COMMUNAUTÉ FRANÇAISE DE BELGIQUE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

OMICS INSIGHTS INTO RUMEN UREOLYTIC BACTERIAL COMMUNITY AND UREA METABOLISM IN DAIRY COWS

Di JIN

Essai présenté en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs: Yves Beckers

Jiaqi Wang (CAAS, China)

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Di Jin (2017). Omics insights into rumen ureolytic bacterial community and urea metabolism in dairy cows

135 p., 14 Tables, 23 Figures.

Abstract

Urea has been used in the diets of ruminants as a non-protein nitrogen source. Ureolytic bacteria are key organisms in the rumen producing urease enzymes to catalyze the breakdown of urea to ammonia (NH_3), and the NH_3 is used as nitrogen for microbial protein synthesis. In the rumen, hydrolysis of urea to NH_3 occurs at a greater rate than NH_3 can be utilized by rumen bacteria, and excess ammonia absorbed into blood may be harmful to the animals. Nowadays, little is known about the information of ureolytic microorganisms in the rumen, and the changes that occur in the rumen microbial and host metabolites induced by urea nitrogen have not been fully characterized. 'Omics' approaches, such as metagenomics and metabolomics have been applied to analyzing rumen microbial community and nutrients metabolism in dairy cows. The objective of this study is to investigate the rumen predominant ureolytic bacteria community and the mechanisms of urea utilization in ruminants using sequencing and metabolomics approaches. Firstly, an *in vitro* experiment trying to explore the ruminal ureolytic bacterial community was performed. Urea or acetohydroxamic acid were supplemented into the rumen simulation systems as the stimulator and inhibitor for ureolytic bacteria, respectively. The bacterial 16S rRNA genes were analyzed by Miseq sequencing and used to reveal the ureolytic bacteria by comparing different treatments. We found that urea supplementation significantly increased the proportion of *ureC* genes. The rumen ureolytic bacteria were abundant in the genera of *Pseudomonas*, *Haemophilus*, *Neisseria*, *Streptococcus*, *Actinomyces*, *Bacillus* and unclassified Succinivibrionaceae. Secondly, an *in vivo* experiment was taken to investigate differences in ureolytic bacterial composition between the rumen digesta and rumen wall based on *ureC* gene classification. Six dairy cows with rumen fistula were assigned to a two-period cross-over trial. One group was fed a total mixed ration without urea and the treatment group was fed rations plus 180 g urea per cow per day. Rumen bacterial samples from rumen content and rumen wall fractions were collected for *ureC* gene amplification and sequencing using Miseq. More than 55% of the *ureC* sequences did not affiliate with any known taxonomically assigned urease genes. The wall-adherent bacteria had a distinct ureolytic bacterial profile compared to the bacteria in the rumen content. The most

abundant *ureC* genes were affiliated with Methylococcaceae, Clostridiaceae, Paenibacillaceae, Helicobacteraceae and Methylophilaceae families. Relative abundance of the operational taxonomic units (OTUs) affiliated with *Methylophilus* and *Marinobacter* genera were significantly higher in the bacteria on the rumen wall than that in the rumen content. Thirdly, based on the *in vivo* experiment, rumen fluid and blood samples were collected and analyzed using nuclear magnetic resonance spectroscopy and multivariate analysis of variance. Concentrations of valine, aspartate, glutamate, and uracil in the rumen, and urea and pyroglutamate in the plasma were increased after urea supplementation. Metabolic pathways include pantothenate and CoA biosynthesis, beta-alanine metabolism, valine, leucine, and isoleucine metabolism in the rumen, and urea and glutathione metabolism in the plasma were significantly increased by urea nitrogen. In conclusion, this study identified significant populations of ureolytic bacterial community that have not been recognized or studied previously in the rumen and provides a basis for obtaining regulatory targets to moderate urea hydrolysis in the rumen. The findings also provided novel information to aid understanding of the metabolic pathways affected by urea nitrogen in dairy cows, and could potentially help to guide efforts directed at improving the efficiency of urea utilization in the rumen.

Keywords: Dairy cow, rumen, ureolytic bactertia, urea, acetohydroxamic acid, 16S rRNA gene, *ureC* gene, high-throughput sequencing, plasma, metabolites, NMR spectroscopy.

Di Jin (2017). Les technologies omiques pour identifier la communauté bactérienne uréolytique du rumen et le métabolisme de l'urée chez les vaches laitières

135 p., 14 Tables, 23 Figures.

Résumé

L'urée est utilisée dans les régimes alimentaires des ruminants en tant que source d'azote non protéique. Les bactéries uréolytiques sont des organismes clés dans le rumen car ils produisent des enzymes du type uréase nécessaires pour catalyser la transformation de l'urée en ammoniac (NH_3). Le NH_3 produit est ensuite utilisé comme source azotée pour la synthèse des protéines microbiennes. Dans le rumen, l'hydrolyse de l'urée en NH_3 se produit à un taux plus élevé que son utilisation par les bactéries, et l'excès d'ammoniac est alors absorbé dans le sang qui peut nuire aux animaux. De nos jours, on connaît peu d'informations sur les microorganismes uréolytiques dans le rumen et les changements dans les métabolites microbiens et hôtes du rumen induits par l'apport d'N non protéique. Les approches «omiques» telles que la métagénomique et la métabolomique ont été appliquées à l'analyse de la communauté microbienne du rumen et du métabolisme des nutriments chez les vaches laitières. L'objectif de cette étude était d'étudier la communauté des bactéries uréolytiques prédominantes dans le rumen et les mécanismes de l'utilisation de l'urée chez les ruminants en utilisant des approches séquentielles et métabolomiques. Tout d'abord, une expérience *in vitro* explore la communauté bactérienne uréolytique ruminale. L'urée et l'acide acétohydroxamique ont été employés dans des systèmes *in vitro* de simulation du rumen an tant que stimulateur et inhibiteur pour les bactéries uréolytiques, respectivement. Les gènes bactériens 16S de l'ARNr ont été analysés par séquençage Miseq et utilisés pour révéler les bactéries uréolytiques en comparant les différents traitements. Nous avons constaté que la supplémentation en urée augmentait de façon significative la proportion de gènes *ureC*. Les bactéries uréolytiques du rumen étaient représentées par les genres Pseudomonas, Haemophilus, Neisseria, Streptococcus, Actinomyces, Bacillus et Succinivibrionaceae non classés. Deuxièmement, une expérience *in vivo* a été effectuée pour rechercher des différences au sein de la composition bactérienne uréolytique associée au digesta du rumen et à la paroi du rumen en se basant sur la classification des gènes *ureC*. Six vaches laitières munies d'une canule au rumen ont été assignées à un essai réalisé en deux périodes. Un groupe témoin a reçu une ration mixte totale sans urée et un groupe expérimental a reçu la ration témoin plus

