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Review Article

Urea transport and hydrolysis in the rumen: A review

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ABSTRACT

Inefficient dietary nitrogen (N) conversion to microbial proteins, and the subsequent use by ruminants, is a major research focus across different fields. Excess bacterial ammonia (NH_3) produced due to degradation or hydrolyses of N containing compounds, such as urea, leads to an inefficiency in a host's ability to utilize nitrogen. Urea is a non-protein N containing compound used by ruminants as an ammonia source, obtained from feed and endogenous sources. It is hydrolyzed by ureases from rumen bacteria to produce NH_3 which is used for microbial protein synthesis. However, lack of information exists regarding urea hydrolysis in ruminal bacteria, and how urea gets to hydrolysis sites. Therefore, this review describes research on sites of urea hydrolysis, urea transport routes towards these sites, the role and structure of urea transporters in rumen epithelium and bacteria, the composition of ruminal ureolytic bacteria, mechanisms behind urea hydrolysis by bacterial ureases, and factors influencing urea hydrolysis. This review explores the current knowledge on the structure and physiological role of urea transport and ureolytic bacteria, for the regulation of urea hydrolysis and recycling in ruminants. Lastly, underlying mechanisms of urea transportation in rumen bacteria and their physiological importance are currently unknown, and therefore future research should be directed to this subject.

1. Introduction

Researchers from animal nutrition, animal physiology, environmental sciences and microbiology fields have long discussed inefficient dietary nitrogen (N) conversion to microbial proteins, and their subsequent use by ruminants (Firkins, 2010; Hackmann and Firkins, 2015). This leads to the potential loss of useful N, and N excretion to the environment, which causes pollution due to excess NH_3 produced from high dietary N degradation. Nitrogen is a major limiting element for living creatures, including bacteria, because they depend on it for growth and survival. Ruminal bacteria obtain N from a wide range of compounds, but also differ in their N sources (Kim et al., 2014, 2017).

For ruminants, the dietary and recycled urea from their liver can be absorbed by microbes in the rumen and metabolized to become microbial protein, which is a good protein source for milk or muscle protein synthesis (Tadele, 2015). Urea is normally added to the diet of a ruminant as non-protein nitrogen, which benefits animal production and saves on nitrogen costs. The research progress concerning urea transporters and hydrolysis will be helpful to guide the regulation of urea utilization.

The urea-N metabolism uses 2 interconnected pathway networks (Arriaga et al., 2009; Sigurdarson et al., 2018). The first hydrolysis pathway is necessary for N release from compounds, to make N available in the surrounding medium. Secondly, assimilatory and biosynthetic pathways produce amino acids and peptides used by the cell. These pathways require various enzymatic activities and accessory proteins; however, compounds for metabolization must reach effective sites, thus requiring transportation mechanisms.

It is accepted that excess NH_3 from urea hydrolysis and other N containing compounds are absorbed and transported to the liver (Abdoun et al., 2006). Here, NH_3 is used for endogenous urea

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synthesis, which is recycled through the ruminal wall and salivary secretion. This process plays a vital role in N utilization and metabolism in ruminants (Long et al., 2004; Reynolds and Kristensen, 2008; Wang et al., 2011; Zhou et al., 2017). Hepatic urea is transported to the rumen via gut epithelia, where ureases are located. According to recent findings (Stewart et al., 2005; Abdoun et al., 2006), humans and different animals express specific urea transporters in various tissues, such as the kidney and gut epithelium.

In our previous research, we investigated efficient urea utilization mechanisms; and factors affecting urease activity such as dairy cow immunization against ureases in the rumen (Zhao et al., 2015), and urease inhibitory compounds (Liu et al., 2020). We have also published a review about ruminal microbial urease activation, ureolytic bacteria diversity and urea recycling, but no integrated review about urea transport and hydrolysis (Jin et al., 2018). Furthermore, we understand that knowing about the factors that affect urea transportation in rumen and ruminal bacteria will provide other possibilities to manipulate urea utilization in the ruminant. The urea transport system in rumen is well studied by a number of researchers such as (Marini and Van Amburgh, 2003; Marini et al., 2004; Recktenwald et al., 2014), however ruminal bacteria is not well understood.

