950. Later, this amino acid, which is only present in bacteria, was identified in oligopeptides linked to peptidoglycans of the bacterial cell wall (Broderick and Merchen, 1992). In addition to the DAP, the amino acid D-alanine and muramic acid, also present in the bacteria cell wall, are microbial compounds used as markers.

DAP was suggested as a microbial marker in 1953 by Synge, and has since been used in various experiments to estimate the synthesis of microbial protein (Broderick and Merchen, 1992). The concentration of DAP present in the duodenal digesta can be obtained according to Czerkawski (1974) and, with the measurement of the duodenal flow, the microbial protein flow can be estimated from the DAP: microbial N relationship.

However, the proportion of DAP in relation to microbial protein varies between different bacterial and protozoa species. The DAP:N ratios (mg/g of N) found by Czerkawski (1974) in small bacteria, large bacteria and protozoa were 7.3, 4.7 and 0.9, respectively. Protozoa are less numerous in the ruminal content; however, due to their larger size, they may represent a significant portion of the microbial biomass in the rumen. Generally, less than 10% (in some cases, more than 40%) of the duodenal flow of microbial N is derived from the N contained in protozoa and, if the DAP:protozoa N ratio is ignored, the accuracy of this marker for determining the synthesis of microbial protein is reduced (Sylvester et al., 2005).

Since it is a constituent of the cell wall, conditions that favor average growth of the bacterial cell may result in the reduction of the cell wall:protoplasm relationship and, consequently, decrease the DAP:bacteria protein ratio, underestimating the synthesis of microbial protein. In addition, it was identified the natural occurrence of DAP in feeds and also demonstrated the presence of DAP bacterial residues adhering to the rumen undegradable feed particles. Therefore, these forms of DAP may alter the estimated flow of microbial protein, making its quantification to increase the efficiency of this method necessary.

D-Alanine

Another constituent of the microbial cell wall, D-alanine, was suggested as a microbial marker by Garrett et al. (1982), since it is not detected in feeds and its concentration is higher in bacteria than the DAP. However, the same problems related to factors that affect the cell wall:microbial protoplasm relationship may limit the use of this marker.

Odd-chain fatty acids (OCFAs)

Bovine milk contains measurable quantities of odd-chain fatty acids (OCFAs), such as pentadecanoic acid (C15:0), heptadecanoic acid (C17:0) and heptadecenoic acid, as well as branched chain isomers (BCFAs). The odd-chain fatty acids are synthesized in negligible amounts by cattle and are not present in vegetables; however, they constitute most of the fatty acids of the microbial lipid membrane. The OCFAs can be determined according to a technique described by Vlaeminck et al. (2005).

The OCFAs, C15:0 and C17:0, are synthesized by the microorganisms through the elongation of the propionate or valerate, while their branched-chain isomers, BCFAs, are synthesized using the branched-chain amino acids (valine, leucine and isoleucine) and their corresponding volatile fatty acids of branched chain (isobutyrate, isovalerate and 2-methylbutyrate) as precursors (Kaneda, 1991).

Keeney et al. (1962), cited by Vlaeminck et al. (2005), suggested the use of OCFAs and their branched isomers as markers of microbial synthesis, however, due to the difference in the OCFAs profile among microorganisms, they would only qualitatively describe the ruminal microbial synthesis (Dewhurst.et al., 2000b).

The different groups of microorganisms have distinct OCFAs quantities and profiles. Cabrita et al. (2003) demonstrated that the fatty acid profile in milk was affected by the levels of N and dietary carbohydrates, and that through this profile, it is possible to distinguish the fatty acids that are synthesized in the mammary gland (short and medium-chain fatty acids), those modified in the mammary gland by the enzyme Δ^9-desaturase (monoenoic fatty acids), those derived from the absorption of dietary fatty acids (long-chain fatty acids) and others, such as those synthesized by the ruminal microorganisms (OCFAs). The same authors report that the anteiso C15:0 fatty acid positively correlates with the level of dietary sugar and that the heptadecanoic acid (C17:0) is an indicative of protein deficiency.

Vlaeminck et al. (2005) demonstrated that the BCFA level was strongly related to the microbial biomass present in the rumen and that the secretion of BCFAs in milk, particularly C17:0, could be used to predict the duodenal flow of microorganisms.

PCR

As previously discussed, the presence of protozoa leads to errors in the determination of microbial protein. Using current methods, neither internal nor external markers distinguish bacteria from protozoa, limiting the measurement of microbial synthesis and the intra-ruminal recycling of N. Molecular techniques, using 18S rDNA as a quantity marker of N derived from protozoa are evolving, and will so allow the distinction of protozoa from bacteria in the total pool of microbial protein synthesized in the rumen.

^{35}S

After infusion of $Na_2{}^{35}SO_4$, ^{35}S is incorporated during the *de novo* synthesis of sulfur amino acids,(cystine and methionine). ^{35}S has a low environmental risk and danger to human health, although it accumulates in the tissue and is secreted in milk; therefore, these cannot be consumed and should be properly discarded. Beever et al. (1974) proposed a technique to estimate microbial synthesis using ^{35}S.

^{15}N

^{15}N has been widely used as an marker to determine the microbial production for the following reasons: it is a stable isotope, has a low environmental risk, has low cost compared to other isotopes, marks all pools of microbial N, is not naturally found in the proteins contained in feed and does not mark the animal protein until the marked microbial amino acids are incorporated into the tissues (Broderick and Merchen, 1992). The ^{15}N becomes well distributed in the microbial cell; thus, in the case of cellular lysis during isolation, the losses of protoplasm, which leads to underestimation of the amount of nucleic acids, is less important when determining the concentration of ^{15}N.

When ammonia salts, $(^{15}NH_4)_2SO_4$ are infused in the rumen, the synthesis of microbial amino acids gradually occurs using the $^{15}NH_3$ as precursors and, therefore, the isotope becomes a constituent of the microbial protein. The protozoa are principally marked after the incorporation of the ^{15}N found in the predated bacteria.

Broderick and Merchen (1992) recommended the continuous infusion of $(^{15}NH_4)_2SO_4$ through the ruminal fistula during 48 hours and determination of ^{15}N concentration according to the method described by Siddons et al. (1985).

Usually, the marker:microbial N ratio has been obtained in isolated bacteria from the liquid phase of the ruminal digesta, considering that it is similar to the relationship with the mixed ruminal microbes, although differences between bacteria in the liquid and solid phases, as well as between bacteria and protozoa, have been widely reported. The fraction of bacteria associated with the solid phase is greater than that associated with the liquid phase, representing more than 90% (Faichney, 1980) of the bacteria isolated from animals receiving forage diets. Therefore, the procedures of bacterial isolation should consider the bacteria associated with particles to determine a more representative marker:total N ratio.

Martín et al. (1994) directly observed different ^{15}N and DAP levels between bacteria in the liquid phase (0.164% and 2.25% of the total N) and the solid phase (0.111% and 11.6% of the total N), possibly due to the higher growth rate and protein synthesis of the bacteria present in the ruminal fluid. Although the contribution of bacteria associated with the particles have not been widely studied, its presence in the determination of the marker:total N relationship may have large impact when estimating the microbial protein flow. Carro and Miller (2002) found higher levels of ^{15}N and purine related to the total N in liquid phase bacteria than in bacteria associated with the particles and intermediate levels in the mixed pellets containing both bacteria. Thus, methods capable of isolating mixed bacteria are recommended.