180 g d'urée par jour. Les échantillons bactériens du rumen ont été extraits à partir du contenu du rumen et de la paroi du rumen pour l'amplification et le séquençage du gène *ureC* en utilisant Miseq. Plus de 55% des séquences de *l'ureC* ne sont affiliées à aucun gène d'uréase taxonomiquement connu. Les bactéries adhérentes à la paroi avaient un profil bactérien uréolytique distinct par rapport aux bactéries extraites du contenu du rumen. Les gènes *ureC* les plus abondants ont été affiliés aux familles Methylococcaceae, Clostridiaceae, Paenibacillaceae, Helicobacteraceae et Methylophilaceae. L'abondance relative des OTU affiliées aux genres Methylophilus et Marinobacter était significativement plus élevée dans les bactéries fixées sur la paroi du rumen que dans celles extraites du contenu du rumen. Troisièmement, sur la base de l'expérience *in vivo*, les échantillons de la phase liquide du rumen et de sang ont été recueillis et analysés en utilisant la spectroscopie de résonance magnétique nucléaire. Les concentrations en valine, aspartate, glutamate et uracile dans la phase liquide du rumen, et l'urée et le pyroglutamate dans le plasma étaient augmentées après la supplémentation en urée. Les voies métaboliques incluent la biosynthèse du pantothénate et du CoA, le métabolisme de la bêta-alanine, le métabolisme de la valine, de la leucine et de l'isoleucine dans le rumen, et le métabolisme de l'urée et du glutathion dans le plasma ont été significativement augmentées par l'ajout d'urée. En conclusion, cette étude a identifié des populations importantes de communautés bactériennes uréolytiques qui n'ont pas été mise en évidence auparavant dans le rumen et elles constituent une base de travail pour moduler l'hydrolyse de l'urée dans le rumen. Les résultats ont également fourni de nouvelles informations pour faciliter la compréhension des voies métaboliques affectées par l'N non protéique chez les vaches laitières et pourraient potentiellement aider à guider les efforts visant à améliorer l'efficacité de l'utilisation de l'urée dans le rumen et par le ruminant.

Mots-clés: Vache laitière, rumen, bactéries uréolytiques, urée, acide acétohydroxamique, 16S rRNA gene, *ureC* gene, séquençage à haut débit, métabolites plasmatiques, NMR Spectroscopie.

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Di Jin

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Abbreviations

ADF, acid detergent fiber

AHA, acetohydroxamic acid

ATP, adenosine triphosphat

BCAAs, branched-chain amino acids

BW, bodyweight

CP, crude protein

CTAB, cetyl trimethylammonium bromide

DDGS, distillers dried grains with soluble

DIP, degradable intake protein

DM, dry matter

DMI, dry matter intake

DSS, dextran sulfate sodium

EE, ether extracts

EN, endogenous nitrogen

GTP, guanosine triphosphate

GDH, glutamate dehydrogenase

GS, glutamine synthetase

LAB, liquid-associated bacteria

N, nitrogen

NH_3, ammonia

NDF, neutral detergent fiber

NMR, nuclear magnetic resonance

NBPT, N-(n-butyl) thiophosphoric triamide

OM, organic matter

OTU, operational taxonomic units

OPLS-DA, orthogonal projections to latent structures-discriminant analysis

P, p-value

PCA, principal component analysis

PCR, polymerase chain reaction

PCoA, principal coordinate analysis

r, correlation coefficient

SAB, solid-adherent bacteria

SEM, standard error of the mean

TMR, total mixed ration

U-CaS, urea-calcium sulphate mixture

VIP, variable importance in the projection

WAB, wall-adherent bacteria

CHAPTER I GENERAL INTRODUCTION

General introduction

1. Context

As the development of the dairy industry in all over the world, the number of ruminants increases rapidly and so as the requirements for feed protein. In ruminant diets, the protein is an expensive dietary nutrient, representing approximately 42% of the cost of lactating cow rations (St-Pierre, 2012). Urea has been recognized for more than a century that may be incorporated in the diets of ruminants, and has been used as a non-protein nitrogen (NPN) in ruminant rations to reduce the supplementation of true protein and the costs of rations (Kertz, 2010). During the 1970s and 1980s, lots of studies were conducted on the utilization of urea as a replacement for protein in ruminant diets, especially its effect on dry matter intake (Wilson et al., 1975; Polan et al., 1976), rumen fermentation (Pisulewski et al., 1981; Kertz et al., 1983), milk yield and reproduction-related parameters (Ryder et al., 1972; Erb et al., 1976). Since then, research trying to understand the mechanisms of urea utilization in dairy cows has been conducted (Balcells et al., 1993; Huntington and Archibeque, 2000; Stewart and Smith, 2005).

Following extensive research on the urea utilization in the rumen, interests began to focus on the rumen urea-degrading microbes and mechanisms involved in urea nitrogen utilization in dairy cows. Rumen ureolytic bacteria play an important role in dietary urea hydrolysis, for they produce ureases which catalyze the breakdown of urea to ammonia (NH_3) and carbon dioxide (Owens et al., 1980). In the rumen, the ammonia can be assimilated by many rumen bacteria for synthesis of microbial protein required to satisfy the protein requirements of ruminants (Milton et al., 1997; Firkins et al., 2007). The rumen redundant NH_3 is subsequently absorbed into the circulation through the rumen wall and is used for hepatic urea synthesis (De Visser et al., 1997; Recavarren and Milano, 2014; Holder et al., 2015). The endogenous urea was recycled for utilization by transfer across the ruminal wall, and salivary secretion (Huntington and Archibeque, 2000). In ruminants, urea that is recycled to the rumen is an important source of N for microbial growth and the reported data indicate that 40 to 80% of endogenously produced urea nitrogen is returned to the gastrointestinal tract (Harmeyer and Martens, 1980; Lapierre and Lobley, 2001).

However, urea is rapidly hydrolyzed to ammonia within 30 min to 2 h by the urease enzyme produced by the ruminal microorganisms (Rekib and Sadhu, 1968), hydrolysis of urea to NH_3 occurs at a greater rate than NH_3 can be utilized, and this is the main cause of limited utilization of urea as a non-protein nitrogen (NPN) source for microbial protein synthesis (Patra, 2015). Due to the vast diversity and extreme complexity of the rumen microbes, and difficulty in cultivating the rumen bacteria, only a small number of rumen bacteria have been isolated (Kim et al., 2011). The lack of sufficient understanding of the ruminal microbiome is one of the major knowledge gaps that hinder effective enhancement of rumen functions (Firkins and Yu, 2006). Therefore, investigation of the rumen ureolytic bacterial community and mechanisms of urea nitrogen utilization in ruminants could provide basis for obtaining regulatory targets to moderate urea hydrolysis in the rumen, and provided novel information to aid understanding the metabolic pathways affected by urea nitrogen in dairy cows, and help to improve the efficiency of urea utilization in the rumen.

Nowadays, there are breakthroughs in molecular strategies for studying the microbiome as well as its host metabolism. The rapid advancement of "~omics" technologies, including metagenomics, metatranscriptomics, metaproteomics, metabolomics, and bioinformatics have been applied for analysis complex rumen microbes and their metabolism as well as functions, and will provide the unprecedented opportunities to disentangle the complex relationships between feed, rumen microbiome, rumen function and host metabolism. In our research, combining the metagenomics, metabolomics and bioinformatic analysis, the rumen ureolytic bacterial community, the microbial and host metabolism induced by urea nitrogen were investigated, these research could give a better understanding of the microbial and molecular mechanism of ruminal urea hydrolysis and utilization, and will provide knowledge for helping to improve the efficiency of urea utilization in the rumen.

2. Objectives

The objective of this study is to investigate the diversity and distribution of rumen ureolytic bacteria community and the mechanisms of urea utilization in dairy cows using sequencing and metabolomics.

(1) To investigate the rumen abundant ureolytic bacterial community and the diversity and distribution of the rumen ureolytic bacteria in different rumen fractions using high-throughput sequencing.

(2) To identify changes in both rumen microbial and host plasma metabolic profiles induced by urea supplementation in dairy cows using metabolomics.

To achieve these goals, the *in vitro* and *in vivo* studies have been designed and completed respectively. These experiments and subsequent published or submitted articles that make up the body of this thesis are briefly depicted in the following section.