Therefore, we reviewed urea transporters and transportation, urea hydrolysis kinetics and mechanism in ruminants and ruminal bacteria, to update knowledge on urea-N metabolism. The review also includes factors affecting urea transportation and hydrolysis processes.

2. Physiological roles and structures of urea transporters

In living organisms, N containing macromolecules are crucial for different biological systems. In large animals such as ruminants, the catabolic processing of N containing compounds, such as true proteins and non-protein N compounds, releases carbon, hydrogen and oxygen and stores them as carbohydrates and fats. However, a nitrogenous compound produces toxic NH_3 which raises the pH of body fluids. Excess NH_3 is excreted from the body after liver detoxification, and converted to the less toxic compound, urea (Weiner et al., 2015; Jin et al., 2018), however a portion is recycled by ruminants (Lapierre and Lobley, 2001). The relationship between dietary crude protein and ruminal degradable protein concentration determines the N balance, the quantities recycled to gastrointestinal tracts, and how much is used by microorganisms in different animals (Weiner et al., 2015; Mutsvangwa et al., 2016; Oliva et al., 2019).

Urea is produced in the liver from the degradation products of N-containing molecules. In most animals, urea produced this way is considered a waste, and is excreted (Hediger et al., 1996). However, in ruminants, urea produced in the liver and ingested with feed is not only a simple waste product of N metabolism, but also an important precursor in protein biosynthesis.

It is accepted that urea enters the rumen from animal feed and endogenous sources as recycled urea and is hydrolysed to produce NH_3 and carbon dioxide by bacterial urease. As different studies indicate, bacteria hydrolyse urea for 2 main purposes. The first is to use NH_3 as a source of N and carbon in amino acid biosynthesis (Pengpeng and Tan, 2013). For the second, particularly for gastric tract dwelling bacteria, NH_3 may be used as a buffering and survival agent against highly acidic environments of the gastrointestinal tract (Arioli et al., 2010). For this purpose, urea must be transported to the gastrointestinal tract and be in contact with active ureases produced by bacteria.

2.1. Urea transporters in rumen epithelium

Numerous studies, as reviewed by Patra and Aschenbach (2018) and Abdoun et al. (2006), have shown that blood urea crosses the rumen epithelium. This process is nutritionally beneficial, because bacteria inside the rumen can use urea N for protein and amino acid biosynthesis (Rodriguez et al., 2007), after hydrolysing it to CO_2 and NH_3 by ureases (Stewart and Smith, 2005). So, different research findings show that for urea to be utilized by ruminal bacteria, it must influx to the area where urea hydrolysing microbes exist and cross the ruminal wall.

Urea influxes into the rumen via several routes (Stewart and Smith, 2015; Alemneh, 2019). The saliva route accounts for 10% to 40% of urea entry, whereas entry via the gastrointestinal wall is the major entry route, particularly across ruminal epithelium (Berends et al., 2014). A minor entry route involves bile and pancreatic juice secretion (Varady et al., 1979).

Work by Alemneh (2019) described urea inflow into the rumen lumen, as urea crossing the ruminal epithelium by simple diffusion into rumen lumen, based on concentration gradients. However, Santos et al. (2015) indicated that because urea had a stronger dipole moment of 4.6 D (debyes), which was greater than that (1.8 D) of water, its diffusion across lipid bilayered ruminal epithelium was very low. This was tested on artificial lipid bilayers by Brahm (2013) and Klein et al. (2011), showing that the rate of urea permeability was low (4×10^{-6} cm/s) for bilayers that lack any urea transport proteins. Many studies have found that urea absorbency supporting proteins, such as aquaporin (AQP) and urea transporters, are present in different cell membranes of different tissues and organs, such as the kidney and red blood cells (Klein et al., 2011; Klein and Sands, 2016). Similarly, in ruminants, urea flow into the rumen lumen is facilitated by transport proteins. Additionally, other studies have confirmed that salivary glands and rumen epithelia express urea transporting proteins (Marini et al., 2008; Dix et al., 2013).

Rumen based urea transporter proteins are generated from 2 closely related genes; solute carrier family 14 member 1 (*SLC14a1*) or the urea transporter B (*UT-B*), and *SLC14a2* or the urea transporter A (UT-A) (Lu et al., 2005; Strugatsky et al., 2013). Walpole et al. (2018) reported that both *AQP* and *UT-B* facilitated urea transportation into the rumen (Fig. 1). Therefore, facilitative urea transport systems function between the bloodstream and rumen, thus playing significant roles in urea-N regulation and salvaging processes (Zhao et al., 2015).

