

## Metabolism and utilisation of non-protein nitrogen compounds in ruminants: a review

### Metabolizam i iskorištenje neproteinskih dušičnih spojeva u preživača: pregled

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#### ABSTRACT

Unique stomach physiology allows ruminants to utilise any form of dietary nitrogen (N) as a protein source. Ruminant N requirements can be divided into N requirements for microbial fermentation and host requirements for amino acids. Ruminant diets often contain non-protein nitrogen (NPN) compounds, a group of components that are not proteins but can be converted to proteins by rumen microbes. Therefore, NPN compounds in ruminant diets serve as alternative sources of ammoniacal nitrogen (NH<sub>3</sub>-N), which is necessary for the synthesis of microbial protein (MCP) in the rumen. MCP is an important component of metabolizable protein for ruminants and, under certain production conditions, may be the only amino acid source for synthesis of tissues and products. In this manner, ruminants utilise NPN compounds without having enzymes for their degradation. The use of NPN compounds in ruminant nutrition allows for an increase in feed consumption and utilisation, as well as the substitution of protein supplements, which in turn leads to more cost-effective production. However, abrupt supplementation or excessive consumption of NPN could negatively affect MCP synthesis, feed digestion, and animal production. On the other hand, it may lead to animal poisoning and the release of large amounts of N into the environment. Knowledge of N metabolism in the rumen, as well as optimization of its utilisation, sets the conditions for the presumed beneficial effects of NPN compounds in ruminant diets, without adverse effects on animal health and the environment. Therefore, the aim of this study was to summarize the recent literature data on the metabolism and utilisation of NPN compounds in ruminants.

**Keywords:** non-protein nitrogen, ruminant animal, nitrogen, metabolism, microbial protein

#### SAŽETAK

Jedinstvena fiziologija želuca omogućuje preživačima korištenje svih oblika dušika (N) iz hrane kao izvora proteina. Potrebe za N kod preživača mogu se podijeliti na potrebe za N za mikrobnu fermentaciju i potrebe domaćina za aminokiselinama. Obroci preživača često sadrže neproteinske spojeve dušika (NPN), skupinu komponenata koje nisu proteini, ali ih mikroorganizmi buraga mogu iskoristiti za sintezu proteina. Stoga, NPN spojevi prisutni u obrocima preživača predstavljaju alternativne izvore amonijskog dušika, potrebnog za sintezu mikrobnog proteina (MP) u buragu. MP važan je dio metaboličkog proteina za preživače koji, u određenim proizvodnim uvjetima, može predstavljati jedini izvor aminokiselina potrebnih za sintezu tkiva i proizvoda. Na taj način preživači koriste NPN spojeve bez posjedovanja enzima za njihovu razgradnju. Upotreba NPN-a u hranidbi preživača omogućuje povećanje konzumacije i konverzije

hrane, kao i supstituciju biljnih proteina, što zauzvrat dovodi do pojeftinjenja proizvodnje. Međutim, naglo uvođenje u obroke ili prekomjerna konzumacija NPN-a može depresivno utjecati na sintezu mikrobnog proteina, probavljivost i proizvodnju. S druge strane, može dovesti do trovanja životinja i izlučivanja velikih količina N u okolinu. Poznavanjem metabolizma N u buragu, kao i optimizacijom njegova iskorištenja, stvaraju se uvjeti za navedene pozitivne utjecaje upotrebe NPN-a u obrocima preživača, bez štetnih učinaka na zdravlje životinja i okolinu. Stoga je cilj ovog istraživanja bio sažeti recentne literaturne podatke o metabolizmu i korištenju NPN spojeva u preživača.

**Ključne riječi:** neproteinski dušik, preživači, dušik, metabolizam, mikrobnii protein

## INTRODUCTION

Ruminants are herbivores characterized by the presence of a voluminous fermentation chamber represented by the rumen and by a wide population of microorganisms adapted to utilize compounds from the feed that have low digestibility or are indigestible to endogenous enzymes. Unlike monogastric animals, in which most digestion occurs in the small intestine through the action of enzymes from the liver and pancreas, intensive microbiological fermentation of substrates in ruminoreticular compartments occurs in ruminants before the feed is passed to the posterior compartments of the digestive system (Bertan Membrive, 2016). Ruminants have established a symbiotic relationship with rumen microorganisms in which the animal provides nutrients and optimal environmental conditions for feed fermentation and the microorganisms break down fibre and synthesize microbial protein (MCP) as a source of energy and protein for the animal (Uddin et al., 2015)

Nitrogen (N) is a component of essential nutrients for ruminant productivity. It has long been known that adequate rumen ammonia levels are required for MCP synthesis and that N deficiency results in decreased protein synthesis, fibre digestion, and feed intake (McDonald et al., 2011; Schwab et al., 2017). An important component of N metabolism in ruminants is the ability of the rumen micropopulation to utilize ammonia for amino acid synthesis when an adequate energy supply is available. Nearly 80% of rumen bacteria use ammonia as their sole N source for growth, 26% of bacteria require only ammonia, while 55% can use ammonia or amino acids. Protozoa cannot utilize ammonia but meet their N requirements by consuming bacteria and particulate matter (Joysowal et al., 2019). Subsequently, MCP synthesized in the rumen

is digested in the small intestine and serves as a source of amino acids for ruminants. Depending on the level of production and efficiency of synthesis, MCP provides between 40% and 98% of the daily protein requirement of ruminants (Stern et al., 1994).