3. Thesis structure

First, a literature review introduces urea hydrolysis by ruminal ureolytic bacterial community and urea utilization in ruminants is presented (Chapter II). This review outlined how urea is hydrolyzed to ammonia with the help of urease that synthesized by rumen ureolytic bacteria, the host urea metabolism, and the regulation of urea hydrolysis and strategies for improving urea utilization efficiency in dairy cows. Chapter II has been submitted for publication to *Annals of Animal Science* (Article 1) and is presently under minor revision.

In Chapter III, we investigated the abundant rumen ureolytic bacterial community using rumen simulation system. Urea and acetohydroxamic acid (AHA) were used as the stimulator or inhibitor for ureolytic bacteria respectively, and the bacterial 16S rRNA genes were sequenced by high-throughput sequencing and used to reveal abundant ureoltyic bacteria composition. The results of Chapter III (Article 2) have been published in *Frontiers in Microbiology* (2016), 7:1006.

The bacterial urease gene (*ureC*) has been the target gene of choice for analysis of the urea degrading microorganisms in various environments. In Chapter IV, we investigated the predominant *ureC* genes of the ureolytic bacteria in the rumen of dairy cows using high-throughput sequencing. We revealed the differences in ureolytic bacterial composition between the rumen digesta and rumen wall based on *ureC* gene classification. This survey has expanded our knowledge of *ureC* gene information relating to the rumen ureolytic microbial community, and provides a basis for obtaining regulatory targets of ureolytic bacteria to moderate urea hydrolysis in the rumen. The results of this experiment are published in Article 3 in *Frontiers in Microbiology* (2017), 8: 385.

Then, in Chapter V, we investigated changes in rumen microbial and plasma metabolite profiles in dairy cows induced by urea nitrogen using a metabolomics approach. The varied metabolites were identified by nuclear magnetic resonance spectroscopy and multivariate analysis of variance. The metabolic pathways of the changed metabolites were also identified based on relative databases. These findings provided novel information to aid understanding

of the metabolic pathways affected by urea nitrogen in dairy cows, and is presented in Article 4 which is undergoing under review for publication in *Livestock Science*.

Finally, Chapter VI presents a general discussion of the results obtained through previous chapters and a perspective for the further study was also presented.

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CHAPTER II

This chapter outlined how urea is hydrolyzed to ammonia with the help of urease that synthesized by rumen ureolytic bacteria, the host urea metabolism, and the regulation of urea hydrolysis and strategies for improving urea utilization efficiency in dairy cows.

Article 1

Urea hydrolysis by ruminal ureolytic bacterial community and utilization in ruminants: a review

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Running headline: Urea hydrolysis and utilization in ruminants

This chapter is adapted from an article that submitted to Annals of Animal Science and is presently under (minor) revision.

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1. Abstract

Urea is used as non-protein nitrogen in the ration of ruminants as an economical replacement for feed proteins. It is rapidly hydrolyzed by rumen bacterial urease to ammonia (NH_3) and the NH_3 is utilized for synthesis of microbial proteins required for the animal growth. Urea transferred from the blood to the rumen is also an important source of nitrogen for rumen microbial growth. Urea has commonly become an accepted ingredient in the diets of ruminants. During the past decades, urea utilization in ruminants has been investigated by using traditional research methods. Nowadays, some modern molecular biotechnologies have also been applied to analyzing the urea-degrading bacteria or the urea nitrogen metabolism in ruminants. Combining the traditional and molecular approach, we can get better information and understanding related to the mechanisms of urea metabolism in ruminants. This review discusses urea hydrolysis by the rumen ureolytic bacteria and urea utilization metabolism in the host. The progress of the accumulated research provides foundations for proposing further new strategies to improve efficiency of urea utilization in ruminants.

Keyword: Rumen, ureolytic bacteria, urease, urea transport, urea recycling.

2. Introduction

Urea has been used as non-protein nitrogen (NPN) in ruminant rations for a long time. Kertz (2010) wrote in his paper that more than one hundred years ago, German workers suggested that urea could be used to replace a portion of dietary protein in ruminants. Thereafter, some studies were conducted on the use of NPN in ruminant diets. During the 1970s and 1980s, multiple studies were conducted on the utilization of urea as a replacement for protein in ruminant diets, especially its effect on dry matter intake (Wilson et al., 1975; Polan et al., 1976), rumen fermentation (Pisulewski et al., 1981; Kertz et al., 1983), milk yield and reproduction-related parameters (Ryder et al., 1972; Erb et al., 1976). Urea use for dairy cattle appeared to be the predominant category for ruminant use. A model has been proposed for predicting efficacy of NPN supplementation that based upon the assumption that NPN is not utilized when ruminal ammonia concentrations exceed the requirement of the ammonia-utilizing bacteria (Roffler and Satter, 1975b). Additions of NPN to rations resulting in predicted ruminal ammonia concentrations greater than 5 mg ammonia nitrogen/100 ml rumen fluid were without benefit. NPN supplementation did not improve milk production if the ration contained more than 13% crude protein prior to supplementation (Roffler and Satter, 1975a). So, use of NPN supplements should be restricted to those dietary conditions which promote conversion of ammonia to microbial protein in the rumen. Following, researches trying to understand the mechanisms of urea utilization in dairy cows have been conducted (Balcells et al., 1993; Huntington and Archibeque, 2000; Stewart and Smith, 2005).

Nowadays, studies for improving urea utilization in dairy cows are ongoing. It is known that performance and metabolism of dairy cows depends upon the amount of urea fed (Sinclair et al., 2012; Giallongo et al., 2015). For example, ruminal nitrogen metabolism and urea kinetics of Holstein steers fed diets containing either rapidly degrading or slowly degrading urea at various levels of degradable intake protein (DIP) were estimated by Holder et al. (2015). They found that the rapidly degrading urea group had higher dry matter digestibility than the slow-release urea group, and gastrointestinal entry of urea-N, urea-N lost to feces and urea-N apparently used for anabolism were not different between treatments while plasma urea concentration was greater in higher DIP diets and higher for the rapidly degrading urea group than the slow release urea group. When 2% of urea was fed to lactating dairy cows as a replacement for soybean meal, both the milk protein content and milk yield decreased, while plasma urea nitrogen increased (Imaizumi et al., 2015). Urea

supplementation could increase nitrogen availability for ruminal microorganisms. A study by Wanapat et al. (2016) showed that when swamp buffaloes were fed rice straw supplemented with urea, the feed intake, nutrient digestibility and microbial protein synthesis increased. More importantly, the author also tried to reveal the effect of urea supplementation on rumen microbes and they found that fungal zoospores, total bacteria and the three predominant cellulolytic bacteria (*Ruminococcus albus*, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens*) were increased by urea supplementation.

Following extensive research on urea utilization in the rumen, interests began to focus on the urea-degrading microbes and urea utilization mechanisms in dairy cows. Researches for regulating bacterial urease for improving urea utilization have also been conducted. The advanced molecular biotechnologies provide new strategies to reveal the mechanisms of urea nitrogen hydrolysis, transportation and utilization in ruminants, and provide more knowledge for the improvement of nitrogen utilization efficiency in practical ruminant production system. This review focuses on ruminal urea hydrolysis by ureolytic bacteria, urea utilization and its regulation for improving the utilization efficiency in ruminants.