RNA

Unicellular organisms have a high concentration of nucleic acids, especially RNA and purine bases, which makes their use as microbial markers attractive. Around 18% of the total nitrogen in ruminal microorganisms is found in nucleic acids and approximately 11% of the total N is present in purine bases (Chen and Ørskov, 2003). According to Broderick and Merchen (1992), the use of nucleic acids as markers has been well established. RNA may be quantified according to the method proposed by Ling and Buttery (1978), and the purine bases according to Ushida et al. (1985).

Most feeds have a low concentration of RNA and, according to McAllan and Smith (1973), extensive degradation of exogenous RNA occurs in the rumen. Therefore, the duodenal flow of RNA is predominantly of microbial origin. However, in the animal protein feeds, the concentration of RNA is similar to that of the

microorganisms, and it is thus not appropriate to use RNA as an marker in animals receiving this type of feed.

The main obstacle of this technique is the difference found in the RNA:microbial protein relationship between bacteria and protozoa and even between bacteria in the liquid phase and bacteria associated with particles of the ruminal digesta (Cecava et al., 1990).

Urinary purine derivatives

The use of purine derivatives (PD) as markers to estimate microbial synthesis in the rumen was first proposed by Blaxter and Martin in 1962 and cited by Fujihara et al. (1987). Topps and Elliott (1965) were the first to demonstrate that the urinary excretion of allantoin was related to the concentration of nucleic acids in the rumen of sheep fed with different energy levels, and were also the first to use the term “purine derivatives”.

In this method, it is assumed that the duodenal flow of nucleic acids is essentially of microbial origin and, after intestinal digestion, the microbial purine bases (adenine and guanine) are catabolized and excreted in the urine as PD, in proportion to the amount of purine bases absorbed.

Allantoin is the most abundant PD, with uric acid, xanthine and hypoxanthine making up the remaining components collectively referred to as PD. In cattle, due to the high activity of the xanthine oxidase enzyme, which converts xanthine and hypoxanthine into uric acid, the excretion of allantoin and uric acid constitutes about 98% of urinary purine derivatives. Therefore, the contribution of the xanthine and hypoxanthine is not significant to determine the total excretion of PD (Rennó et al., 2000). In dairy cows, the excretion of purine derivatives in milk is composed mostly of allantoin, but also occurs some contribution of uric acid and, in cases where the excretion of PD in the milk is disregarded, the absorption of purines may be underestimated. The ratio of PD absorbed in the small intestine:PD recovered in the urine can be modified if the PD excretion by the mammary gland is altered by the production of milk (Gonzalez-Ronquillo et al., 2003).

Several authors (Chen et al., 1990; Balcells et al., 1991; Giesecke et al., 1994; Orellana Boero et al., 2001 and Gonzalez-Ronquillo et al., 2003) confirmed the relationship between the duodenal flow of purine bases and the urinary excretion of PD. Therefore, the flow of microbial N may be calculated from the amount of absorbed purines, which are estimated from the excretion of the PD (Chen and Gomes, 1992).

Traditionally, by this method, the intestinal flow of microbial nitrogen compounds (MN, g N/day) is calculated based on the absorbed microbial purines (AP, mmol/day) using the following equation: $MN = (70 \times AP) / (0.83 \times 0.116 \times 1000)$, where 70 represents the amount of N in the purines (mg N/mmol), 0.83 is the digestibility of the microbial purines, and 0.116 is the purine N:total N ratio in ruminal microorganisms (Chen and Gomes, 1992). The absorbed microbial purines (AP, mmol/day) are calculated from the total excretion of purine derivatives (DP, mmol/day), using the equation: $AP = (PD - 0.385 \times BW^{0.75}) / 0.85$, where 0.85 is the recovery of absorbed purines as urinary purine derivatives, and $0.385*BW^{0.75}$ is the endogenous contribution in the urinary excretion of PD (Verbic et al., 1990).

However, some parameters used in the model are not completely defined, such as the purine N:total N relationship in ruminal microorganisms, the recovery of absorbed purines, the excretion of purine derivates of endogenous origin and the intestinal digestibility of purine bases (RNA). Cecava et al. (1990) and Reynal and Broderick (2005) evaluated the purine N:total microbial N ratio and observed

differences in the relation between the isolated pellets of bacteria associated with the particles, ruminal fluid bacteria and protozoa.

Clark et al. (1992), Chen and Gomes (1992), Valadares Filho (1995), Carvalho et al. (1997), Valadares et al. (1999), Dias et al. (2000), Rennó et al. (2000), Leão et al. (2002), Rennó et al. (2003) and Pina et al. (2009) obtained purine N:total N percentages in the dry matter basis of the bacteria pellet of 13.7, 11.6, 17.6, 15.3, 13.4, 11.3, 11.7, 24.0, 19.5 and 10.0%, respectively.

Incomplete recovery of the absorbed purines has been described, and saliva and milk are the main non-renal pathways for PD excretion. Recovery of the purines absorbed in steers was 0.87 (Beckers and Théwis, 1994) and 0.85 (Chen and Gomes, 1992). In dry cows, Orellana Boero et al. (2001) reported a recovery of 0.84, while Vagnoni et al. (1997) found recoveries of 0.83 to 0.86. In dairy cows, Gonzalez-Ronquillo et al. (2003) reported recoveries ranging from 0.56 to 0.70. According to Chen and Ørskov (2003), the recovery of purines absorbed as urinary PD is similar among the genetic groups *Bos taurus* and *Bos indicus*. Pimpa et al. (2001) conducted duodenal infusion of purine bases in *Bos indicus* cattle and found urinary PD recovery of 0.85.

In animal tissue, a cyclic and continuous process of degradation and *de novo* synthesis of nucleic acids occurs, in which a small fraction of the purines is degraded to PD, and is later excreted in the urine. The existence of an endogenous fraction in the excreted PD was confirmed in several experiments, using different techniques, and it appeared to be variable. Values, in mmol/kg $BW^{0.75}$, ranging from 0.259 to 0.530 for dairy cows were cited by Gonzalez-Ronquillo et al. (2003), who found an average of 0.512 in cows at different stages of lactation. Orellana Boero et al. (2001) estimated lower endogenous excretion in dry cows (0.236 mmol/kg $BW^{0.75}$) when compared to those of dairy cows. In steers, Verbic et al. (1990), Fujihara et al. (1987) and Giesecke et al. (1994), cited by Orellana Boero et al. (2001), found averages of 0.365, 0.455 and 0.489 mmol/kg $BW^{0.75}$, respectively. Beckers and Théwis (1994) reported an average of 0.531 mmol/kg $BW^{0.75}$ in Belgian Blue bulls. The differences among the observations found in the literature are attributed to the use of different techniques and possible variations in the metabolism of nucleic acids in animals at different physiological stages (growth, lactation, pregnancy and maintenance).