Stewart et al. (2005) indicated that urea influx was reduced by UT-B inhibitors such as phloretin. Their findings confirmed that UT-A or UT-B transporters were associated with urea transport in ruminal epithelia. Furthermore, when dietary treatments contain urea, *AQP-3* gene expression is down-regulated, suggesting a portion of urea flux occurs via facilitated diffusion through AQP (Saccà et al., 2018). The expression of *UT-B* and *AQP-3* is up-regulated as an incremental supplementation of calves with solid feeds with minimal nitrogen contents, which is also an indication of facilitated urea recycling in ruminants (Berends et al., 2014).

Bovine UT-B forms a trimer whose interface is formed by equivalent protein helices, revealing a quaternary structure (Fig. 1A). At the center of the trimer interface is a large cavity sealed off from solvent, which is packed with partially ordered lipid or detergent molecules (Levin et al., 2012). The UT fold contains 2 homologous halves with opposite orientations in the membrane, giving the structure an internal pseudo-2-fold symmetry axis. Each half contains 5 transmembrane helices, and one tilted reentrant helix spanning roughly half the membrane. An amphipathic

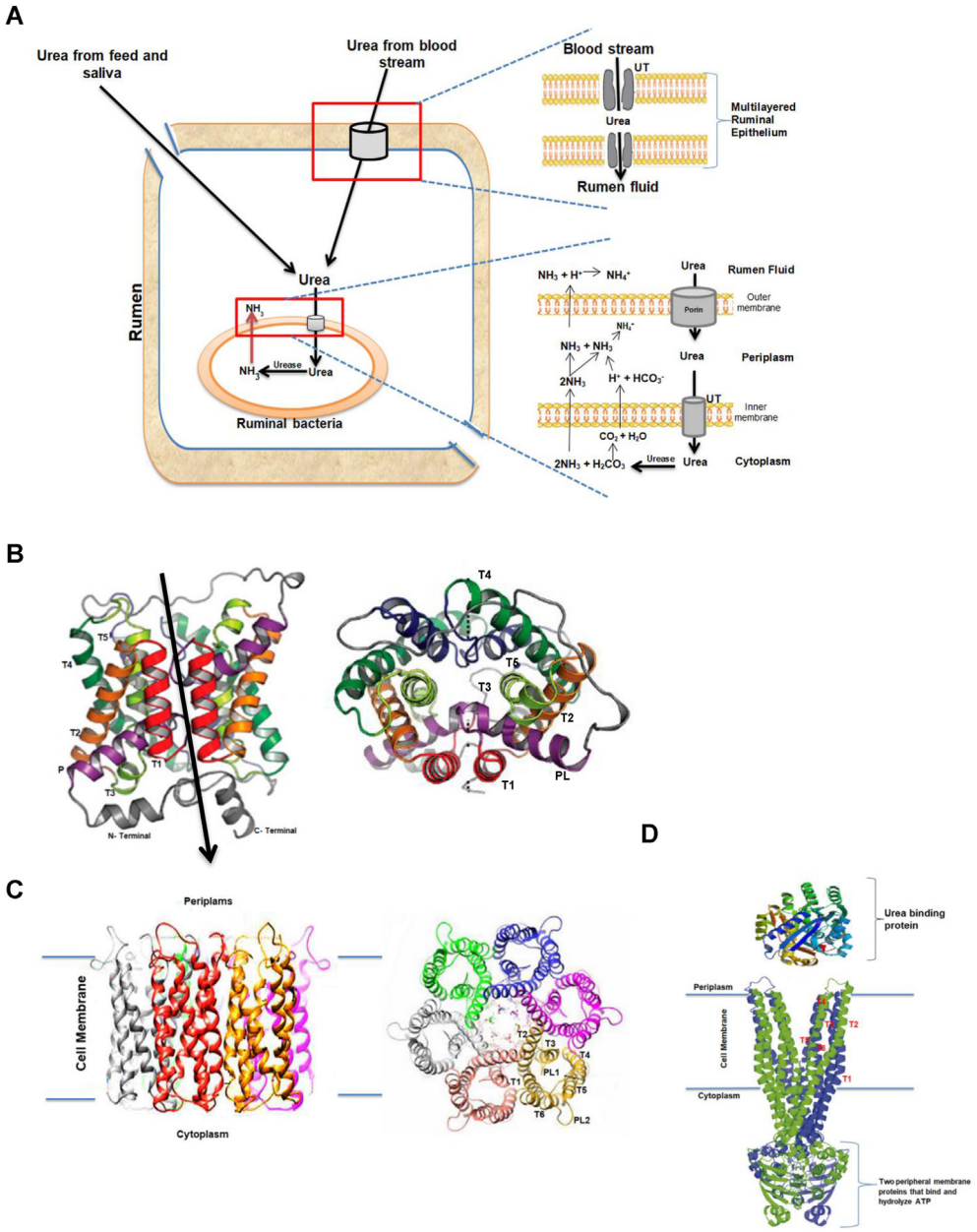

Fig. 1. The ruminal epithelium and bacterial urea transporters. (A) Schematic showing the relationship between the rumen and ruminal bacteria in terms of urea transporters. (B) The structure of a ruminal epithelium urea transporter B protomer, as viewed from within the plane of the membrane (left), and extracellular membrane view (right). The black arrow passing through 2 T3 (green) shows the urea permeation pathway (Levin et al., 2012). (C) Structural model of a bacteria proton gated urea transporter (*urel*) (left). The ribbon diagram (right) shows the closed urea transporter, PL1 (yellow)(Sachs et al., 2006). (D) Structure of an ATP-dependent ABC urea transporter. PL (1 and 2) = periplasmic loops, T (1 to 6) = transmembrane helices (Lu et al., 2005).

membrane-spanning pore is formed at the interface of the 2 halves in each promoter, and is lined by residues from conserved urea signature sequences (Levin et al., 2009). This pore has a restricted region which serves as a selective filter that opens into 2 wide vestibules on both sides and is a designated urea permeation pathway.

2.2. Urea transporters in ruminal bacteria