3. Urea nitrogen recycling in ruminants

For ruminants, ammonia and urea arise in the rumen from the diet. Urea in the rumen is immediately hydrolyzed to ammonia and CO_2 by the bacterial enzyme urease. Ammonia from urea or from degraded dietary protein is used by the ruminal microbiota for synthesis of microbial proteins which are subsequently digested in the intestine. The excess ammonia is transported to the liver for endogenous urea synthesis, and urea recycling via the ruminal wall, and salivary secretion (Figure 1). Urea recycling to the rumen is an evolutionary advantage for ruminants because it provides a source of N for microbial protein synthesis and enhances survival (Reynolds and Kristensen, 2008).

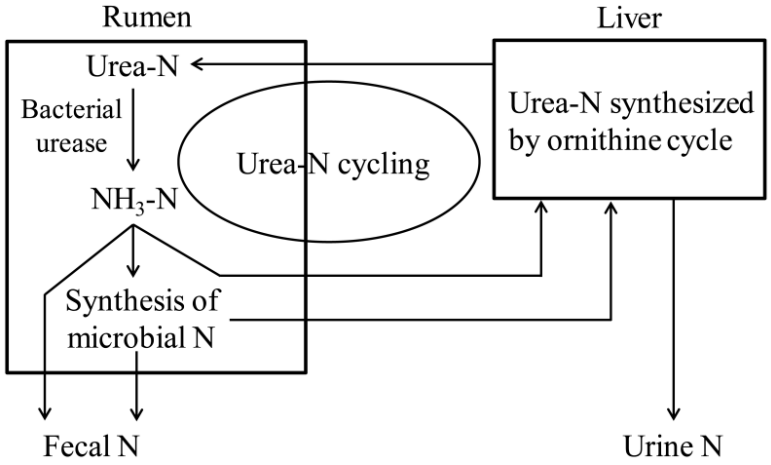

Figure 1 Urea nitrogen (Urea-N) recycling in ruminants

3.1. Reutilization of endogenous urea

Ruminants fed on diets with high NPN had higher portal blood flow, greater hepatic uptake of NH_3 and increased rates of urea synthesis (Symonds et al., 1981; De Visser et al., 1997; Holder et al., 2015). Redundant NH_3 transported to the liver is likely to enter the ornithine cycle (Zhou et al., 2015). Therefore ammonia detoxification in the liver likely results in part to increased plasma urea concentration (Law et al., 2009). Blood urea nitrogen concentrations are influenced by many parameters, especially dietary nitrogen intake (Puppel and Kuczynska, 2016), and it also has been used to predict nitrogen excretion and efficient nitrogen utilization in cattle and several different species of farm animals (Kohn et al., 2005). Ruminants recycle substantial amounts of nitrogen as urea by transfer of urea across the ruminal wall, and salivary secretion (Huntington and Archibeque, 2000). In ruminants, urea that is recycled to the rumen is an important source of N for microbial growth and the reported data indicate that 40 to 80% of endogenously produced urea nitrogen is returned to the gastrointestinal tract (Harmeyer and Martens, 1980; Lapierre and Lobley, 2001). The transfer of endogenous urea through the rumen wall increased when a high concentrate diet was fed compared to the a alfalfa hay diet, and this may be due to increased numbers and activity of ureolytic bacteria adhering to rumen epithelium and decreased concentration of ammonia in the rumen (HUNTINGTON, 1989). Wickersham et al. (2008) evaluated the effect of increasing amounts of rumen-degradable intake protein (DIP) on urea kinetics in steers consuming prairie hay with jugular infusions of $^{15}N^{15}N$-urea. They found that the amount of urea-N entering the gastrointestinal tract was greatest for the high DIP diet and decreased linearly with the decreased DIP concentration. Similarly, Zhou et al. (2015) also used $^{15}N^{15}N$-urea to detect urea kinetics and nitrogen balance in Tibetan sheep when fed oat

hay. Urea-N entry rate, gastrointestinal tract entry rate, return to ornithine cycle and fecal urea-N excretion all increased linearly with an increase in dry matter intake.

Currently, some meta-analytical approaches have been applied in order to get better understanding of the efficiency of urea utilization in ruminants. In the study of Marini et al. (2008), by utilizing a statistical approach and data obtained from studies reporting duodenal, ileal, and fecal N flows in cattle, the endogenous N (EN) losses and true digestibility of N were estimated for different segments of the gastrointestinal tract of cattle. The N transactions for the reference diet (24.2 g of N/kg of organic matter (OM), 32% neutral detergent fiber and carbohydrates of medium fermentation rate) were estimated. The results showed that the minimal contribution of EN to the N available in the rumen was 39%. The free EN represented 13% of the duodenal N flow, and when bacterial N of EN origin was considered, EN contributed 35% of the total N flow. Besides, Batista et al. (2017) also estimated the urea kinetics and microbial usage of recycled urea N in ruminants by combining data from studies with ruminants (beef cattle, dairy cows and sheep) which were published from 2001 to 2016 and analyzed according to meta-analysis techniques using linear or non-linear mixed models. They concluded that urea N synthesized in the liver and urea N recycled to the gut linearly increased as N intake (g/$BW^{0.75}$) increased, with increases corresponding to 71.5% and 35.2% of N intake, respectively. However, increasing dietary crude protein (CP) intake led to decreases in the fractions of urea N recycled to the gastrointestinal tract and of recycled urea N incorporated into microbial N. Therefore, a better understanding of the factors involved in EN losses will allow for a more accurate estimation of both N supply and N requirements. Since urea-N recycling to the gut is influenced by many dietary and ruminal factors, some modulation could be made in the ration of ruminants in order to improve the efficiency of utilization of endogenous urea.

3.2. Urea transport across the rumen epithelium

Urea produced in the liver, is transferred across the rumen wall from the blood and then it is hydrolyzed to ammonia by resident bacteria (Lapierre and Lobley, 2001). As is already known, urea transport across the ruminant wall is mediated via urea transporters in the epithelium membrane. These transporters allow the passage of urea across cell membranes, down a concentration gradient (Smith and Rousselet, 2001). Facilitative urea transporters are derived from the UT-A and UT-B genes (Bankir et al., 2004). UT-B mRNA or protein expressions have been characterized in the rumen epithelium (Stewart et al., 2005; Simmons et al., 2009; Lu et al., 2015). UT-B transporters were also identified to be specifically

localized to certain regions of tissue in the bovine gastrointestinal tract (Coyle et al., 2016). In addition to the UT-B transporters, some alternative transport mechanisms are also involved in urea transport across the rumen epithelium. The aquaporins (AQP), a family of membrane-spanning proteins predominantly involved in water movement, AQP-3, -7, -9 and -10 are also involved in urea uptake or transport (Rojek et al., 2008; Litman et al., 2009). Rojen et al. (2011) showed that messenger RNA expression of AQP3, AQP7, and AQP10 and abundance of AQP8 increased with increasing nitrogen intake, but their findings do not point to these proteins as the cause of increased rumen epithelial urea permeability in dairy cows fed a low N diet. Walpole et al. (2015) have determined the functional roles of UT-B and AQP in the serosal-to-mucosal urea flux across rumen epithelium using Ussing chambers. The urea flux markedly decreased when Phloretin and $NiCl_2$ were added to inhibit UT-B- or AQP-mediated urea transport, respectively. Gene transcript abundance for UT-B and AQP was observed to be significantly correlated with the ruminal serosal to mucosal urea flux. However, the mechanism by which the increased gene expression occurred is unclear. Nowadays, transcriptome analysis has been used to analyze the rumen epithelium metabolic pathway changes under various conditions (Baldwin et al., 2012; Naeem et al., 2014), and this approach may provide better means to understand the regulation of these urea transport mechanisms across the rumen wall.