Regarding to genetic groups, differences in the endogenous urinary excretion of PD between *Bos taurus* and *Bos indicus* have been reported. Pimpa et al. (2001), extrapolating the consumption of purine bases at zero intake, found a PD endogenous excretion of 0.147 mmol/kg $BW^{0.75}$ in Kedah-Kelantan cattle (*Bos indicus*), while Osuji et al. (1996) reported a PD endogenous excretion of 0.17 mmol/kg $BW^{0.75}$ in fasting Zebu cattle (*Bos indicus*). According to Chen and Ørskov (2003), the amount of endogenous PD for Zebu cattle should be considered lower (0.147 mmol/kg $BW^{0.75}$) than the value of 0.385 mmol/kg $BW^{0.75}$ suggested by these authors for *Bos taurus*.

To determine the endogenous contribution of the PD, the recovery percentage of absorbed purines excreted as PD in urine and the intestinal digestibility of RNA in zebu cattle, Barbosa (2009) conducted two experiments with Nelore heifers, fistulated in the rumen, abomasum and ileum. In the first experiment, the heifers (258 ± 20 kg) were fed with a single diet with four levels of dry matter offer (DMO) 1.2, 1.6, 2.0 and 2.4% of the body weight (BW). The endogenous losses and the recovery of purine bases as PD were estimated by the regression between the daily excretion of PD in the urine (Y) and the purine bases flowing in the abomasum (X), both expressed in mmol/kg $BW^{0.75}$, or by the regression between the excretion of PD (Y), in mmol/kg $BW^{0.75}$, and the DM intake (X), in g/kg $BW^{0.75}$. In the second experiment, the same Nelore heifers were used (296 ± 15 kg) and were fed the same diet as those in the first experiment at a maintenance level (1.4% of the body weight). The treatments

were composed of infusions of RNA in the abomasum at dosages of 0, 33, 66 and 100 mmol/day.

In the first experiment, the apparent digestibility of RNA in the small intestine was not affected (*P* > 0,05) by the DMO, obtaining an average value of 75.63%. The true digestibility of 92.78% was obtained by the regression between the amount of absorbed RNA ($\hat{Y}$) in function of RNA flow in the abomasum (X), expressed in mmol/kg $BW^{0.75}$. The average purine N:total N relationship of the bacteria isolated in the rumen was 0.137. PD excretion expressed in mmol/kg $BW^{0.75}$($\hat{Y}$) = 0.0196DMI + 0.242, where 0.242 mmol/kg $BW^{0.75}$ is the endogenous excretion of PD. The daily excretion of PD (mmol/kg $BW^{0.75}$), based on RNA flow in the abomasum (mmol/kg $BW^{0.75}$), adjusted to the regression $\hat{Y}$= 0.860X + 0.460, where 0.86 is the recovery of purines in the urine and 0.460 mmol/kg $BW^{0.75}$ is the fraction of endogenous PD.

In the second experiment, the daily excretion of purine derivatives ($\hat{Y}$, mmol/kg $BW^{0.75}$) based on RNA flow in the abomasum (X, mmol/kg $BW^{0.75}$) was adjusted to the regression: $\hat{Y}$ = 0.741X + 0.301, where 0.301 mmol/kg $BW^{0.75}$ is the endogenous loss and 0.74 is the recovery of the purines infused in the abomasum as PD in the urine. Dividing the value 0.74 by the true digestibility in the small intestine of 0.93, the absorbed purines recovered as PD in the urine was found to be 0.80.

Using 116 observations with 24 bulls fistulated in the rumen, abomasum and ileum in five experiments, Rennó et al. (2000) verified that the production of microbial protein obtained by PD in the urine or by the purine bases in the abomasum did not differ, and concluded that the method of urinary excretion of PD may be used to estimate the production of microbial protein (Figure 1).

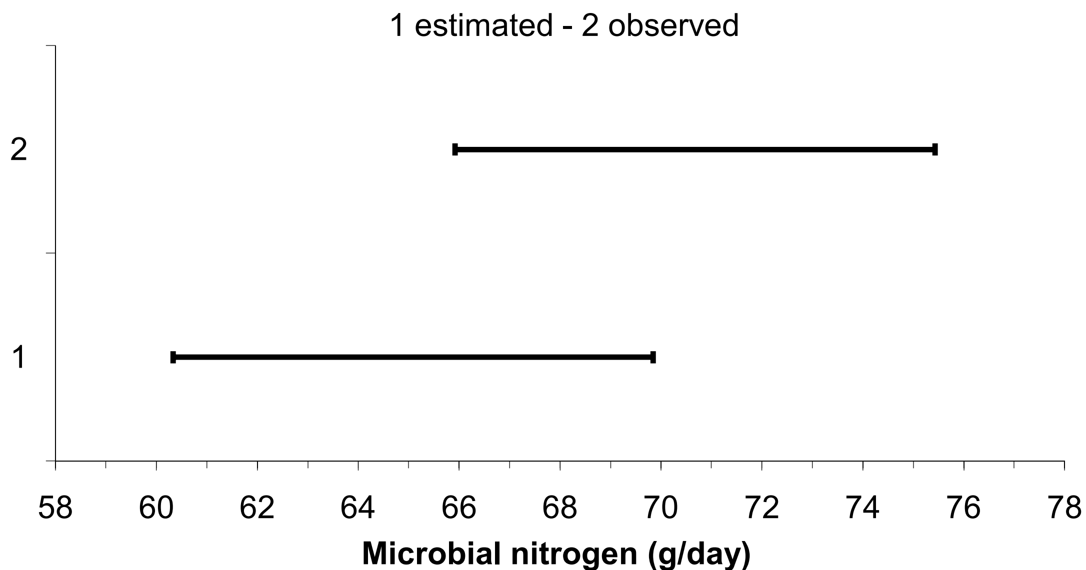

Figure 1 - Confidence interval (95%) to microbial nitrogen flow estimated using urinary PD (1) and obtained by the abomasum purine bases (2),(Rennó, 1999). The values were obtained from 106 observations provide by cattle.

The method based on excretion of PD requires total collection of urine to quantify the daily excretion of PD in urine. The urinary volume has been obtained through collections using periods of 24 to 120 hours, however, studies have been conducted to estimate the daily excretion volume of urine and PD of a single sample

collected throughout the day (spot urine collection) to enable the use of this method in field situations and facilitate collections (Valadares et al., 1999; Oliveira et al., 2001).

Creatinine (C) is formed in the muscle by the removal of water from creatine phosphate, originating from the metabolism of muscle tissue (Harper et al., 1982). The creatine phosphate molecule is degraded spontaneously in relatively constant rates forming creatinine. Creatinine is a metabolite that is no longer used by the organism to form new molecules, and is excreted by the kidneys. The daily production of creatine and, consequently, the excretion of creatinine depends on the muscle mass in proportion with the weight of the animal (Koren, 2000). Thus, since the daily excretion of creatinine based on the weight of the animal is determined, and considering that its urinary concentration is constant throughout the day, the urinary volume can be estimated using a spot urine sample collected from an animal of known weight.

Creatinine excretion is barely affected by the level of protein, non-fibrous carbohydrates or non-protein nitrogen in the diet (Susmel et al., 1994; Vagnoni et al., 1997; Valadares et al., 1997; Oliveira et al., 2001; and Rennó et al., 2003). However, variations due to the diet are not expected. Thus, if collecting a single urine daily sample, known as spot sample, and determine the creatinine concentration, the urinary volume and the excretion of other urinary compounds may be estimated, eliminating the discomfort caused by funnels or catheters used in the total collection and making it simple to obtain experimental data.