Most bacterial species in the rumen have counterparts in other areas of the mammalian digestive system, including the human gut. There is limited information regarding urea transporters and their role in ruminal bacteria so in this review we provide some of the description by taking bacteria of the other mammalian animals as a model. In most bacterial species, ureases are found in the bacterial cytoplasm, although they are considered extracellular in some bacteria (Mobley and Hausinger, 1989). Thus, urease location in ureolytic bacteria is controversial. Hawtin et al. (1990) indicated that bacterial ureases were located on cell surfaces, and in materials shed from these surfaces. In other research, the hydrolysis of urea molecules surrounding the bacteria have produced ammonium H ions associated with a consequent rise in local pH (Mobley and Hausinger, 1989). This observation may suggest urease activity is localized outside the cell. However, in a study investigating whether ureases were surface associated or not, the results showed that urease activity was located in the cytoplasm of fresh log-phase cultures, but as

the cultures aged, urease activity was found on the cell surface, or shed into the medium (Bode et al., 1993; Phadnis et al., 1996). This may have been due to cell autolysis. Dunn et al. (1997) and Mobley and Hausinger (1989) also concluded that ureases were cell membrane bound, when human gastric biopsies and urinary specimens were examined. In ruminal bacteria, cyto-chemical localization studies of ureases by McLean et al. (1985) confirmed that urease in *Staphylococcus* sp. H3-22 was located in the cytoplasm. Thus, for bacterial urea hydrolysis, urea must enter the cytoplasm.

In rumen bacteria, even though it is controversial, members of the UT protein family are involved in the selective and speedy transport of urea across concentration gradients. Currently, 2 evolutionarily distant, but distinct UT have been identified (Levin and Zhou, 2014). These UT form common UT folds, involving 2 structurally homologous domains which appear as a continuous membrane-spanning pore, suggesting urea is transported by UT via a channel-like mechanism (Levin and Zhou, 2014). This finding has underpinned the concept of urea transport, and its role in urea entry into the cytoplasm.

Rumen bacteria express 3 different UT, which have distinct functional activities. The first is a pH-independent UT, e.g. Yut (Sebbane et al., 2002). Yut is a pH-independent protein found in *Yersinia*, and is homologous to mammalian UT, with a sequence identity to human UT-B (Levin et al., 2009).