Because of their unique ability to convert N into high-quality animal proteins, various non-protein nitrogen (NPN) compounds are added to ruminant diets as a source of ammonia for rumen microorganisms. NPN stands for a group of compounds that are not proteins but contain N in their structure. These include urea, ammonium salts, nitrates, alkaloids, asparagine, purine, choline, uric acid, amines, amides, amino acids, and nucleic acids. The nutritional value of NPN compounds is based on N utilization by rumen microorganisms, which break it down to ammonia and then bind ammonia to carbon chains for amino acid synthesis through further metabolic processes (Kalivoda, 1990). Their usage emphasizes the economic and nutritional aspects. For example, about 37% of terrestrial areas are covered with pastures that are partially or totally unsuitable for crop production (Loveland et al., 2000). However, ruminants can be fed forages, and desired production levels could be achieved by supplementation with appropriate feeds and additives (de Carvalho et al., 2019; O'Mara, 2012). On the other hand, protein-rich feeds (e.g., legumes) are not grown in all areas grazed by ruminants, while tropical grasses have poor protein quality. Protein supplements and other alternative sources are often expensive or unavailable, while forages and concentrates with inconsistent composition and poorer nutrient quality do not always meet the nutritional needs of ruminants (Tadele and Amha, 2015). Commercial NPN sources can replace up to 30% of dietary protein, with the same efficiency of MCP synthesis from cheaper N sources (Chalupa, 1972).

The concentration of ammonia nitrogen ( $\text{NH}_3\text{-N}$ ) has been used as a qualitative reference to understand the adequacy of the rumen environment in relation to microbial activity on fibrous carbohydrates (Detmann et al., 2009). It was found that the efficiency of hydrolysis and utilization of NPN compounds by microorganisms, or the amount of protein that can be replaced, depends on the following factors: rumen pH, amount and type of carbohydrates and crude protein (CP) in the diet, the amounts of certain mineral elements, feed intake, and passage. In addition, abrupt supplementation or excessive consumption could increase the risk of NPN toxicosis and associated adverse effects on ruminant health, and for this reason, dietary NPN supplementation is usually limited. An understanding of NPN metabolism in ruminants, as well as the factors that influence their utilization, will allow for maximal MCP synthesis, which is an essential component of metabolic protein in ruminants. Therefore, the objective of this review was to describe the metabolism and utilisation of some NPN compounds in ruminant diets based on recent findings in the available literature.

## NITROGEN METABOLISM IN RUMINANTS

### *Microbial degradation of non-protein nitrogen sources*

NPN sources are hydrolysed to ammonia in the rumen. Ureolytic rumen bacteria produce ureases that catalyse the degradation of urea to ammonia and carbon dioxide, while biuret is degraded to urea and ammonia by the enzyme biuretase (Tadele and Amha, 2015; Getahun et al., 2019). Urease activity is mainly localized in adhesive bacteria on the rumen wall and in rumen fluid (Rybosová et al., 1984). The high urease activity of bacteria adhering to rumen walls is believed to be one of the mechanisms for enhanced hydrolysis and utilization of urea from blood under low protein dietary conditions (Patra and Aschenbach, 2018; Puniya et al., 2015). Neither ciliated protozoa nor fungi have ureolytic activity (Nagaraja, 2016).

Among other sources, ruminant diets most commonly contain nucleic acids, nitrates, choline, and ethanolamine.

The microbial species involved in the fermentation of nucleic acids or nitrates are poorly understood (Nagaraja, 2016). Nucleic acids make up 5 to 10% of the dietary N. Nucleic acids are degraded to nucleotides by the action of nucleases (deoxyribonucleases and ribonucleases), which are further hydrolysed to nucleosides by the action of nucleotidases and nonspecific phosphatases. Nucleosides are degraded by the action of nucleosidases to purine and/or pyrimidine bases, ribose, and deoxyribose, while phosphatases release ribose-1-phosphate. Purines are sequentially converted to uric acid, allantoin, allantoic and glycolic acid, urea, and glycoylates. Pyrimidine bases are metabolized to  $\beta$ -alanine,  $\beta$ -aminoisobutyrate, ammonia, and carbon dioxide. *Paracolobactrum aerogenoides* is the predominant ureolytic bacterium, while *Selenomonas ruminantium* can utilize both adenine and uric acid as N sources (Nagaraja, 2016; Vogels and Van der Driet, 1976; Wu, 2018).

In the rumen, nitrates are reduced to nitrites by nitrate reductase, which are then converted to ammonia by nitrite reductase. Nitrate reduction involves dissimilation and assimilation processes. The assimilation process consumes energy to reduce nitrate to ammonia (Moreno-Vivián et al., 1999). In contrast, the dissimilation process generates energy and is common in anaerobic and facultative anaerobic bacteria (Thauer et al., 1977). The dissimilation process consists of two steps in which nitrates are reduced to nitrite and subsequently accumulated prior to conversion to ammonia (Latham et al., 2016). Nitrate-utilizing rumen bacteria include *Wolinella succinogenes*, *Veillonella parvula*, and *Selenomonas ruminantium* (Yang et al., 2001).