However, since creatinine is synthesized in the muscle tissue and the proportion of animal tissues varies, the daily excretion of creatinine, expressed on the body weight may be different in animals of different ages and weights. Chizzotti at al. (2006), working with Holstein steers, developed equations to estimate the daily creatinine excretion (CE) based on the body weight or metabolic body weight, where CE (mg/kg BW)= 32.2 ± 0,59 – 0.0109 ± 0.0016 × BW (R^2 = 0.70, RMSE = 1.07, n=22) and the CE (mmol/kg $BW^{0,75}$) = 0.83 ± 0.04 + 0.0029 ± 0.0004 × $BW^{0.75}$ (R^2 = 0.68, RMSE = 0.05, n=22).

Working with Nelore steers of different ages and body weights (four with an average weight of 127 kg ± 24, three weighing 221 kg ± 22 and eight weighing 434 kg ± 29), confined in individual stalls, Pereira (2009) evaluated the effect of 6 periods of total urine collection (four hours each) on the PD:C ratio, verifying that the collection time had no effect on the PD:C ratio, which was around 2.05 (Table 2). These observations indicate that the collection of a single spot urine sample, at any given time of the day may be used to determine the urinary excretion of purine derivatives. Confirming the observations described by Valadares et al. (1999), spot urine collection used to quantify the volume of urine and the production of microbial N, based on the excretion of creatinine, was similar to the total collection of the urine.

The average excretion of creatinine was 26.35 mg/kgBW or 0.95 mmol/$kgBW^{0.75}$, with confidence intervals of CI(μ)0.95= [25.56 ≤ μ ≤ 27.14] and CI(μ)0.95= [0.89 ≤ μ ≤ 1.02] for the excretion of creatinine expressed in mg/kgBW and mmol/$kgBW^{0.75}$, respectively. This value (26.35 mg/kgBW) is similar to the one found by Barbosa et al. (2006) of 27.11 mg/kgBW for Nellore heifers, being this value included in the CI obtained by Pereira (2009).

Considering the results of the experiments conducted with Nellore animals (Barbosa et al., 2006; Barbosa, 2009; Pereira, 2009), it is suggested that the estimate of ruminal microbial protein synthesis may be achieved from the collection of a single spot urine sample obtained at any given period of the day, that the absorbed purines (AP) may be estimated from the excretion of the purine derivatives (PD) in urine by the equation: AP = [PD – (0.301 mmol/kg $BW^{0.75}$)] / 0.80, and that the production of microbial nitrogen compounds (MN) may be estimated from the absorbed purines, using the equation: MN = (70 × AP) / (0.93 × 1000 × 0.137), where 70 is the amount of

N in the purines (mg N/mmol), 0.93 is the true digestibility of the purines, and 0,137 is the average purine N: total N ratio in the bacteria isolated in the rumen (Barbosa, 2009).

Table 2 - Variation in the urinary compounds excretion obtained in Nellore heifers

Item	Collection Period (hours)						*P*-Value	SEM
	24-4	4-8	8-12	12-16	16-20	20-24		
Creatinine								
g	1.29	1.23	1.31	1.27	1.24	1.22	0.1430	0.0403
mg/BW	4.30	4.12	4.35	4.28	4.14	4.10	0.1802	0.0939
mmol/$BW^{0.75}$	0.155	0.148	0.157	0.154	0.149	0.147	0.1469	0.0035
Purine Derivatives								
g	2.95	3.07	3.29	3.12	3.26	3.11	0.1242	0.1008
mg/BW	11.14	11.50	12.49	11.63	12.02	11.55	0.1257	0.5008
mmol/$BW^{0.75}$	0.27	0.28	0.31	0.29	0.30	0.29	0.1102	0.0101
mmol/day	118.98	119.97	132.92	133.76	126.17	119.82	0.4329	7.5715
Relationship								
PD:C[1]	1.99	2.01	2.21	2.15	1.99	1.97	0.5443	0.1403

Adapted from Pereira (2009).
[1] – Ratios calculated from the concentration in mmol/L.
SEM = standard error of the mean.

MICROBIAL EFFICIENCY

To maximize efficiency of animal production and reduce the need for true protein in the diet, increases in production and the passage of ruminal microbial protein to the gastrointestinal tract are required. The output of microbial organic matter from the rumen is a function of the amount of organic matter digested in the rumen (production of ATP) and the efficiency with which the ruminal microorganisms use the available energy for growth (Owens and Goetsch, 1993). According to Russell (1984), ruminal microorganisms derive most of their energy from the fermentation of carbohydrates, and ruminal bacteria may be divided into two groups according with the type of carbohydrate used: the cellulose and hemicellulose fermenting microorganisms (BFFC) (fibrous carbohydrates) grow slower and use ammonia to synthesize protein, while the pectin, starch and sugar fermenting microorganisms (BFNFC) (non-fibrous carbohydrates) grow faster when compared to the BFFC and use ammonia, peptides and amino acids for protein synthesis (Russell et al., 1992).

The microbial growth efficiency (MGE) may be expressed in terms of DM of microbial cells produced per mole of fermented substrate (Y_{SUB}) or per mole of available ATP (Y_{ATP}). The total microbial production in the rumen generally increases with the rise of the amount of organic matter fermented in the rumen, whereas the MGE does not depend on the microbial production, and these two terms should not be confused (Owens and Goetsch, 1993).

Microbial production is usually measured in batch or continuous culture systems, which are most similar to the ruminal conditions. At steady state conditions of continuous systems, the bacteria have a specific growth rate (replication rate minus the lysis rate) equal to the rate of passage. In this system, the bacteria grow until they reach the limit of nutrient and energy availability, and the energy (ATP) is usually the limiting factor.

The MGE varies according to the ionic composition of the medium, the composition of the cell and the rate of microbial growth. The average composition of ruminal bacteria is approximately 62.5% CP, 21.1% carbohydrates, 12% fat and 4.4% ash based on DM (Russell et al., 1992). Similar to animals, the bacteria require

energy for maintenance and are more efficient when they grow or multiply more rapidly. The maintenance costs include the nutrients and the energy used for mobility, the turnover of cell constituents, active transport, etc. Bacterial species differ in the maintenance cost, and the BFFC and BFNFC require 0.050 and 0.150 g of carbohydrate per gram of bacteria per hour, respectively (Russell et al., 1992).

Two different bases have been used to calculate the MGE, which may be expressed in relation to the true organic matter or organic matter apparently digested in the rumen. The disappearance of feed in the rumen differs from the quantity of feed truly digested in the rumen, since between 24 and 50% of the digesta weight is incorporated in microbial cells. The true fermentation exceeds apparent fermentation by the amount of OM incorporated inside the microbial cells, and the truly fermented OM is calculated by adding microbial OM to the OM disappearing from the rumen by digestion, which increases the fermented OM and decreases the MGE.

The expression of MGE in terms of grams of microbial nitrogen per energy unit available in the rumen is justified because energy is the most limiting factor related to microbial growth. Thus, by maximizing the amount of microbial CP synthesized per unit of fermented energy, maximum microbial growth is achieved. However, according to Bach et al. (2005), although the MGE measured in relation to the availability of energy is an efficient measurement of the use of energy by the microorganisms (Figure 2), it is not a good indicative of the efficiency of the use of available nitrogen (Figure 3).