The second is a proton-gated (pH-dependent) UT, common in *Helicobacter pylori*. It has a channel like structure, which is closed and opened at neutral and acidic pH, respectively (Fig. 1C). When the channel opens at an acidic pH, it allows rapid urea entry to access cytoplasmic ureases (Levin and Zhou, 2014; Tanaka et al., 2018).

The structure of the pH-dependent UT, as described by Cui et al. (2019), contains oligomers of 6 channel protomers, arranged in a hexamer, with a lipid core at the center. Six-fold symmetry provides a 3-dimensional (3D) reconstruction, and extends the resolution of the closed and open channel. Each channel is roughly divided into 3 sections: the first one is a periplasmic domain and vestibule formed by N and C termini, the second periplasmic loop 1 (PL1) and PL2, and the third one is transmembrane helices on the periplasmic side of the urea filter (Fig. 1C). The urea filter near the center of the membrane is composed of a ring of side chains from several hydrophobic residues, and a cytoplasmic domain and vestibule composed of transmembrane helices and loops on the cytoplasmic side of the filter. The urea gating or filtration mechanism is accomplished by conformational changes in PL1, PL2 and the C terminus (Fig. 1C).

The third transporter is an ATP-activated UT (Jahns et al., 1988). Some ruminal bacteria, such as *Corynebacterium glutamicum* allow urea transport into the cytoplasm, crossing cell membranes accompanied by ATP hydrolysis. For this process, the ATP binding cassette, the ABC-type transporter, encoded by *urtABCDE* genes, is vital (Leng and Nolan, 2010; Jin et al., 2017). Furthermore, evidence (^{14}C urea uptake) shows that an energy-dependent UT exists in *Alcaligenes eutrophus* H16 and *Klebsiella pneumoniae* M5al (Jahns et al., 1988). Thus, UT in these organisms facilitate urea as a N source (Weeks and Sachs, 2001; Sebbane et al., 2002; Valladares et al., 2002; Beckers et al., 2004).

Structurally, ABC-transporters consist of a urea binding protein (UBP) which delivers urea to the transporter. The UBP is located in the periplasm of Gram-negative bacteria, but is tied to the cytoplasmic membrane or transporter in Gram-positive bacteria (Poolman and van der Heide 2002). The other component of ABC-transporters are transmembrane domains (TMD), which are embedded in lipid bilayers to form translocation channels and nucleotide-binding domains (NBD) for ATP hydrolysis (Nicholas and Yung, 2018) (Fig. 1D).

Most UT protein and transportation studies have focussed on bacteria in humans, other non-ruminants or bacteria from soil and water (Li et al., 2012; Strugatsky et al., 2013; Esteva-Font et al., 2015). However, there is a dearth of information on the precise sites where urea hydrolysis occurs in ruminal bacteria, and the presence of UT proteins. Some information exists in bacteria, e.g. *Succinovibrio dextrinosolvens* strain 22B, has a *urtE* gene, and *S. dextrinosolvens* strain Z6 have *urtABCDE* genes (Hailemariam et al., 2020), suggesting the presence of UT proteins. Those genes are the subunit for urea ABC-transporter (ATP-binding) as indicated in NCBI assembly results in GenBank accession GCA_900114195.1 and CP047056, respectively. However, the exact function of this gene is not known. Furthermore, UT mechanisms into the cytoplasm of ruminal bacteria are also unknown. Studying such mechanisms can be a potential area for regulation of urea hydrolysis, efficient utilization and tackling the impact of nitrogenous compound pollution to the environment.