### *Urea cycle*

Unlike most mammals, in which large amounts of urea are excreted in the urine, ruminants have evolved a mechanism to circulate urea in the digestive system. The urea cycle is the result of the adaptation of ruminants to the inefficient use of proteins in the rumen to prevent the toxicity of ammonia molecules and to use the N that is subsequently released (Rodríguez et al., 2007). In sheep, for example, 30 to 50% of the urea that enters

the digestive tract is returned to the host as ammonia, whereas in cattle this value is between 25 and 40% (Lapierre and Lobley, 2001). Under normal physiological conditions, ammonia is removed from the rumen by incorporation into MCP, absorption through the rumen wall (35-65% of the total in the rumen), and passage through the rumen contents into the omasum (about 10% of the total in the rumen; Nolan and Strachin, 1979; Siddons et al., 1985; Obara et al., 1991). The liver plays a central role in N metabolism in ruminants. Ammonia transported through the rumen epithelium and the wall of the small and large intestines enters the portal circulation through which it is transported to the liver. Nearly 50% of the ammonia that arrives in the liver is derived from N and ammonia ingested from the forestomach (Reynolds and Huntington, 1988; Huntington, 1989; Theurer et al., 2002). In hepatocytes, ammonia is neutralized by its conversion to urea or glutamine. Through the action of ornithine cycle enzymes, ammonia combines with carbon dioxide and is converted to urea. In addition, perivenous cells possess the enzyme glutamine synthetase, which catalyses the condensation of ammonia with glutamate to produce glutamine, which is another way to remove ammonia from the circulation. The above enzymes are found in mitochondria as well as in the cytoplasm of periportal and perivenous hepatocytes (Getahun et al., 2019; Hakvoort et al., 2017). There are several ways to utilize circulating urea in ruminants. Some of the synthesized urea is excreted in urine and faeces, while up to 70% of urea is "recycled" through the circulation, depending on the CP content of the feed (Wu, 2013). About 20% of circulating urea enters the intestine, where it is degraded to ammonia and carbon dioxide by the action of bacterial urease. Between 30 and 60% of circulating urea is returned to the rumen, with the majority transported from the blood through the rumen epithelium and the remainder excreted through the salivary glands (Wu, 2018).

#### *De novo synthesis of amino acids*

Ammonia is the key intermediate in N degradation and assimilation in the rumen. Rumen ammonia concentration ranges from 0 to 130 mg N/100 mL,

depending on N source and postprandial time. The optimal concentration for MCP synthesis ranges from 5.6 to 10.0 mg ammonia/100 mL rumen fluid when energy availability does not limit the rumen ecosystem (Rodríguez et al., 2007; Van Soest, 1994). Ammonia in the rumen is taken up by bacteria, which are quantitatively the most important microbes for protein synthesis, followed by rumen fungi. Protozoa cannot utilise ammonia as a source of N (McDonald et al., 2011). Rumen microorganisms synthesise their own protein from ammonia and volatile fatty acid (VFA) carbon chains, the formation of which is possible through the inclusion of carbohydrates in the feed (Cholewińska et al., 2019). Optimal bacterial growth in the rumen occurs when the efficiency of MCP synthesis is 29 g bacterial N/kg fermented organic matter (OM) and the efficiency of N utilisation is 69%, implying that bacteria require about  $1.31 \times N$  available in the rumen per unit bacterial N (Bach et al., 2005; Uddin et al., 2015). After uptake into cells, rumen bacteria assimilate ammonia to amino acids primarily via reductive amination of glutamate by NAD-linked glutamate dehydrogenase. Other enzymes involved in ammonia assimilation include NADP-linked glutamate dehydrogenase, glutamine synthetase-glutamate synthase coupled reactions, and alanine dehydrogenase. Once assimilated into glutamate, the N from ammonia is rapidly distributed to other amino acids via aminotransferase activities (Chalupa et al., 1972; Pfeffer and Hristov, 2005; Wallace, 1979). Glutamate is then used to synthesize glutamine, alanine, asparagine, and aspartate by glutamine synthetase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, and asparagine synthetase. The above glutamine family of amino acids serves as a substrate for the synthesis of all other amino acids by microorganisms in the presence of fermentable carbohydrates and sulphur in the rumen. Sulphur is required for the synthesis of methionine and cysteine. Rumen microorganisms reduce sulphates to sulphites, while sulphites are reduced to sulphides, with hydrogen sulphide required for the conversion of serine to cysteine. Ruminant diets should contain N and sulphur at a ratio (g/g) of 12:1 to 16:1, depending on desired growth and production levels (Wu, 2018).

### **Factors affecting utilization of non-protein nitrogen sources**

The efficiency of hydrolysis and utilization of NPN compounds depends on appropriate rumen conditions and nutritional factors. The optimal rumen pH for ureolytic bacterial activity is between 6.8 and 7.6, while at values above or below, utilization of urea decreases linearly. At low pH, energy in the rumen is used to maintain a neutral environment in bacterial cells rather than for growth and synthesis of MCP (Strobel and Russel, 1986). Wanapat et al. (2013) studied the effects of different ratio of urea-treated rice straw to concentrate (R:C; 3.5%) on rumen pH, fermentation, and bacterial population in dairy steers. The authors reported that rumen pH decreased linearly with increasing dietary concentrates, with mean pH values of 6.4, 6.3, 6.2, and 5.9 at R:C ratios of 80:20, 60:40, 40:60, and 20:80, respectively. The greatest decrease in pH (5.7) was observed 4 h after consumption of a diet with an R:C ratio of 20:80. The highest MCP synthesis (397 g/day) was observed in diets with an R:C ratio of 40:60, while a ratio of 20:80 reduced protein synthesis (302 g/day).