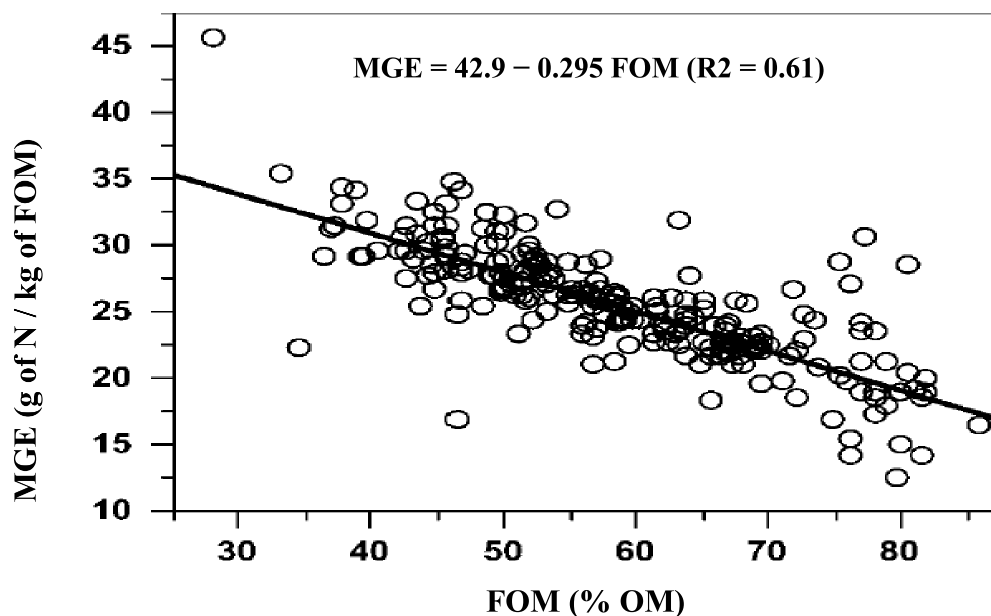

Figure 2 - Relationship between the organic matter fermented (FOM) in the rumen and the microbial efficiency. Adapted from Bach et al. (2005).

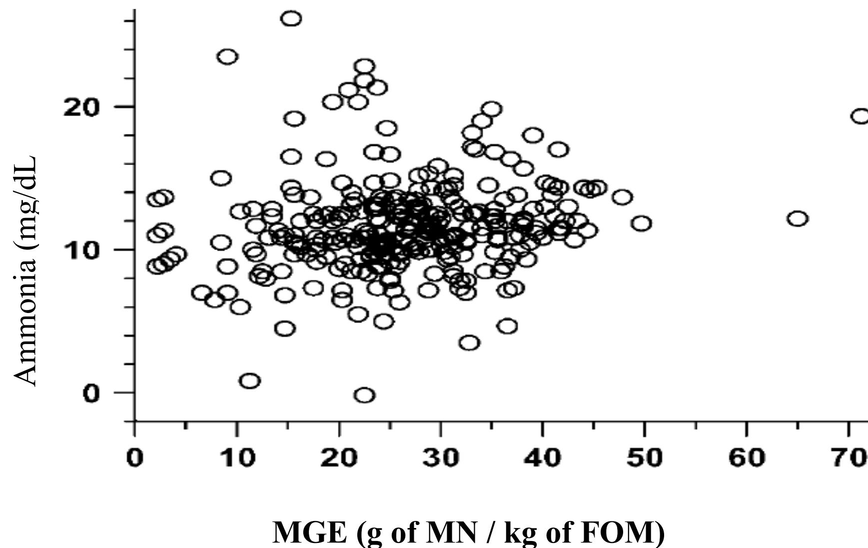

Figure 3 - Relationship between the microbial efficiency and the ruminal concentration of ammonia. Adapted from Bach et al. (2005).

The lack of a correlation between the MGE estimated through the available energy and the ruminal concentration of ammonia, indicates the inability of using ammonia as a factor to predict the efficiency with which the microorganisms capture the N available in the rumen. Therefore, nitrogen use efficiency (NEf) by the microorganisms should be calculated as NEf = (g of MN / g of available N) × 100. Bach et al. (2005) found a correlation between the NEf and the ruminal concentration of ammonia, described by the equation: NH_3 (mg / dL) = 43.6 – 0.469 NEf (R^2 = 0.78). Thus, the MGE and the NEf appear to be complementary measures of nutrient use efficiency by the microorganisms.

Bach et al. (2005) reported a quadratic relationship between the MGE and the NEf ($\hat{Y}_{NEf}$ = 15.31 + 3.724* MGE – 0.0643* MGE^2), and the optimum for MGE of 29 g of MN / kg of FOM occurred with a NEf of 69 g of MN / 100 g of available nitrogen. This implies that the microorganisms captured 69% of the available nitrogen and synthesized 29 g of MN / kg of FOM, under ideal growth conditions. Therefore, ensuring the supply of 42 (29/0.69) g of nitrogen compounds available in the rumen per kg of true organic matter seems to be a reasonable goal.

FACTORS AFFECTING THE MICROBIAL EFFICIENCY

The literature regarding the synthesis of microbial protein is often contradictory, since complex factors are involved and there are many difficulties in measuring it properly (Dewhurst et al., 2000a). Among the factors that affect the synthesis of microbial protein, the availability and synchronization between energy and nitrogen compounds (N) in the rumen has been recognized as the most important factor (Russell et al., 1992). Considering that proteins are rapidly and extensively degraded in the rumen, it is possible that the rate at which the energy is made available represents the most limiting factor regarding the synthesis of microbial protein, since fibrous carbohydrates have a low digestion rate. Therefore, the supply of moderate amounts of non-fibrous carbohydrates usually increases the flow of microbial N to the abomasum, as long as there is no limitation of the availability of N in the rumen. According to Stern et al. (1994), the main compounds needed for ruminal microbial

growth are crude protein and carbohydrates, which may be fermented to provide ammonia nitrogen, amino acids, carbon skeletons and energy in the form of ATP, for the microbial synthesis.

Energy sources

The supply of energy is usually the first factor limiting ruminal microbial growth. Therefore, the majority of nutritional systems use direct or indirect estimates of the energy supply for animals to predict microbial synthesis. As a result, the production of microbial protein may be estimated based on the metabolizable energy (ME), the net energy of lactation (NE_L), the digestible carbohydrates (DC), the fermented organic matter (FOM), or on the total digestible nutrients (TDN), (INRA 1988; AFRC, 1992; Tamminga et al., 1994; Madsen et al. 1995; and NRC, 2001). According to the NRC (1985), the production of microbial crude protein (MCP) is related to the energy fermented in the rumen, commonly expressed as a function of the organic matter apparently digested in the rumen (RADOM). In all of these systems, the synthesis of MCP per unit of energy is considered fixed, although in some systems it is already considered that not all nutrients available for animals may be used by the ruminal microorganisms.

It has been demonstrated that in diets containing high levels of concentrates, the efficiency of ruminal microbial synthesis is lower than in diets containing an adequate forage:concentrate ratio (ARC, 1984). However, according to Clark et al. (1992), in experiments where a variation of 24 to 81% of forage in the diet was observed, there was no significant alteration in the flow of non-ammonia nitrogen (NAN) and amino acids to the small intestine of dairy cows, which prevented establishment of an adequate concentrate:forage relationship.