4. Urea hydrolysis by rumen ureolytic bacteria

Rumen ureolytic bacteria play an important role in dietary urea hydrolysis, for they produce ureases which catalyze the breakdown of urea to NH_3 and carbon dioxide (Owens et al., 1980). In the rumen, the ammonia can be assimilated by many rumen bacteria for synthesis of microbial proteins (Owens et al., 1980; Milton et al., 1997). However, efficiency of urea nitrogen utilization in ruminants is low and this is attributed to the rapid hydrolysis of urea to NH_3 which occurs at a higher rate than NH_3 utilization by rumen bacteria (Patra, 2015). Due to the difficulty in cultivating the rumen bacteria, only a small number of bacteria have been isolated (Kim et al., 2011). The lack of sufficient understanding of the ruminal microbiome is one of the major knowledge gaps that hinder effective enhancement of rumen functions (Firkins and Yu, 2006). Also, limited information about the rumen urea-degrading bacteria makes regulation of the urea hydrolysis rate by targeting the predominant ureolytic bacteria difficult.

4.1. Ureolytic bacteria isolated using culture-dependent methods

Early studies have isolated some ureolytic bacteria from the rumen (Cook, 1976; On et al., 1998). Wozny et al. (1977) described a rapid qualitative procedure to detect urease in strains isolated from the bovine rumen, and found that many species including *Succinivibrio dextrinosolvens*, *Treponema* sp., *Ruminococcus brom*i, *Butyrivibrio* sp., *Bifidobacterium* sp., *Bacteroides ruminicola*, and *Peptostreptococcus productus* had urease activity and most *P. productus* strains contain urease. Kakimoto et al. (1989) assayed about 16,000 isolates from animal feces and intestines for the production of acid urease and found that most of the selected strains belonged to the genera *Streptococcus* and *Lactobacillus*. In a similar study by Laukov á and Koniarov á (1994), they tested 909 strains from the rumen of 104 domestic and wild ruminants for urease activity, and their results showed that some *Selenomonas ruminantium* strains and *lactobacilli* manifested medium urease activity and most of the *Enterococcus faecium* and all of the *E. faecalis* isolates expressed urease activity. In addition, *Howardella ureilytica*, a Gram-positive bacterium has been isolated from the rumen fluid of sheep, it was strongly ureolytic and generated ATP through the hydrolysis of urea (Cook et al., 2007). All these above studies were conducted using culture-based methods, and limited ureolytic bacteria were isolated, also the research only identified the urease activity of the isolated bacteria. Information about the urease genes which express the urease activity was not achieved.

4.2. Culture-independent methods in studying the ureolytic bacteria

In order to get further information about the function of rumen microbes, sequencing and phylogenetic analysis of 16S rRNA genes and functional genes have been extensively carried out in studies focused on members of the uncultivable bacteria (Chaucheyras-Durand and Ossa, 2014). For the ureolytic bacteria, the *ureC* subunit is the largest of the genes encoding urease functional subunits and contains several highly conserved regions that are suitable as PCR priming sites (Mobley et al., 1995). Previously, Reed (2001) successfully designed the urease PCR primers that can be used to amplify a 340 bp fragment of the *ureC* gene from a variety of urease producing bacteria. Then, primers for *ureC* gene have been developed and applied to the analysis of urea-degrading microorganisms in various environments, including the open ocean (Collier et al., 2009), sponges (Su et al., 2013), and soil (Singh et al., 2009) (Table 1). Zhao et al. (2015) attempted to examine rumen ureolytic bacterial diversity by cloning and sequencing *ureC* genes, and found that among the total 317 *ureC* sequences from the rumen digesta, some were about 84 % identical (based on amino acid sequence) to the

ureC gene of *H. pylori*. They also developed a vaccine based on *ureC* of *H. pylor*i, vaccinated cows had significantly reduced urease activity in the rumen compared to the control cows that were mock immunized. Jin et al. (2016) have attempted to reveal abundant ureolytic bacterial communities by high-throughput sequencing when treated with an activator (urea) or inhibitor (acetohydroxamic acid, AHA) of ureolytic bacteria *in vitro*, and results from 16S rRNA gene sequencing showed that rumen ureolytic bacteria were abundant in the genera of *Pseudomonas*, *Haemophilus*, *Neisseria*, *Streptococcus*, *Actinomyces*, *Bacillus*, and unclassified Succinivibrionaceae. Recently, Jin et al. (2017) studied the differences in ureolytic bacterial composition between the rumen digesta and rumen wall based on *ureC* Gene Classification, and found that more than 55% of the *ureC* sequences did not affiliate with any known taxonomically assigned urease genes, and the most abundant *ureC* genes were affiliated with Methylococcaceae, Clostridiaceae, Paenibacillaceae, Helicobacteraceae, and Methylophilaceae families. They also found that relative abundance of the ureolytic bacteria affiliated with *Methylophilus* and *Marinobacter* genera were significantly higher in the wall-adherent bacteria compared with bacteria in the rumen content. Studies which target the *ureC* genes provide a basis for obtaining the full-length urease functional gene information (Yuan et al., 2012). Further insights into abundant ureolytic bacteria communities could provide the basis for designing strategies to efficiently manipulate the rumen bacteria and improve urea utilization in ruminants.

5. Bacterial urease

5.1. Urease activity in the rumen

Three distinct bacterial populations in the bovine rumen are conventionally defined: the liquid-associated bacteria, the solid-associated bacteria, and bacteria adherent to the epithelial wall (Cheng et al., 1977; Cheng and Costerton, 1980). The urease activities are contributed by the rumen ureolytic bacteria in these three fractions. In the early days, Rahman and Decker (1966) had suggested that in ruminant species, urease activity is greatest within the stratified layers of the rumen epithelium and the urease in the rumen mucosa originated from bacteria. The distribution and changes of urease activity have been investigated by Czerkawski and Breckenridge (1982) using a heterogeneous fermentation system. The ureolytic activity was found higher in space occupied by micro-organisms that are loosely associated with the solid than in strained rumen contents or space occupied by microbial population that cannot be washed out of the solid matrix. Javorsk ý et al. (1986) found that *in*

vitro ureolytic activity was highest in the bacteria adhered to the rumen wall, intermediate in rumen fluid bacteria and lowest in bacteria adherent to feed particles in rumen of sheep. It is thought that ureolytic bacteria attached to the rumen epithelium facilitate the movement of urea across the rumen wall (Wallace, 1979; Cheng and Costerton, 1980). Ruminal urease activity is likely a major modulator for urea transfer across the rumen wall by producing a urea gradient into the rumen (Abdoun et al., 2006). The predominant populations of bacteria adhering to the rumen wall have proven to be different from the luminal microorganisms (Chen et al., 2011; Petri et al., 2013), and this may be the reason why the urease activities varied among different rumen fractions. While research of Moharrery and Das (2001) showed that no clear difference of urease activity was found among strained rumen fluid without protozoa, cell free rumen fluid and enzymes associated with the bacteria cell.