Ammonia utilization depends largely on the availability of fermentable carbohydrates and the balance between their release and the availability of ammonia. Forages entering the rumen provide a source of energy, while the yield of adenosine triphosphate (ATP) ranges from 1.5 to 4.4 mmol ATP/mmol substrate (Russell and Strobel, 2005). In fact, not all energy sources are available for fermentation in the rumen. Microorganisms cannot utilize lipids as well as the energy contained in bypass protein (Hynd, 2019). The highest yields come from fermentable polysaccharides, which contain 6.2 hexose equivalents/kg, giving a total of 27.3 moles ATP/kg. Thus, 27.3, 27.3, 23.9, 23.9, and 13.7 mol ATP/kg substrate can be obtained from structural polysaccharides (cellulose, hemicellulose), non-structural polysaccharides (starch), sugars, oligosaccharides, and proteins, respectively (Hynd, 2019; Tamminga et al., 2007). Soluble sugars and starches provide higher amounts of ATP than structural carbohydrates up to 4 hours after feeding, but after 4 hours after feeding they provide almost no ATP for

microbial growth. On the other hand, degradation of cellulose and hemicellulose begins about 3 to 4 h after feeding and continues for a long period (up to 96 h) after feeding, providing ATP for subsequent microbial growth. Therefore, feeding a mixture of forage and concentrate results in higher MCP synthesis compared to feeding only concentrate or forage (Pathak, 2008). Azizi-Shotorkhoft et al. (2016) investigated the effects of different carbohydrate sources on rumen microbial enzyme activity and N retention in sheep fed diets containing recycled poultry bedding (RPB). The three experimental diets consisted of alfalfa hay, wheat straw, and RPB. In the first diet, maize and barley were used as carbohydrate sources, while they were replaced by 50 and 100 g/kg of sugar beet molasses, respectively, in the other two treatments.

The results of the study showed that the activity of carboxymethyl cellulase and microcrystalline cellulase in the extracellular fraction of the rumen contents increased linearly when molasses was included in the diet. The authors also reported higher N retention in diets containing 50 and 100 g/kg molasses (4.75 and 5.82, respectively) compared to diets containing maize and barley as energy sources (3.94). In addition, results from the Lu et al. (2019b) study showed that MCP synthesis in the rumen increased by 15% when goats were fed iso-nitrogenous diets containing 28% non-fibre carbohydrate (NFC) compared to a group fed diets containing 14% NFC.

The efficiency of utilisation of NPN compounds is also influenced by the content of CP in the diet, with a content of more than 14% of total digestible nutrients reducing their utilisation (Pugh and Baird, 2012). Lu et al. (2019a) investigated the effects of NFC and CP content on  $\text{NH}_3\text{-N}$ , MCP, and VFA content in the rumen. A total of 24 goats were divided into three groups receiving an NFC-rich diet (10.0% of CP and 28.3% NFC), a protein-rich diet (15.6% CP and 16.3% NFC), and a basal diet (9.6% CP and 14.1% NFC). The highest ruminal  $\text{NH}_3\text{-N}$  concentration was obtained in the high-protein diet (12.51 mM), followed by the NFC-rich and basal diets (8.73 and 3.04 mM, respectively). The MPC synthesis achieved was 11.49,

9.31, and 8.47 g/day for NFC-rich, protein-rich, and basal diets, respectively. Moreover, the total amount of VFAs increased significantly in both the NFC-rich and protein-rich groups compared with the basal group (78.67, 67.66, and 53.01mM, respectively).

In addition to N and energy content, sulphur and phosphorus contents are also important. Sulphur is used as a substrate for the synthesis of sulphur-containing amino acids, while phosphorus is required for ATP and MCP synthesis (Pathak, 2008; Pugh and Baird, 2012). Supamong et al. (2019) investigated the effects of 1% and 2% sulphur content in fermented total mixed rations and ensiling times of zero and 7 days on rumen characteristics, MCP synthesis, and blood metabolites in cattle. The MCP synthesis determined at zero and 7 days of ensiling was 271.5 and 465.3 g/day for the 1% sulphur group and 345.2 and 478.5 g/day for the 2% sulphur group, respectively.

Among other factors, utilisation of NPN compounds is influenced by feed consumption and passage rate. Alves et al. (2014) studied the effects of replacing conventional urea with slow-release urea (SRU) on N metabolism and MCP synthesis in feedlot sheep. Replacement of conventional urea with SRU had no effect on MCP synthesis, which was 23.79, 24.82, 23.23, 24.13, and 18.54 g/day at substitution levels of 0, 20, 40, 60, and 80%, respectively. The authors explained that the low MCP production could be related to the low content of neutral detergent fibre (NDF) in the diet (on average 18.7%). Higher passages rates of digesta combined with a lower rate and extent of degradation resulted in a lower amount of substrate available for fermentation in the rumen, and thus reducing MCP synthesis.

## NON-PROTEIN NITROGEN TOXICITY

### *Ammonia toxicity*

Urea is very rapidly hydrolysed in the rumen to ammonia, which can override utilization by rumen microorganisms and the ability of the liver to convert it to nontoxic urea, resulting in ammonia toxicity (Patra and Aschenbach, 2018; Patra, 2015). In most cases, ammonia

(urea) poisoning occurs in animals that are not adapted to urea-containing diets or in animals that are given access to a diet that contains an excessive amount of urea. When urea is abruptly ingested and hydrolysed to ammonia, the ammonia concentration in the rumen can reach 100 mg/dL and in the peripheral blood more than 10 mg/L (McDonald 1958; Owens and Basalan, 2016). The buffering capacity of rumen fluid for bases is limited above a pH of 6.9, so ammonia as a weak base ( $pK = 9.3$ ) slightly raises the pH in the rumen. This increase in rumen pH is relevant because absorption in the rumen is always greater for nonionized compounds (ammonia, free fatty acids) than for their ionized counterparts (ammonium, ionized fatty acids; Owens and Basalan, 2016).