According to Clark et al. (1992), the organic matter intake (OMI) is an important factor that contributes to the determination of the quantity of microbial N passing to the small intestine (MN (g/day) = 14.69 OMI + 21.94, r^2 = 0.62), where the organic matter truly degraded in the rumen (RTDOM), (MN (g/day) = 79.29 RTDOM – 3.38 $RTDOM^2$ – 152.89, R^2 = 0.49) is a more accurate predictor when compared to the organic matter apparently degraded in the rumen (MN (g/day) = 121.09 RADOM – 8.87 $RADOM^2$ – 131.92, R^2 = 0.25).

However, the organic matter intake was more correlated to the passage of microbial N to the small intestine than the RTDOM and the RADOM, suggesting that factors other than the RADOM contribute to the amount of MCP synthesized in the rumen. The low coefficient of determination obtained for the relationship between the RTDOM and the MGE, expressed in g of microbial N / kg of RTDOM (MN = 6.58 RTDOM - 0.41 $RTDOM^2$ + 7.30, R^2 = 0.16), indicates that other factors affect the MGE, and these factors are possibly the proportion of other nutrients provided in the diet, synchronizing degradation of the diet ingredients making nutrients available at all times to supply the microbial requirements and the ruminal environment conditions (Clark et al., 1992).

The fermentation of the various components of NFC, such as starch, sugars, or soluble fiber, differs in the characteristics and the profile of the organic acids produced. Differences between the carbohydrates purified in the production of microbial cells in mixed cultures have been reported (Strobel and Russell, 1986), but the differences between the NFC in the production of microbial mass or MCP in mixed microorganism cultures generally have not been considered (Hall and Herejk, 2001).

By evaluating the effect of different fractions (pectin, sucrose and starch) of the NFC and the NDF on the production of MCP in *in vitro* fermentation systems, with the pH maintained above 6.49 in the fermentation tubes, Hall and Herejk (2001) observed

increased production for the starch, pectin, sucrose and NDF, respectively. It was also observed that the maximum MCP production was achieved 15.6, 13.5, 12.6 and 19.3 hours after the start of fermentation for starch, pectin, sucrose and NDF, respectively.

An interesting aspect of the use of sugars in the diet of ruminants is related to its effect on nitrogen metabolism and microbial growth. A reduction in the concentration of ruminal ammonia has been observed in almost all studies in which sugar was added to the diet. This reduction suggests an increase in the microbial growth and in the efficiency of use of protein compounds of fast ruminal degradation.

Chamberlain et al. (1993) demonstrated that the soluble sugars (sucrose, lactose and fructose) are a better energy source than starch for microbial nitrogen utilization in the rumen. These observations suggest the existence of an optimal relationship between the available sugars and the soluble nitrogen. Hoover and Miller-Webster (1998) reported an average increase of 25% in the microbial growth, when the protein / soluble sugar relationship increased from 1:1 to 2 or 3:1.

Since sugars usually represent less than 10% of the total NFC, starch becomes the main source of carbohydrates for microbial growth (Hoover and Miller-Webster, 1998). The fermentation rate of all carbohydrates determines their destination in the digestive tract and the efficiency with which the microorganisms may use them (Van Soest et al., 1991). Knowledge about the variation of the effective degradability (DE) of the many sources of starch, which may be used as ingredients, to synchronize the availability of energy and protein to maximize the ruminal fermentation, is an interesting strategy in the formulation of diets for ruminants.

The variability in DE may be caused by several factors, such as the proportion between molecules constituents of starch (amylase and amylopectin), the structure (presence or absence of pericarp), presence, absence, or proportion of endosperms (aleurone, peripheral, corneum, vitreous and farinaceous), and processes such as grinding,crushing, flaking and pelleting.

According to Dewhurst et al. (2000a), the inclusion of starch in ruminant diets may affect ruminal microorganisms in many ways, making it difficult to predict the final result. However, a moderate inclusion benefits the ruminal microorganisms through the increase in the substrate and in the growth rate of the bacteria associated with the liquid phase.

Hristov et al. (2005), when evaluating the effect of dextrose, starch, NDF and a mixture of carbohydrates on the microbial N flow to the duodenum, observed that this was depressed by the NDF when compared to other sources of carbohydrates, and the flow of microbial N formed from ammonia was higher for corn starch when compared to other sources of carbohydrates. The concentration and size of the NH_3 pool were also reduced by the ruminal infusion of dextrose and cornstarch. The proportion of bacterial N synthesized from the ruminal NH_3 was higher for the diet containing cornstarch and, consequently, the loss and efflux were lower, demonstrating that the supply of readily fermentable carbohydrates may decrease the concentration of ruminal ammonia by decreasing the production of ammonia or by increasing the retention of ammonia as microbial matter.

When reviewing some experiments on the effect of starch sources on the ruminal microbial metabolism, Clark et al. (1992) suggested that, proportionally, a higher amount of MCP and lower quantity of non-ammonia and non-microbial nitrogen passed into the small intestine when the fermentation of OM was increased in the rumen. Thus, the use of sources of readily degraded carbohydrates, such as barley, in the feeding of dairy cows was associated with an increase in the synthesis of ruminal microbial protein, which is possibly due to a greater availability of energy. Corn-based diets increase the amount of non- ammonia and non-microbial compounds moving to

the small intestine, resulting in small changes in the passage of non-ammonia nitrogen, when different grains were supplied in the diet of dairy cows.

Ruminal pH

The pH value is a factor that may alter the production of ruminal MCP. Low pH values may be harmful to the microorganisms and are also related to a reduction in the digestibility of the fibrous compounds in plants (Verbic, 2002). According to Strobel and Russell (1986), low values of ruminal pH lead to the diversion of available energy for microbial growth to the maintenance of the internal pH of microorganisms, thereby reducing energy use efficiency in microbial synthesis

A generalization is that pH values lower than 6 inhibit the degradation of cellulose. Under normal conditions, the cellulolytic microorganisms grow well at a 6.7 pH and substantial deviations from this value are inhibitory. A pH variation where the activity remains next to normal would be of 0.5 units. Values of pH lower than 6.2 inhibit the digestion rate and increase the lag time for the degradation of the cell wall (Van Soest, 1994).

The rumen is well buffered by salivary secretion; however, if the amount of dietary NDF is restricted and the rate of carbohydrate fermentation is fast, the pH may decline. Some studies indicate that when diets contain less than 40% of forage (20% NDF), a low microbial growth is observed. A mixture of ruminal bacteria that was incubated *in vitro* produced 50% less microbial protein at a pH of 5.7 when compared to pH 6.7 (Strobel and Russell, 1986). The CNCPS adjusts the production of microbial protein using the pH, which is predicted from the content of dietary NDF. When the content of NDF in the diet is equal to or lower than 20% of the dry matter, the production of microbial protein decreases by 2.5% for each 1% of decrease in the NDF (Russell et al., 1992).

Although pH has a pronounced effect on the synthesis of microbial protein, it is not correlated with the efficiency of microbial synthesis. The negative correlation between the ruminal flow of microbial N and the pH is the result of a higher availability of OM degraded in the rumen, implying in a greater availability of energy and, consequently, of microbial growth (Bach et al., 2005).