3. Composition of ruminal ureolytic bacterial communities

Ureolytic bacteria are the most important organisms in the rumen (Leng and Nolan 2010; Jin et al., 2017). They produce ureases which breakdown urea to NH_3 for microbial protein synthesis. However, little is known about the diversity and distribution of rumen ureolytic microorganisms, by using different microbiological mechanism ruminal bacteria from diverse taxa possess urease enzymes synthesis system. Previously, approximately 35% of rumen bacteria detected by culture dependent methods belonged to ureolytic species, e.g. *Staphylococcus* spp., *Lactobacillus casei* and *Klebsiella aerogenes* (Mobley et al., 1995). Jin et al. (2016) identified abundant ureolytic bacteria, using urea and urease inhibitors and selection methods, from *Pseudomonas*, *Haemophilus*, *Neisseria*, *Streptococcus*, *Actinomyces*, *Bacillus* genera, and unclassified genera, Succinivibrionaceae. Using recent microbiological and molecular technology, the new bacteria species and strains can be identified. Jin et al. (2017) used the *ureC* gene as a biomarker in their phylogenetic analyses to identify ruminal ureolytic bacteria. They obtained better compositional estimates of ureolytic bacteria in the rumen. Importantly, more than 55% of sequenced bacterial samples were not assigned to any known phylum, suggesting the rumen may contain more undiscovered urease producing bacteria.

The ureases produced by ruminal ureolytic bacteria rapidly hydrolyze urea to NH_3. In nature, urea is hydrolyzed by urea aminohydrolase, which is a multi-subunit nickel dependent metalloenzyme. The rate of urea hydrolysis by ureases is approximately 10^{14} times faster than uncatalyzed reactions (Kafarski and Talma, 2018a). As described by Callahan et al. (2005), uncatalyzed urea degradation will take an elimination time of up to 40 years at 25 °C. Urea hydrolysis yields NH_3 and carbamate, which is an unstable compound, and spontaneously hydrolyses to produce more NH_3 and carbonic acid. However, urease activity levels in different ureolytic bacteria are variable. For instance, levels are generally higher in bacterial species loosely adhered with the solid feed particles than in bacteria species tightly bound with solid feed particles (Kumar and Rudolf, 2018). The urea kinetics constant shows differences in the same bacterial species and different strains. Breitenbach and Hausinger (2015) and Jin and Murray (2010) reported that various *Proteus mirabilis* strains exhibited urease K_m values ranging from 13 to 60 mmol/L. These observations show urea hydrolysis rates and quantities depend on bacterial species and strains. Therefore, the isolation and identification of ruminal ureolytic bacteria may provide regulatory targets to mitigate urea hydrolysis, and increase urea N efficiency in ruminants (Jin et al., 2016).

4. Factors affecting urea hydrolysis and transportation in the rumen

The rate of urea hydrolysis in ruminants is variable and depends on different factors. Mechanisms that regulate urea hydrolysis could lead to improved N utilization, support efforts to reduce N excretion, and improve environmental sustainability of animal production.

Urea is hydrolyzed by ureases and therefore the presence of any factors that influence urease synthesis and activity directly affects urea hydrolytic processes. In most bacteria, urease synthesis and activity are regulated by several factors, such as hydrolysis product concentrations i.e., NH_3 and N levels consumed by host ruminants, urea concentrations, and the pH of the surrounding medium where hydrolysis occurs. The following sections include brief analyses of these factors and provide more information on urea metabolism and host ruminants.

4.1. Urea, NH_3 and other N concentrations

To regulate efficient urea use in ruminants, the effects of urea concentrations on urea hydrolysis rates and the relative accumulation of NH_3 must be understood. It is accepted that for different enzyme reactions, the concentration of the substrate affects enzyme biosynthesis and activity. Many studies have indicated that urea hydrolysis by bacterial ureases follows simple Michaelis–Menten kinetics, whereby increasing substrate (urea) concentrations increase reaction rates, until the concentration satisfies urease saturation (Kurtz, 1970). As indicated by Patra and Aschenbach (2018), in the rusitec system, urea hydrolysis was increased, by increasing urea infusion rates from 10 to 170 mg/d for a forage-based diet, and 40 to 170 mg/d for a concentrate-based diet. In addition to the other required condition for urea hydrolysis to take place, the concentration of urea in the medium is a determinant. In a study by Pearson and Smith (1943), the effect of urea concentration on the rate of urea hydrolysis was known by liberated NH_3. Their results confirmed a direct relationship between liberated NH_3. The study conducted by Marini and Van Amburgh (2003) and Recktenwald et al. (2014) also indicated that ruminal ammonia concentration increased by increasing N intake. Furthermore, several research papers have indicated that controlling the release rate of ammonia from dietary urea hydrolysis allows more efficient incorporation of nitrogen into ruminal microbial protein (Jones and Milligan, 1975; Makkar et al., 1981; Berends et al., 2014; Wang et al., 2018). However, as the concentration of urea increased beyond the maximum ammonia production level, the rate of urea hydrolysis was either unchanged or decreased, because the medium was saturated with accumulated ammonia (Patra and Aschenbach, 2018). In certain bacteria, urease is inducible, and is synthesized and activated in the presence of urea. Thus, urea in the surrounding medium initiates urease synthesis. In this type of bacteria there are regulatory genes, whose product is induced by the presence of urea (Konieczna et al., 2013). For these bacteria, if the regulatory genes were initiated, the urease structural and accessory genes were activated to form the active urease.