Indicators of ammonia toxicity include a rumen ammonia concentration greater than  $1 \times 10^3$  mg/L, a rumen pH greater than 8, and a blood ammonia concentration greater than 20 mg/L (Owens and Bergen, 1983; Patra, 2015). Death of animals usually occurs when blood ammonia concentration exceeds 40 mg/L. Ammonia exerts toxic effects on the central nervous system, kidneys, and heart. Signs of urea or ammonia toxicity include dull or depressed behaviour, muscle tremors, frequent urination and defecation, excessive salivation, increased respiration, ataxia, and tetanic convulsions. In addition, high ammonia concentrations lead to acid-base imbalance and alteration of electrolyte balance (Owens and Basalan, 2016; Patra, 2015; Rankins and Pugh, 2012; Salih et al., 2018).

Treatment includes neutralizing the high alkalinity of the rumen by infusing an acidic solution (acetic, propionic, or lactic acid), while intravenous calcium and magnesium solutions may be effective in reducing tetany. In severe cases, rumenotomy and fluid therapy may be required. However, acute ammonia poisoning sometimes leads to animal death so quickly that there is usually not enough time for many of these treatments (Patra, 2015; Rankins and Pugh, 2012).

### *Nitrate/nitrite toxicity*

Nitrates are often present in feeds consumed by ruminants (clover, sorghum, sunflower hay, oat hay).

They are the primary source of N in the soil and are accumulated in plants through their conversion to plant proteins. Conditions such as drought, cold, herbicide application, or wilting cause excessive amounts of nitrates to accumulate. In addition, nitrate levels in plant tissue depend on factors such as the type of plant species, the stage of maturity, and the part of the plant. Immature plants contain higher concentrations of nitrate than mature plants. The leaves and flowers contain less nitrate because most of the nitrate is found in the lower third of the plant (Basso and Ritchie, 2005; Chamizo-Ampudia et al., 2017; Haritha et al., 2019; Kamra et al., 2015; Rashid et al., 2018).

Nitrate itself is not toxic to ruminants, but nitrites are very toxic. When ruminants eat a diet high in nitrate, the activity of nitrite reductase does not match that of nitrate reductase. Therefore, the nitrite content in the rumen exceeds the ability of the microbes to convert it to ammonia and is absorbed through the rumen wall into the bloodstream. Nitrite combines with haemoglobin to form methaemoglobin, which cannot carry oxygen to the tissues, and the animal develops anaemic anoxia. In the rumen, there is an enzyme called methaemoglobin reductase that converts methaemoglobin back to oxyhaemoglobin, but its activity is very low. As methaemoglobin content increases, the colour of the blood changes: light brown at about 20% conversion, dark brown at 50%, and dark brown to black at 80%. When about 80% of haemoglobin is converted to methaemoglobin, the animal may die (Haritha et al., 2019; Kamra et al., 2015; Mohini et al., 2017).

Symptoms of acute nitrate poisoning usually do not occur because the animal dies before they become apparent (several hours after feeding), or they occur when the conversion of oxy- to methaemoglobin is 80-90% complete. Chronic toxicity occurs when animals are fed diets containing moderate amounts (0.5-1.0% DM) of nitrate, while subclinical poisoning occurs at 10 to 20% conversion rate. Clinical signs of nitrate poisoning may be associated with hypoxia and include dyspnoea, tachycardia, cyanotic mucous membranes, exercise

intolerance, excessive salivation, frequent urination, vomiting, muscle tremors, low body temperature, decreased weight gain, decreased milk yield, and sudden death. Abortions can also occur days to a week after sublethal exposure. If a calf is born alive, nitrate toxicity can be transferred to the newborn calf, and it may suffer from convulsions and seizures (Haritha et al., 2019; Kamra et al., 2015; Plummer et al., 2012).

Animals with a low degree of toxicity (indicated by a methaemoglobin concentration of 40 to 50%) may recover spontaneously. Treatment is by intravenous administration of methylene blue, which converts methaemoglobin to haemoglobin, while implementation of preventive measures reduces nitrate toxicity (Haritha et al., 2019; Kamra et al., 2015; Plummer et al., 2012).

#### USAGE OF NON-PROTEIN NITROGEN COMPOUNDS IN RUMINANT FEEDING

Two main factors support the use of NPN in ruminant diets: from a nutritional point of view, it adjusts the amount of rumen-degradable protein in the diet, and from an economic point of view, NPN is less expensive compared to plant protein sources (Cappellozza, 2012). Ruminant diets worldwide are dominated by locally grown forages that are either consumed by animals on pasture or fed as silage or hay when pasture is limited or unavailable due to adverse weather conditions (Wilkinson and Lee, 2018). However, the chemical composition of fibrous feeds (e.g., pastures, crop residues) is influenced by seasonality and is characterized by high volume, low energy content and especially small amount of proteins (Aruwayo, 2018; Tadele and Amha, 2015). Due to the high proportion of crude fibre, the digestibility of these feeds is considerably low.