Table 1 Environmental ureolytic bacteria community investigated using sequencing methods

Environment	*UreC* primers (5'-3') for sequencing	New discovery	Reference
Groundwater	Forward: L2F (ATHGGYAARGCNGGNAAYCC) Reverse: L2R (ATHGGYAARGCNGGNAAYCC)	Amplified novel *ureC* sequences from groundwater isolates in the genera Hydrogenophaga, Acidovorax, Janthinobacterium, and Arthrobacter.	(Gresham et al., 2007)
Open-ocean and estuarine plankton	Forward: HEDWG primer (GCTATCGGTCTCAAACTTCAYGARGAYTGGGG) Reverse: cTINP primer (GCAATACCATGCGCAATCGCNGCNGGRTTDATNGT)	709 urease gene fragments from 31 plankton samples collected at both estuarine and open-ocean locations were sequenced, and 423 amplicons were not closely enough related to named organisms to be identified, and belonged to 96 distinct sequence types of which 43 types were found in two or more different samples.	(Collier et al., 2009)
Grasslandsoil	Forward: ureC-F (TGGGCCTTAAATHCAYGARGAYTGGG) Reverse: ureC-R (GGTGGTGGCACACCATNANCATRTC)	The ureolytic community comprised of members from a range of phylogenetically different taxa including Bradyrhizobium, Bacillus, Methylobacter spp., Flavobacterium johnsoniae, and Methylobacterium spp.	(Singh et al., 2009)
Marine Sponge Xestospongia testudinaria	Forward: L_2F (ATHGGYAARGCNGGNAAYCC) Reverse: L_2R (ATHGGYAARGCNGGNAAYCC)	Most of the *ureC* sequences were similar with the urease alpha subunit of members from *Proteobacteria*, which were the predominant component in sponge X. testudinaria, and the remaining *ureC* sequences were related to those from Magnetococcus, Cyanobacteria, and Actinobacteria.	(Su et al., 2013)
Rumen bacteria of dairy cows	Forward: ureC forward (TGGGCCTTAARMTHCAYGARGAYTGGG) Reverse: ureC reverse (GTGRTGRCAMACCATNANCATRTC)	Most alpha subunit of rumen urease (*UreC*) proteins shared very similar amino acid sequences, which were also highly similar to that of H. pylori.	(Zhao et al., 2015)

Some studies have focus on the location of urease in the bacteria which could help us to understand the varied urease activity in different fraction. The urease in a rumen *Staphylococcus* sp. has been described to be a membrane-bound urease (McLean et al., 1985). For the *Helicobacter pylori*, the enzyme was located on the cell surface (Hawtin et al., 1990). But to date, the urease has not been localized for many other rumen bacteria cells. So, in view of the relative importance of urease activity in the nitrogen metabolism of the rumen, it is of great interests to ascertain the location of ureolytic activity in the cells of the ruminal bacteria, and more work have to been taken to progress this areas.

5.2. Characterization and activation of bacterial ureases

Microbial ureases (urea amidohydrolases, EC 3.5.1.5) are nickel-dependent enzymes and commonly composed of two or three submits complexes (encoded by genes *ureA*, *ureB*, and *ureC*), and require up to several accessory proteins for activation (Mobley et al., 1995a). For example, the urease of *Klebsiella aerogenes* has three subunits $(UreABC)_3$ (Jabri et al., 1995). The urease of *Helicobacter pylori* consists of two subunits $((ureAB)_3)_4$, and *ureB* in the *Helicobacter* species is equivalent to *ureC* in the organisms possessing a three-subunit enzyme (Hu and Mobley, 1990). Urease accessory genes (such as *ureD*, *ureE*, *ureF*, *ureG*, *ureH*, and *ureI*) are required for synthesis of catalytically active urease when the gene clusters are expressed in a recombinant bacterial host. Some of the accessory genes were shown to play a role in activation of the apoenzyme, and these genes are known to be required for assembly of the nickel metallocenter within the active site of the enzyme (Mehta et al., 2003; Witte et al., 2005; Boer and Hausinger, 2012). All purified ureases that have been analyzed for metal content have been shown to possess nickel, and the presence of urease activity in ureolytic organisms uniformly exhibits a dependence on nickel in the growth medium. So bacterial ureases universally appear to contain nickel, but the nickel content varies among the different enzymes (Mobley et al., 1995b). Taking the urease activation of *Klebsiella aerogenes* as an example, the UreD, UreF, UreG, and UreE are sequentially complexed to UreABC as required for its activation (Farrugia et al., 2013) (Figure 2).

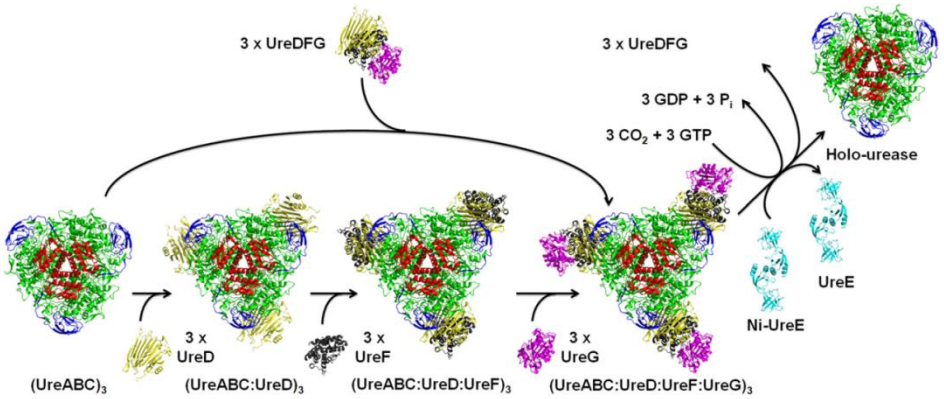

Figure 2 Model of K. aerogenes urease activation.

The trimer-of-trimers urease apoprotein (UreA, red; UreB, blue; UreC, green) either sequentially binds UreD (yellow), UreF (gray), and UreG (magenta) or binds the UreDFG complex (only one protomer of each protein is shown, but the isolated complex contains two protomers of each). Formation of the active enzyme requires CO2 to carbamylate Lys-217 at the native active site, GTP binding to and hydrolysis by UreG, and nickel delivery by dimeric UreE (cyan). It remains unclear whether the accessory proteins are released as a UreDFG unit or as individual proteins (Farrugia et al., 2013).

Some studies have been done to explore the structures for this activation complex (Biagi et al., 2013; Fong et al., 2013). Ligabue-Braun et al. (2013) provide an atomic-level model for the (UreABC–UreDFG)$_3$ complex from *K. aerogenes* by employing comparative modeling associated to sequential macromolecular dockings, validated through small-angle X-ray scattering profiles. The resulting model included a putative orientation for UreG at the (UreABC–UreDFG)$_3$ oligomer. Fong et al. (2013) have proposed a mechanism on how urease accessory proteins facilitate maturation of urease. They reported the crystal structure of the UreG/UreF/UreH complex in *Helicobacter pylori*, which illustrates how UreF and UreH facilitate dimerization of UreG, and assembles its metal binding site by juxtaposing two invariant Cys66-Pro67-His68 metal binding motifs at the interface to form the (UreG/UreF/UreH)$_2$ complex. Further, Zambelli et al. (2014) identified the nickel binding properties of *Helicobacter pylori* UreF in the nickel-based activation of urease. UreF binds two Ni^{2+} ions per dimer, with micromolar dissociation constant. Two nearly identical and symmetric tunnels were found, going from the central cavity in the UreG/UreF/UreH complex, and UreF was involved in the metal ion transport through these tunnels during urease activation. Currently, many aspects of the urease metallocenter assembly still remain

obscure. The activation mechanism and roles of each accessory protein in urease maturation still need to be answered.