In many bacterial species, urease biosynthesis and activity appears to be tightly regulated by factors related to different N containing compounds, and N regulatory systems (Konieczna et al., 2013). However, there are pronounced differences between bacterial species. In some bacteria, the presence of NH_3 and/or N rich compounds which release NH_3 upon degradation, inhibit urease synthesis and activity; and are derepressed under N-limiting or N starvation conditions (Morou-Bermudez and Burne, 1999). As cited by Patra and Aschenbach (2018), urea hydrolysis by *Selenomonas ruminantium* ureases was low when cells were grown at high NH_3 levels. Hydrolysis processes increased several folds in cells grown under the condition of low urea; conditions where NH_3 did not accumulate in the medium. Thus, ruminal NH_3 concentrations impact negatively on ruminal urea clearance rates.

The rate of urea degradation/hydrolysis per plasma urea concentration is affected in steers given different diets containing different nitrogen sources (Holder et al., 2013; Batista et al., 2016). More specifically, the rate of urea hydrolysis by ruminal urease activity is affected by crude protein levels fed to animals. As the animal is fed higher proteins levels, urease activities reduce. Kappaun et al. (2018) reported that rumen bacteria showed lower urease activities when sheep were fed a high protein diet (137 g protein/d), but when fed a low protein diet (23 g protein/d), the greatest urease activity was found in some bacteria.

The activity of urea transporters are also affected by different dietary nitrogen contents. Saccà et al. (2018) and Røjen et al. (2011) showed that mRNA expression of the urea transporters, *AQP3*, *AQP7, AQP10* and *UT-B* genes, appeared responsive to dietary N treatments. Furthermore, the transport of urea-N across rumen epithelia was determined by NH_3 absorption from the rumen, and by urea influx into the rumen (Abdoun et al., 2006). As indicated by Kristensen et al. (2010) arterial urea extraction across the rumen increased from 7.1% to 23.8% when cows were changed from high-N to low-N, respectively. This is used to balance the level of nitrogen by using the endogenous sources. Kristensen et al. (2010) concluded that urea transport across gut epithelia in cattle adapts to N status, which is regulated by the expression or activity of facilitative urea transporters.

4.2. Fermentable carbohydrates or microbial activity

As indicated earlier, urea hydrolysis rates are inhibited by NH_3 accumulation in the surrounding medium. However, NH_3 is assimilated and incorporated into cells. In an energy-rich (glucose-containing), N-poor environment, the action of glutamine synthetase and glutamate synthase forms an NH_3 assimilatory cycle, where NH_3 is incorporated into L-glutamate, to form L-glutamine. Therefore, when sufficient fermentable carbohydrates are available, NH_3 is converted to amino acids, which may create additional space for urea hydrolysis. Studies have confirmed that highly fermentable carbohydrate supplementation increases urea and urea-N hydrolysis influx into the rumen (Abdoun et al., 2006). Other research conducted by Seram et al. (2019) found that ruminal ammonia-N concentration decreased linearly as the total sugar content of the diet fed to dairy cows increased. In steers fed hay diets supplemented with 0, 150, or 300 g sucrose per d, the rate of urea disappearance from the rumen significantly increased as the sucrose levels were increased. This improved fermentation status could create a higher demand for NH_3-N by ruminal bacteria. Various studies have confirmed that ruminal fermentable carbohydrate supplementation decreases NH_3 levels in the rumen through the enhanced uptake of NH_3 for microbial protein synthesis (Hristov et al., 2019).