Consequently, when forage is used as a basal diet, it requires supplementation with protein N (e.g., oil seed cakes) or NPN sources that provide ammonia for MCP synthesis. Although concentrate feeds are used as prominent rich source of nutrients such as energy, protein, minerals, and vitamins, their increasing price leads to higher production costs (Joysowal et al., 2019).

More so, protein-rich leguminous forages are not widely grown in many areas grazed by ruminants, and plant protein supplements are either expensive or unavailable (Salami et al., 2021; Tadele and Amha, 2015).

Urea is the most common NPN added to poor quality forage, as well as a partial substitute for plant protein sources. The results of the study by Gunun et al. (2016) showed that beef cattle fed 4% urea-treated sugarcane bagasse (SBU) and 2% urea- and 2% calcium hydroxide-treated sugarcane bagasse (SBUC) had higher total dry matter (DM) intake compared to those fed untreated sugarcane bagasse (5.2, 5.1, and 4.1 kg/d, respectively). More so, apparent digestibility of OM and CP was highest in cattle fed SBU, while digestibility of NDF and acid detergent fibre (ADF) was most improved in cattle fed SBUC. The authors also reported higher ruminal  $\text{NH}_3\text{-N}$  concentration in cattle fed SBU and SBUC, followed by the groups fed untreated sugarcane bagasse and rice straw. The results indicate that treatment with urea or urea and calcium hydroxide could improve the nutritional value of sugarcane bagasse and its potential use as a high-quality roughage source for cattle. Also, alkaline treatment could improve digestibility and feed intake by altering the structure through the removal of lignin from the lignocellulose and causing swelling of the cellulose fibres, which increases the internal surface area and allows cellulases to have greater contact with the substrate.

Hallajian et al. (2021) replaced soybean meal (SBM) with SRU to investigate the effects on milk production, milk composition, and rumen fermentation in Holstein dairy cows. Treatments consisted of a control diet containing 57% SBM and three additional treatments in which 0.5%, 0.75%, and 1% of SBM were replaced with SRU. Although the authors did not find an increase in DM intake among groups (20.76 - 21.42 kg/day), the addition of SRU controlled the release rate of ammonia and improved rumen fermentation by providing more  $\text{NH}_3\text{-N}$  for MCP synthesis in the rumen. In addition, the levels of milk production (33.28 - 34.27 kg/day) were not significantly different among groups, however, SRU inclusion increased milk fat concentration. In agreement, Corte et al. (2018)

also found no increased feed consumption with increased SRU content in the diet, while Xin et al. (2010) stated that the possible cause of increased fat content in milk in cows fed protected urea could be related to higher production of propionic and acetic acids in the rumen. Similar results were obtained in goats. Campos et al. (2020) evaluated the effects of substituting SBM with 25% urea protected in a matrix of different lipids on the performance and quality of milk from dairy goats. The highest DM (1502 g/day) and OM (1411 g/day) intakes were obtained with meals containing urea protected by carnauba wax, while the highest NDV intake (423 g/day) was obtained with a treatment containing urea encapsulated in beeswax.

Moreover, milk production decreased in the order beeswax > vegetable fat > carnauba wax (1319, 1207 and 1097 g/day, respectively). The highest levels of milk protein and fat were obtained in the beeswax treatment (39 g/day and 55 g/day, respectively). The authors noted that encapsulation of urea with carnauba wax provided higher DM and OM intakes which could be related to its high melting point (85 °C), leading to slower urea diffusion and increased biodegradation. In comparison, beeswax has a lower melting point (65 °C), which allows an easier and more adequate urea release rate, explaining the highest NDF intake. In addition, the improved efficiency of NDF digestion increases the availability of acetate for the production of milk fat and is associated with a greater N availability, which explains the higher content of milk protein and fat in the beeswax group.

Pereira et al. (2020) reported that urea can replace SBM up to 24.0 g/kg in sheep fed spineless cactus-based diets without altering intake, nutrient digestibility, and ruminal parameters. Moreover, the study showed the highest intake of DM (2.22 kg/day), OM (2.06 kg/day), and CP (0.33 kg/day) at a urea concentration of 16 g/kg, while at a concentration of 24 g/kg, the authors determined the highest DM digestibility (832.32 g/kg). Similarly, Lins et al. (2017) reported that spineless cactus with urea could replace up to 80% of wheat bran in sugarcane-based diets for sheep, promoting higher intake of DM and total digestible nutrients. Furthermore, Bhatt and Sahoo



(2017) studied nutrient utilization, body condition, and carcass characteristics of Malupa ewes fed three different dietary regimens based on a complete feed block (65% concentrate, 30% roughage, and 5% molasses) containing: rumen-protected protein (RPP; C), RPP and urea (6 g/kg diet; CU), or RPP, urea and rumen-protected fat (40 g/kg diet; CUF). The results of the study showed that the CU group had the highest DM intake, followed by the CUF and C groups (1179, 1112 and 1105 g/day, respectively). The highest OM and CP digestibility coefficients were obtained in groups CUF and CU compared to group C (on average 0.723, 0.690 and 0.670 for OM and 0.681, 0.664 and 0.598 for CP, respectively), while NDF digestibility coefficients decreased in the order CU > C > CUF (0.653, 0.615 and 0.552, respectively). The authors note that urea supplementation enabled better synchronization of soluble carbohydrates and N required for microbial growth. Although urea supplementation increased digestion rate, resulting in higher feed intake, the high energy content in the CUF group resulted in reduced consumption. More so, the average daily gain was 86.7, 93.2, and 111 g for CU, C, and CUF, respectively. The higher average daily gain in the CUF group was due to the higher energy content of the diet and OM digestibility. In addition, the lack of amino acid N and metabolizable protein could explain the poorer results of the CU group compared to CUF.