5.3. Regulation of bacterial urease synthesis

The regulation of urease synthesis in ureolytic bacteria is complex. In some organisms such as *Bacillus pasteurii* and *Morganella morganii*, urease synthesis is constitutive (Mörsdorf and Kaltwasser, 1989; Burbank et al., 2012). However, urease synthesis in some bacteria is regulated by environmental conditions, such as concentration of urea and nitrogen or pH (Weeks and Sachs, 2001; Dyhrman and Anderson, 2003; Belzer et al., 2005; Liu et al., 2008). Urease activity of *Providencia stuartiiin*, for example, is induced by the presence of urea (Armbruster et al., 2014), while *Klebsiella pneumoniae* can use urea as the sole source of nitrogen, and the urease expression is regulated by the supply of nitrogen in the growth medium (Liu and Bender, 2007). The regulation of urease gene expression of *Actinomyces naeslundii* under different environmental conditions has been investigated by Liu et al. (2008). They found that the conditions of neutral pH, fast dilution rate, increased carbohydrate supply or low amino acid nitrogen supply in the medium all resulted in the enhancement of urease activity in *Actinomyces naeslundii*. In research comparing the regulation of urease activity in *Helicobacter hepaticus* and *Helicobacter pylori*, the urease activity of *H. hepaticus* was found to be acid-independent, which contrasts with the acid-induced urease system of *H. pylori* (Belzer et al., 2005). When the model rumen *Firmicutes* organism *Ruminococcus albus* 8 were supplied with different nitrogen sources (urea, ammonia and peptides), the urease activity was higher in the presence of urea than in the presence of ammonia and peptides (Kim et al., 2014). But urease transcript abundance in *R. albus* 8 is not predicated on the presence of urea in the medium. This urease activity may demonstrate that *R. albus* 8 expresses urease to acquire urea as an alternative nitrogen source when the ammonia concentration in the medium is limited. Since the regulation of urease activity is complex and the rumen harbors a large diversity of ureolytic bacteria, the mechanisms controlling urease synthesis in the complicated rumen environment need further research.

6. Utilization of urea in the rumen: ammonia assimilation

In the rumen, great amounts of ammonia are produced during both the protein and NPN degradation, and ammonia is both a satisfactory and essential source of nitrogen for most of

rumen bacteria (Patra, 2015). It has been suggested that NH_3 uptake is mediated by an active carrier for the translocation of NH_3 into the cell (Thomas et al., 2000). Ammonia assimilation is the process of ammonia incorporation into carbon skeleton which is the first step for NH_3 utilization (Wang and Tan, 2013). There are two classic routes for ammonia assimilation in bacteria, one pathway fixed $NH4^+$ through the action of an NADPH-linked glutamate dehydrogenase (GDH). The second pathway fixed NH_4^+ into the amide of glutamine by an ATP-dependent glutamine synthetase (GS) (DANIEL, 2009). The GDH is the main mechanism of NH_3 assimilation, GS activity was highest in cells grown under nitrogen limitation (Wallace, 1979). Most of the rumen bacteria such as *Selenomonas ruminantium, Ruminococcus flavefaciens* and *Streptococcus bovis* were found to possess two pathways for ammonia assimilation that resulted in glutamate synthesis (Griffith and Carlsson, 1974; Pettipher and Latham, 1979; Smith et al., 1980; Duncan et al., 1992). In some rumen bacteria, asparagine synthetase also participates in the ammonia assimilation (Ciustea et al., 2005). The rumen bacteria could also possess effective mechanisms for alanine synthesis from ammonia by alanine dehydrogenase (Morrison and Mackie, 1996; Oba et al., 2005). Hence, the rumen bacteria use ammonia to synthesize amino acids and peptides required for synthesis of microbial protein (Pfeffer and Hristov, 2005). The detailed pathways and regulation mechanisms of ammonia assimilation in ruminal bacteria have been demonstrated by Wang and Tan (2013).

7. Strategys for improving urea utilization in rumen

For ruminants, reducing the rate of rumen urea hydrolysis is of great importance for improving feed urea utilization and minimizing ammonia wastage. Some strategys such as urea inhibitors and some new forms of urea have been developed to slow ammonia release in the rumen.

7.1. Urease inhibitors

Urease inhibitors are one of the available options proven to be an effective way to reduce feed urea hydrolysis. Supplementation of urease inhibitors have proven to be an effective way to reduce urea hydrolysis in the rumen, and several urease inhibitors, including acetohydroxamic acid (AHA) (Brent et al., 1971; Jones and Milligan, 1975), phenylphosphorodiamidate (Voigt et al., 1980a; Voigt et al., 1980b; Whitelaw et al., 1991), and N-(n-butyl) thiophosphoric triamide (NBPT) have been investigated (Ludden et al., 2000). Zhang et al. (2001) also studied the effect of hydroquinone on ruminal urease activity

and found the hydroquinone at concentrations of 0.01 ppm to 10 ppm inhibited urease activity of intact rumen microbes *in vitro* by 25% to 64%. Urease inhibitors also provide insight to understanding the mechanism of enzyme catalytic activity present at the active site of enzyme and the importance of nickel to urease, the metalloenzyme (Upadhyay, 2012). The mechanism of *Bacillus pasteurii* urease inhibition with acetohydroxamic acid was solved, the inhibitor anion symmetrically bridging the two Ni ions in the active site through the hydroxamate oxygen and chelating one Ni ion through the carbonyl oxygen (Benini et al., 2000). Although, recent studies have already evaluated the function of different urease inhibitors in improving urea utilization efficiency (Ludden et al., 2000; Giallongo et al., 2015), further research is needed to investigate the response of the rumen bacteria community, especially the ureolytic bacteria to these inhibitors.

7.2. Slow-release urea

Slow release urea is another solution to control urea hydrolysis rate so that NH_3 release more closely parallels carbohydrate digestion (Pinos-Rodr ́guez et al., 2010). Slow release urea compounds include biuret, starea, urea phosphate, coatings based on oil, formaldehyde treated urea and polymer-coated urea have been fed to ruminants (Cherdthong and Wanapat, 2010). More recently, Cherdthong and Wanapat (2013) have investigated the influence of urea-calcium sulphate mixture (U-CaS), a kind of slow release urea, in feed blocks on rumen micro-organisms and microbial protein synthesis in Thai native beef cattle. Results showed that microbial crude protein yield and efficiency of microbial nitroge synthesis were linearly increased with different levels of U-CaS addition, so as the concentrations of total bacteria and *Fibrobacter succinogenes*. The U–CaS not only contains urea, but also contains CaSO4, a good available source of sulphur, which is an essential element for rumen bacterial growth and its metabolism is closely related to N metabolism. Thus, the continuous availability of N and sulphur for ruminal fermentation is important. What's more, research of Giallongo et al. (2015) showed that total-tract apparent digestibility of crude protein was increased with the addition of slow release urea (Optigen) to the metabolizable protein-deficient diet. Besides, supplementation of urea and cassava hay for buffaloes fed rice straw improved rumen ecology and increased fermentation end products and microbial protein synthesis while reducing protozoal populations (Ampapon et al., 2016). Slow-release urea products provide constant supply of ammonia to rumen microorganism for their growth, which also improves nutrition utilization for low-quality forages (Patra, 2015).

8. Summary

Urea is one of the major non-protein nitrogen feeds for ruminants and the optimal utilization of urea in feed can alleviate to some extent the cost of dietary protein. Urea is hydrolyzed quickly by ureolytic bacteria in the rumen. Since about 90% of rumen microbes have not been pure-cultured to date, only limited information about active ureolytic bacteria communities are known, which limits the regulation and efficient application of urea in ruminant production. The rapid advancement of "~omics" technologies, including metagenomics, metatranscriptomics, metabolomics, and bioinformatics could give a better understanding of the microbial and molecular mechanisms of ruminal urea hydrolysis and utilization, and will provide the knowledge for improving urea utilization efficiency in ruminants.