During the adaptation to diets containing high levels of concentrate, the pH exerts a selective pressure on sensitive microorganisms. When the pH drops, the amylolytic bacteria resistant to acidity increase, while the cellulolytic microorganisms decrease. Therefore, the relative activity of amylase in relation to cellulase increases. It has been suggested that the optimum pH for ruminal amylase is around 5.6 (Kaufmann et al.1980, cited by Owens and Goetsch, 1993).

The inhibition of the fiber digestion may become a problem in the adaptation of animals to a concentrate diet due to its possible accumulation in the rumen. Little fiber is present in diets with high levels of concentrate and, since the forage is often processed, the rate of passage may be increased unless the selective ruminal retention extends the residence time of ruminal fiber digestion in this compartment. Depending on this balance, even if the rate of fiber digestion may be depressed, the extent of its ruminal digestion may not be substantially reduced until the pH drops and remains lower than 6.0.

When the pH is lower than 6 (between 5.0 and 5.5) many ruminal microorganisms stop growing, although they can survive in high concentrations of H^+. The cellular membrane of these microorganisms does not allow for the entrance of H^+ or OH^-, however some other nutrients, such as lactic acid, enter and alter the proton-motive force, which is responsible of the generation of ATP from the ion gradient between membranes. When the pH is altered, the concentration of ions and cellular

permeability may change, reducing the potential to generate ATP. Certain lipophilic acids, such as lactic acid, act as proton conductors, balancing the internal and external H^+ concentrations and reducing the proton-motive force (Bergen and Bates, 1984). The microbial growth per unit of fermented energy is lower when the pH is low, possibly due to a reduction in the generation of ATP through the proton-motive force. In addition, most of the acids that are similar to lactic acid inhibit microorganisms at high concentrations, which is very effective when the pH is low; this is probably due to easier penetration in the cell membrane of a non-ionized form of these acids (Owens and Goetsch, 1993).

Sources of N

Ruminal degradation of protein is one of the main reasons for its inefficient use by ruminants. Moreover, the nitrogen compounds that are released in the rumen during protein degradation are essential for ruminal microbial growth (Verbic, 2002). The ruminal protein degradation in feeds is an important factor that affects the uptake of amino acids to the small intestine. The speed and the amount of protein degraded in the rumen may condition the amount of MCP synthesized in the rumen and determine the total amount of undegraded protein in the rumen that reaches the duodenum (Stern et al., 1994).

The requirements of ruminal microorganisms for nitrogen compounds are met by the rumen degraded protein (RDP) and by the endogenous metabolic nitrogen derived from the oxidation of amino acids in tissues and organs, which is recycled to the rumen through blood or saliva. Some nutritional systems (INRA, 1988; AFRC, 1992 and NRC, 2001) propose that the capture of rumen-degraded protein is not complete, therefore an excess of RDP is needed.

According to Hoover and Stokes (1991), there is a strong correlation between the level of RDP in the diet and the synthesis of MCP. Maximum efficiency and a greater contribution of MCP to the duodenum is obtained in diets containing from 10 to 13% RDP in the DM for dairy cows. However, these levels may be altered according to factors such as the production level, physiological stage and animal category. Therefore, these levels should not be taken as definitive.

The amount of crude protein ingested by cows significantly influences the passage of non-ammonia nitrogen to the small intestine. However, differences in the passage of MCP to the small intestine were not significantly influenced by the level of CP in the diet when it varied from 11 to 25%. According to Clark et al. (1992), the passage of MCP to the duodenum was more influenced by the intake of DM and other dietary factors than by the level of CP.

The effect of the level of dietary CP was evaluated by Stern et al. (1986), who fed cows with increasing amounts of corn gluten in order to alter the level of CP in the diet (13.1 to 22.9% DM) without altering the organic matter truly degraded in the rumen (RTDOM). They also observed that there was an increase in the concentration of ammonia in the ruminal fluid (9.6 to 14.4 mg/dL); however, the RTDOM and the flow of amino acids derived from the ruminal microorganisms were not altered, suggesting that the availability of energy limited the microbial synthesis more than the concentration of ruminal NH_3.

Another important consideration is that when the availability of nitrogen increases in relation of the RTDOM, the efficiency of the microbial synthesis decreases, that is, if the availability of ruminal N is relatively high in relation to the RADOM, the amount of microbial N synthesized per unit of RADOM decreases. This indicates that the use of energy to synthesize MCP becomes less efficient because the excess N is not used by the ruminal microorganisms.

An important factor influencing the synthesis and flow of MCP to the duodenum is the source of dietary nitrogen. Santos et al. (1998), when reviewing studies where sources of RUP substituted soybean meal, concluded that the increase of dietary RUP did not improve the performance of dairy cows, suggesting that the inclusion of RUP sources may have resulted in a reduction of the ruminal microbial synthesis. Ipharraguerre and Clark (2005), evaluating the effect of different sources of dietary protein on the flow of MCP to the duodenum of cows, observed a reduction of 7% in the flow of MCP when the soybean meal was replaced by sources of RUP, supporting the findings of Hoover and Stokes (1991), Clark et al. (1992) and Firkins (1996), who showed that the deficiency of energy, amino acids, peptides or ammonia in the rumen can decrease ruminal microbial growth when sources of RDP are replaced by RUP.

Due to the capacity of ruminal microorganisms to efficiently use the sources of non-protein nitrogen as a substrate for growth, these sources become an important factor in the formulation of diets. Russell et al. (1992) indicated that the microorganisms that ferment fibrous carbohydrates require only ammonia as a source of N, while species degrading NFC require pre-formulated amino acids.

Wallace (1996) observed that the proportion of nitrogen derived from ammonia varies according to the available source of N, indicating that the minimum contribution of microbial N from NH_3 was 26%, when high concentrations of peptides and amino acids were present; with a maximum potential of 100% when NH_3 was the only source of N. Griswold et al. (1996) demonstrated that forms of N other from NH_3 are necessary not only to maximize microbial growth, but also for better ruminal digestion of fiber.

Rate of passage

The rate of passage is another factor affecting the efficiency of ruminal microbial synthesis. Under conditions where the rate of passage is high, a reduction in the costs of maintenance is expected due to a reduction in the ruminal residence time. From a theoretical point of view, it is expected that maximum microbial growth may occur when the dilution rate is the same as the microbial replication rate (Ørskov, 1992). This theory has been confirmed experimentally (Harrison et al., 1975; Kennedy and Milligan, 1978; Dewhurst and Webster, 1992 and Murphy et al., 1994).

The AFRC (1992) proposes that the efficiency of microbial synthesis is increased by approximately 20% when the rate of ruminal passage increases from 0.02 to 0.08 h^{-1}. The rate of passage is a function of the dry matter intake. Thus, we can assume that the efficiency of ruminal microbial synthesis may be increased by the dry matter intake (Verbic, 2002).

The rate of passage also influences the standards of ruminal fermentation and, consequently, microbial synthesis. Fast rates of passage of ruminal fluid are usually associated with high concentrations of acetate. The duration and intensity of rumination are greatly determined by the level and form of the fiber intake. When ruminants increase the production of saliva, they also increase the buffering and dilution of the rumen contents. The dilution causes the ruminal acid concentration to be lower in diets based on forages when compared to diets based on concentrate (50 to 100 versus 80 to 150 mmol/L).