Another and effective way to provide urea for the rumen microbes is to mix it with readily fermentable carbohydrates. Mohamed et al. (2020) reported that Friesian calves fed a diet based on peanut hulls supplemented with 10% peanut cake, 1.5% urea, and 8.5% molasses achieved an average live weight gain of 0.43 kg/day and a total feed intake of 3.78 kg/day. In the control group, the values achieved for the above-mentioned performances were 0.14 kg/day and 2.95 kg/day, respectively. However, the utilization of feed-grade urea in ruminant nutrition is limited because it is rapidly hydrolysed to ammonia in the rumen, which often exceeds the rate of carbohydrate fermentation in the rumen. To alleviate the limitations associated with the use of feed-grade urea, urea is often substituted with SRU in the diet.

SRU degrade less rapidly in the rumen, with potential claims of improved synchronisation of ruminal ammonia with energy digestion (Salami et al., 2020; 2021). Sevim and Önel (2019) investigated the effects of adding SRU and/or non-structural carbohydrates (NSC) to groundnut straw on digestibility and some rumen parameters in sheep and goats. The treatments consisted of four experimental groups: control group fed basal diet (99.4% chopped groundnut straw, 0.5% salt and 0.1% vitamin-mineral premix), group fed basal diet + 10 g/day SRU, group fed basal diet + 10 g/day SRU + molasses at 10% of ration on DM basis and group fed basal diet + 10 g/day SRU + starch at 5% of ration on DM basis. The results of the study showed that supplementation of SRU and NSC enhanced OM digestibility, however, the authors did not determine the effect of NSC on the digestibility of NDF and ADF. Also, molasses supplementation had a positive effect on N retention (1.46 g/day) and N digestibility (56.39%) in sheep and N digestibility in goats (61.39%). However, Miranda et al. (2018) reported that the inclusion of 0.75% of SRU in maize silage-based diets (47% maize silage, 2% Tifton hay, 51% concentrate, 16.8% CP) decreased feed intake compared to the control group fed without SRU addition (18 and 20.9 kg/day, respectively). Although SRU could decrease feed and N intake due to low palatability, Salami et al. (2020) reported that inclusion of SRU increased feed and N utilization, milk yield, and decreased N excretion.

Other forms of NPN have also been investigated. Ammoniation is one way to improve feed quality by providing urea as NPN. Low-protein feed is allowed to react with the ammonia, usually under high pressure and temperature, whereupon the ammonia becomes chemically bound and released only when the feed is fermented in the rumen (Tadele and Amha, 2015). Kaunang and Pudjihastuti (2020) studied the effects of feeding goats with ammoniated local forage (*B. mutica* grass or maize straw) and urea palm sugar block (UPSB). The authors reported DM intakes of 430.25 and 470.90 g/head per day when fed a diet containing ammoniated maize, i.e., *B. mutica* grass, 1% concentrate of body weight and 300 g UPSB. Daily weight gains in

the same treatments were 98.50 and 110.40 g/head per day, respectively. The authors note that the increased consumption and weight gain in the UPSB group could be related to the presence of palm sugar, which acts as an appetite stimulant and also stimulates microbiological activity in the rumen to more efficiently ferment the high-fibre feed. Similarly, de Oliveira Franco et al. (2017) investigated the effects of N supplementation combined with different starch levels on voluntary intake, digestibility, and rumen and metabolic characteristics of cattle fed low-quality tropical forage (*Brachiaria decumbens* hay, 7.4% CP). Treatments consisted of control (forage only), supplementation with 300 g CP /day (0:1), supplementation with 300 g starch and 300 g CP /day (1:1), supplementation with 600 g starch and 300 g CP /day (2:1), and supplementation with 900 g starch and 300 g CP /day (3:1). A mixture of N compounds was provided 1/3 from true protein (casein) and 2/3 from NPN (mixture of urea and ammonium sulphate, 9:1). Maize starch was used as the energy source. The study results showed that N and starch addition increased DM intake (on average 16.2, 17.5, 18.2, 22, 21.4 g/kg BW, respectively) and OM digestibility. In addition, the authors reported a positive linear effect of the amount of starch added on OM digestibility. Furthermore, the increased body anabolism, supported by higher serum concentration of insulin-like growth factor, suggested that supplements increased N balance and utilisation efficiency.

Nitrate salts have been evaluated as alternative hydrogen sinks in the rumen, competing with methanogens and resulting in a considerable reduction in methane production (Adejoro et al., 2019). In addition, nitrate is also a useful NPN source for ruminants and can be used as a substitute for urea when provided to animals below the toxic level (Silveira et al., 2019). Adejoro et al. (2019) reported that the addition of urea (10 g/kg) or calcium nitrate (32 g/kg) in a total mixed ration (43:57 roughage to concentrate) did not affect DM, OM, NDF and ADF intake of lambs. While DM, OM, and ADF digestibility were not affected by N source, nitrate-containing diets had higher NDF digestibility than urea-containing diet (565 vs. 499 g/day, respectively). Although N source did

not affect feed conversion ratio, the nitrate-containing diet improved average daily gain by 20% compared to lambs fed urea-containing diet (149 vs. 182 g/head per day). The authors note that more efficient MCP synthesis likely occurs when nitrate replaces urea in ruminant diets. Similarly, potential energy loss in the form of methane may have been converted to energy for greater weight gain in lambs fed nitrate-containing diets, as confirmed by lower methane emission in lambs fed nitrate-containing diets. Furthermore, goats fed diets containing 12.5 g/kg and 25 g/kg of protected calcium nitrate achieved an average DM consumption of 1306 and 1141 g/day and OM consumption of 1239 and 1080 g/day, while values obtained for the SBM group were 1205 and 1189 g/day, respectively (Silveira et al., 2019). However, the authors reported that nitrate inclusion above 9.5 g/kg DM resulted in a decrease in DM intake due to the bitter taste, suggesting that the maximum inclusion level for goats is lower than the recommended level for cattle, which has been reported to decrease DM intake above 20 g/kg DM (Lee et al., 2015).