4.3. Application of inhibitors

Urea hydrolysis to NH_3 is rapid (Chalupa et al., 1964) and can surpass its utilization by the ruminal microorganisms to produce microbial protein, leading to NH_3 toxicity, wastage of nitrogen and environmental pollution (Jonker et al., 2002; Kumar and Rudolf, 2018). Therefore, balancing hydrolysis rates with NH_3 assimilation rates are crucial for efficient ruminant utilization of any N source. Different methods have been employed to slow down urea hydrolysis rates, such as urease inhibitors (Modolo et al., 2015;

Kafarski and Talma, 2018b) and slow the release of urea (Taylor-Edwards et al., 2009).

Urease inhibitors decrease urease activity in the rumen. Several compounds are available and have been reviewed by Kumar and Rudolf (2018), including acetohydroxamic acid (AHA), phosphoric phenyl ester diamide (PPD), N- (n-butyl) thiophosphoric triamide (NBPT), boric acid, bismuth compounds and hydroquinone.

Natural products (mostly secondary metabolites from plants such as tannins, saponins and essential oils) are sources of potential compounds for urease inhibition (Modolo et al., 2015). Most recently, Liu et al. (2020) reported that Biochanin A (a natural compound) effectively inhibited rumen urease and subsequent urea degradation, thereby reducing rumen NH_3 production. The use of urease protein vaccination also slowed down urease activity in ruminants (Zhao et al., 2015). Zhao et al. (2015) reported that cows vaccinated with *UreC* from *H. pylori* caused a 17% reduction in urease activity. The other option to limit rapid urea hydrolysis in the rumen can be achieved by manipulating the factors that affect urea transporter activity. Even if there is no research conducted directly on ruminal bacteria and rumen in other parts of ruminant and non-ruminant animals, the use of urea transporter inhibitors are used as means to reduce urea hydrolysis (Knepper and Miranda, 2013; Sands, 2013). This might be one of the possibilities to manage the rate of urea hydrolysis in the rumen to achieve efficient utilization.

5. Conclusions

Ruminants obtain urea from feedstock and endogenous sources, which is recycled urea in the rumen. Ruminal microorganisms have developed mechanisms to exploit urea hydrolysis. For ruminants to utilize urea, it has to be converted to NH_3 and eventually to microbial protein by those ureolytic bacteria and others. Approximately 35% of rumen bacteria, belonging to ureolytic species, can be detected by culture dependent methods. However, be using molecular methods, more than 55% of new ureolytic bacteria were identified, which were not previously found in any phylum. Ureases produced by ureolytic bacteria such as *Pseudomonas, Haemophilus, Neisseria, Streptococcus, Actinomyces, Bacillus and* Succinivibrionaceae in the rumen, are the most important elements of urea utilization. Urea used by microbes in the rumen, especially endogenous urea, must pass rumen epithelia, however the results are controversial. Some studies argue that urea enters the rumen from the bloodstream by simple diffusion, whereas others state urea requires a dedicated UT for rumen entry. Most recently, researchers have confirmed that urea transport proteins are responsible for the influx of urea into the rumen and are dependent on the type and concentration of nitrogen sources in the rumen. These studies have also confirmed that urea transport across rumen epithelia is mediated by diffusion down a concentration gradient, via transport proteins, such as UT-B and certain AQP family members that are known to transport urea. The other controversy relates to the exact site of urease activity in bacteria. Two conflicting ideas have been proposed; the first suggests that urease activity occurs outside the cell, and the other suggests that urease activity is completely cytosolic. However, recent research has indicated that bacteria, especially some environmental bacteria and bacteria in the human body, have 3 different urea transporters, i.e., pH-independent, proton-gated and ATP-activated transporters.

6. Future research directions

Future research should focus on investigating the structure, expression, and regulation of urea transporters, thus confirming the physiological role of these entities in urea hydrolysis and NH_3 utilization. The regulation of urea transporters in bacteria might play a great role in controlling the rate of urea hydrolysis in the rumen and have the potential for enhancing efficient urea utilization.

Author contributions

Samson Hailemariam and **Shengguo Zhao** initiated the idea and outline of this review paper. **Samson Hailemariam**, **Shengguo Zhao**, **Yue He** and **Jiaqi Wang** studied and analyzed all of the publications cited in this paper and prepared the initial manuscript. All authors read and approved the final manuscript.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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