Microbial growth and efficiency improve in high rates of passage. It is known that the rate of passage depends on the feed intake (Evans, 1981), and the improvement of growth and microbial efficiency is due to a reduction in the maintenance requirements of the microorganisms (Meng et al., 1999). Therefore, ensuring an adequate intake of dry matter is a way of increasing the production of MCP and reducing the need of RUP in the diets (Evans, 2003).

PREDICTION OF MICROBIAL PROTEIN FLOW

The most recent nutrition models for ruminants have recognized the biological need for a balance between fermented carbohydrates and RDP to satisfy the requirements of microorganisms for pre-formulated amino acids and ammonia. These models may vary from empirical (better statistical adjustment) to mechanistic (attempt to quantitatively describe biological processes), and are used for the prediction of events from which the feeding recommendations are based (Firkins, 2002).

Mathematical equations that describe the relationship between the access of feed and the synthesis of MCP are needed to quantify and optimize microbial synthesis. Several equations have been successfully formulated and applied to predict microbial synthesis in systems evaluating feed proteins and in large mechanistic models of ruminal fermentation process, such as the CNCPS (Dijkstra et al., 1998).

In the current systems of protein evaluation for ruminants, the production of MCP is predicted mostly through empirical equations. Although the terminology and details are different in these systems, they are conceptually similar in their objectives to predict the amount of microbial N available for the metabolism of the host animal (Beever and Cottrill, 1994). The synthesis of MCP is calculated from the amount of energy or degraded organic matter, applying fixed or variable factors to the microbial production per unit of energy or degraded organic matter. None of these systems explicitly consider the effect of the interactions between microorganisms in the rumen and the variables of microbial activity in the OM degradation (Dijkstra et al., 1998).

The synthesis of MCP has been estimated from the supply of energy to the microorganisms through the intakje of TDN, net energy, organic matter, or carbohydrates fermented in the rumen. In the NRC (1989), the supply of microbial protein was predicted from the net energy intake used for lactation (NE_l) through the equation: $6.25(30.93 + 11.45\ NE_l)$. In recent publications from this committee (NRC, 2000 and 2001) a fixed microbial efficiency factor of 13% was used in relation to the TDN, when the supply of degraded protein in the rumen is not limiting. Therefore, the MCP (g/day) in these publications is estimated by TDN intake (kg/day) × 130, and of this amount, approximately 64% is metabolizable (80% of true microbial protein and 80% of intestinal microbial protein digestibility).

Such mechanistic calculations ignore the digestion site (rumen x intestine), which influences the availability of energy for ruminal microbial growth. However, this variable, resulting from the digestion site appears to have a relatively low impact on the prediction of energy for microbial growth when compared to the dry matter intake (Firkins, 2002). The use of empirical methods to predict microbial synthesis may be a way to summarize the research data, and an important tool used to formulate diets (Dijkstra et al. 1998).

In mechanistic models such as the CNCPS, the prediction is more complex. Microorganisms in the rumen are classified according to the types of carbohydrates that they ferment. The microorganisms fermenting fibrous and non-fibrous carbohydrates have different maintenance requirements (CNCPS uses 0.05 and 0.15 g of carbohydrate per g of microorganism per hour, respectively), and the growth efficiency of bacteria that digest the NFC is optimized in the presence of 14% peptides as a percentage of NFC. Therefore, the requirement of degradable protein is considered to meet the use of non-fibrous and fibrous carbohydrates. The rate of microbial growth of each category is directly proportional to the digestion rate of carbohydrates, provided that an adequate source of nitrogen is available. The extent of digestion in the rumen depends on the digestion of the carbohydrate fractions and the rate of passage in the rumen. Thus, the extent of digestion depends on factors

such as DM intake, particle size, hydration rate, lignification, as well as the characteristics of each fraction of carbohydrate and protein.

In Brazil, complete information obtained from 42 researches (including master's dissertations and doctoral theses) carried out at the DZO (Department of Animal Science) at UFV (Universidade Federal de Viçosa), in which animals were used for the production of meat and milk and submitted to different feeding conditions, were summarized and their values of synthesis and efficiency in the use of TDN for the production of MCP are summarized in Table 3.

From these data meta-analysis was performed (St-Pierre, 2001), which has been the most appropriate procedure to evaluate the data from the various studies aiming to build quantitative models. These models may explain the effect of one or more independent variables on the dependent variable of interest. Since there are usually differences between studies, and if these differences are not considered during the data analysis, they may cause biased estimates for the parameters. Therefore, during the analysis procedure, the effect of the experiment and its interaction with the independent variables was considered as a random component in a mixed linear model (St-Pierre, 2001), and the solution for the model was determined with PROC MIXED SAS (version 9.1, SAS Institute Inc, Cary, NC).

In Table 3, the influence of the animal category ($P = 0.0016$) and the TDN intake ($P < 0.0001$) can be observed on the microbial efficiency (g of MCP / kg of TDN). However, no effect was observed for the interaction between the animal category and the TDN intake ($P = 0.6949$); therefore, it may be concluded that the inclination of the linear model obtained by the relationship between the synthesis of MCP (g/day) and the TDN intake (kg/day), which represents the efficiency of the synthesis of MCP (g of MCP / kg of TDN) does not change in relation to the animal category.

The average value obtained for the microbial crude protein efficiency was 115.58 g of MCP / kg of TDN. From the confidence interval ($\alpha = 95\%$) established for the parameter β_1 of the analyzed model (115.58 ± 6.26), we may conclude that it does not differ from the efficiency proposed previously. Therefore, we recommend the use of the same value of 120g MCP / kg of TDN obtained in the previous publication, as a reference to tropical conditions.

Table 3 - Descriptive statistics for the values related to the efficiency of the synthesis of microbial crude protein (g of MCP / kg of TDN), obtained in experiments conducted at the DZO / UFV

Variables	Category	N	Average (μ)	Minimum	Maximum	PD	SE	CI
DMI (kg/day)	Dairy	316	17.31	8.28	27.07	3.40	0.19	μ ±0.38
	Beef	585	5.31	1.58	12.71	1.88	0.078	μ ±0.16
	All	901	9.52	1.58	27.07	6.26	0.21	μ ±0.41
TDNI (kg/day)	Dairy	412	11.56	5.50	16.84	2.19	0.11	μ ±0.21
	Beef	585	3.45	9.02	0.905	1.43	0.059	μ ±0.12
	All	997	6.80	0.905	16.84	4.38	0.14	μ ±0.27
MCP (g/day)	Dairy	412	1426.25	364.73	3007.94	468.34	23.07	μ ±45.36
	Beef	585	394.99	51.13	1173.03	178.70	7.39	μ ±14.51
	All	997	821.15	51.13	3007.94	606.11	19.20	μ ±37.67
MGE (g/kg TDN)	Dairy	412	125.16	29.71	274.01	39.07	1.92	μ ±3.78
	Beef	585	117.02	30.73	273.63	35.97	1.49	μ ±2.92
	All	997	120.38	29.71	274.01	37.48	1.19	μ ±2.33

Meta-analysis

Independent Variable	F	*P* - Value
TDN intake	44.31	<0.0001
Category	9.97	0.0016
TDN intake x Category	0.15	0.6949
Equation (MCP g/day = β_1*TDNI kg/day)		
Variable	β_1 (± EP)	*P* - Value
TDNI	115.58 (± 3.1939)	<0.0001

MGE = microbial growth efficiency; TDNI = TDN intake; PD = purine derivatives; SE = standart error, CI = confidence interval.

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