Animal manure can be added to ruminant diets in dry, ground, and pelleted forms. For example, dried poultry waste contains true protein and high amount of NPN in the form of rumen-degradable uric acid. Uric acid can be utilized by rumen microbes for protein production. Therefore, since uric acid is not readily soluble in rumen fluid and ammonia is only slowly released, it is efficiently utilized (Tadele and Amha, 2015). Goats fed cassava peel meal supplemented with either urea or poultry manure had a digestibility of 93.85 - 95.42% for DM, 89.98 - 91.49% for OM, while the CP digestibility ranged from 89 - 94.22%. Also, N retention ranged from 83.10% for diets without supplemental N to 90.70% for diets with 40% poultry manure (Ajagbe et al., 2020). Meeting the protein requirements of ruminants fed silage can also be achieved by adding NPN sources to the silage mass during production to provide ammonia for microbial growth and produce a high quality silage with high protein content. Thus, poultry manure could be used as an ensiling agent that not only increases the CP content of the silage, but also serves as a buffer to prevent the pH of the silage

mass from dropping too much (Morbos et al., 2017). The results of the in vitro experiment showed that the use of 1% walnut shell biochar (WSB) and 1.5% chicken manure biochar (CMB) on a DM basis reduced the total extent of methane production by 42% and 54%, respectively. Ammonia concentration decreased linearly as the level of WSB and CMB in the diet increased (from 0.5 to 1.5% in DM). Also, different levels of WSB and CMB in the TMR diet (0.5, 1, and 1.5% diet DM) did not change the volume of gas production and total VFA or the proportion of acetate, propionate, and butyrate (Mirheidari et al., 2019). In the second part of the experiment, the same authors investigated the effects of adding 1% WSB and 1.5% poultry manure on the performance of milking ewes. Despite similar DM intakes, dietary inclusion of WSB and CMB resulted in an increased milk yield and milk protein content compared to the control group (312.1, 336.3, and 269.4 g/day for milk yield and 4.38, 4.42, and 3.86% for milk protein, respectively). The addition of WSB and CMB increased the digestibility of DM, OM, and NDF. Furthermore, Morbos et al. (2017) evaluated the acceptability and digestibility of Napier grass silage to which dried poultry manure (DPM) was added as NPN additive in sheep. The effect of the following treatments was evaluated: Napier grass alone containing 8% CP (C), Napier grass with DPM containing 10% CP (T1), and Napier grass with DPM containing 12% CP (T2). The authors reported significant differences in the nutrient composition of the silages with and without DPM. The contents of DM and CP were significantly higher in the silages containing DPM (T1 and T2) than in the C group (20.80, 24.90, and 17.07% for DM and 7.81, 8.67, and 5.58% for CP, respectively). However, the OM content was significantly lower in the silages containing DPM (63.57, 51.68, and 41.48 for C, T1, and T2, respectively). The addition of DPM did not affect the fibre content of the silages. Nutrient intake results showed increased DM intake in T1 and T2 groups compared to C (1.49%, 2.22%, and 1.17% of BW, respectively). Similarly, the addition of DPM improved CP, OM, and fibre intake, with the highest

values observed in the T2 group. The results of the study also showed that the digestibility of DM, NDF, and ADF tended to decrease, and that of CP and OM tended to increase with increasing DPM levels.

## CONCLUSIONS

The use of NPN compounds in ruminant diets provides N essential for MCP synthesis. NPN metabolism in ruminants consists of complex metabolic processes involving degradation of dietary NPN, ammonia uptake by microbes, synthesis of MCP, ammonia absorption through the rumen wall, and leaching into the omasum. Changes in any of these factors could alter rumen ammonia concentrations and negatively affect animal health and production. Previous studies have shown that conditions for optimal MCP synthesis can be influenced by pH, a source of energy, protein content, nutrients important for growth (e.g., minerals), feed intake, and passage rate. In this context, studies have shown that not all energy sources have the same effect on MCP synthesis. Therefore, the energy released in the rumen during the fermentation of carbohydrates to organic acids is one of the most important factors limiting MCP synthesis. In addition, diets containing a mixture of forages and concentrates have been shown to increase MCP synthesis due to better synchronization of nutrient release. A high CP content in the diets could decrease MCP synthesis and lead to the formation of high ammonia concentrations. However, the resistance of proteins to microbial degradation should also be considered. Although the required minerals are sufficient under many feeding conditions, they could limit MCP synthesis in some cases. As indicated by several studies, the addition of NPN compounds increases feed intake, nutrient digestibility, MCP synthesis, and N retention when N supply is inadequate relative to animal requirements, and also has stimulatory effects on ruminant growth and production. In addition, NPN compounds can be safely fed to ruminants to replace part of the dietary protein.

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