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CHAPTER III

Urea, a kind of non-protein nitrogen for dairy cows, is rapidly hydrolyzed to ammonia by urease produced by ureolytic bacteria in the rumen, and the ammonia is used as nitrogen for rumen bacterial growth. Ureolytic bacteria play important role in urea hydrolysis in the rumen. This study revealed abundant ureolytic bacterial community by high-throughput sequencing in a rumen simulation system when treated with an activator (urea) or inhibitor (AHA) of ureolytic bacteria.

Article 2

Insights into abundant rumen ureolytic bacterial community using rumen simulation system

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Running head: Abundant rumen ureolytic bacteria

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1. Abstract

Urea, a non-protein nitrogen for dairy cows, is rapidly hydrolyzed to ammonia by urease produced by ureolytic bacteria in the rumen, and the ammonia is used as nitrogen for rumen bacterial growth. However, there is limited knowledge with regard to the ureolytic bacteria community in the rumen. To explore the ruminal ureolytic bacterial community, urea or acetohydroxamic acid (AHA, an inhibitor of urea hydrolysis) were supplemented into the rumen simulation systems. The bacterial 16S rRNA genes were sequenced by Miseq high-throughput sequencing and used to reveal the ureoltyic bacteria by comparing different treatments. The results revealed that urea supplementation significantly increased the ammonia concentration, and AHA addition inhibited urea hydrolysis. Urea supplementation significantly increased the richness of bacterial community and the proportion of *ureC* genes. The composition of bacterial community following urea or AHA supplementation showed no significant difference compared to the groups without supplementation. The abundance of *Bacillus* and unclassified *Succinivibrionaceae* increased significantly following urea supplementation. *Pseudomonas*, *Haemophilus*, *Neisseria*, *Streptococcus*, and *Actinomyces* exhibited a positive response to urea supplementation and a negative response to AHA addition. Results retrieved from the NCBI protein database and publications confirmed that the representative bacteria in these genera mentioned above had urease genes or urease activities. Therefore, the rumen ureolytic bacteria were abundant in the genera of *Pseudomonas*, *Haemophilus*, *Neisseria*, *Streptococcus*, *Actinomyces*, *Bacillus* and unclassified *Succinivibrionaceae*. Insights into abundant rumen ureolytic bacteria provide the regulation targets to mitigate urea hydrolysis and increase efficiency of urea nitrogen utilization in ruminants.

Keywords: Rumen, ureolytic bacteria, urea, acetohydroxamic acid, high-throughput sequencing.

2. Introduction

The use of urea in feeds of ruminants is increasing to reduce the supplementation of true protein and the costs of rations. The recommendations of urea would be for no more than 1% in the concentrate, approximately 135 g/cow daily (Kertz, 2010). In the rumen, ureolytic bacteria produce urease to hydrolyze urea to ammonia, which is subsequently used for the synthesis of amino acids and microbial protein. Normally, the rate of urea hydrolysis exceeds the rate of ammonia utilization, which leads to poor efficiency of urea utilization in the rumen and increase the toxic ammonia in the blood (Patra, 2015). Acetohydroxamic acid (AHA), an inhibitor of urease activity that prevents the rapid hydrolysis of urea and consequent explosion of ammonia in rumen, is commonly applied in the rations of ruminants (Upadhyay, 2012).

Ureolytic bacteria play an important role in the hydrolysis of urea in the rumen. Previous studies have isolated some ureolytic bacteria from the rumen including *Succinovibrio dextrinosolvens*, *Treponema* sp., *Ruminococcus bromii*, *Butyrivibrio* sp., *Bifidobacterium* sp., *Prevotella ruminicola*, and *Peptostreptococcus productus* (Wozny et al., 1977). However, due to the difficulty in cultivating the rumen bacteria, those that have been isolated represent only 6.5% of the community (Kim et al., 2011). Thus, sequencing and phylogenetic analysis of 16S rRNA genes and functional genes have been extensively used in studies focused on members of the uncultured bacteria. By sequencing, ureolytic bacterial diversity has been observed in the environment including open oceans (Collier et al., 2009), groundwater (Gresham et al., 2007), sponges (Su et al., 2013), and soil (Singh et al., 2009). We have previously studied rumen ureolytic bacteria using a urease gene clone library, and found that ureolytic bacterial composition in the rumen was distinct from that in the environment (Zhao et al., 2015). Therefore, it is interesting and meaningful to explore the rumen ureolytic bacterial communities further.

Rumen simulation systems have been developed and used in the evaluation of feeds nutrients degradation and rumen fermentation manipulation in order to avoid the use of animals or decrease study costs (Hristov et al., 2012). We invented a dual-flow continuous rumen simulation system with real-time monitoring of pH, temperature, gas production, methane and carbon dioxide concentration (Figure 3). We demonstrated that the conditions of microbial fermentation in the system were similar to those in the rumen of dairy cows (Shen et al., 2012), making it a powerful and practical tool for the study of rumen microbes or fermentation.

The objective of this study was to reveal abundant ureolytic bacterial community by high-throughput sequencing in a rumen simulation system when treated with an activator (urea) or inhibitor (AHA) of ureolytic bacteria.

Figure 3 Appearance of the rumen stimulation system used in this study

3. Materials and methods

3.1 Experimental design and continuous cultivation

The rumen simulation system with eight fermenters were used in two replicated periods of 10 d each (7 d for adaptation and 3 d for sampling) (Shen et al., 2012). The basic total mixed ration (TMR) was ground down to 1 mm for subsequent use. Fermenters were assigned to four treatments: U0_A0 (basic diet only), U0_A0.45 (basic diet plus AHA of 0.45 g/kg dry matter (DM)), U5_A0 (basic diet plus urea of 5 g/kg DM), U5_A0.45 (basic diet plus urea of 5 g/kg DM and AHA of 0.45 g/kg DM). Two fermenters were randomly assigned to each treatment in each period. A total of 40 g feed (DM based) was placed into each fermenter daily in two equal portions at 09:00 and 21:00. The dilution rate for the liquid is 8%/h and for the solid is 200 ml/d during the fermentation. Urea and AHA were dissolved in artificial saliva referenced to Weller and Pilgrim (1974) with some modification ($NaHCO_3$ 9.8 g, $Na_2HPO_4.12H_2O$, NaCl 0.47 g, KCl 0.57 g, $MgSO_4.\ 7H_2O$ 0.12 g, $CaCl_2$ 0.04 g per litre) and

were added directly into the fermenters after each feeding. The basic diet (DM based) primarily consisted of alfalfa hay (17.72 %), corn silage (17.50 %), oaten hay (5.09 %), cotton seed (5.61%), apple pulp (3.74%), sugar beet pulp (6.71%), and compound packet (40.95 %). The compound packet provided the following per kg of diets: steam corn 180.39 g, soybean skin 55.84 g, soybean meal 64.43 g, extruded soybean 38.66 g, distillers dried grains with soluble (DDGS) 24.48 g, double-low rapeseed meal 25.77 g, $Ca(HCO_3)_2$ 2.58 g, $CaCO_3$ 2.58 g, NaCl 3.44 g and $NaHCO_3$ 6.01 g (Table 2).

Table 2 Composition and nutrient levels of basal diets (Dry matter based)

Item	**Content (%)**
Ingredients	
Alfalfa hay	17.72
Corn silage	17.50
Oaten hay	5.09
Cotton seed	5.61
Apple pulp	3.74
Sugar beet pulp	6.71
Molasses (30%)	2.68
Compound packet [a]	40.95
Nutrient levels	
CP	16.50
NDF	35.46
ADF	21.71
EE	6.46
Ca	0.97
P	0.35

[a] The compound packet provided the following per kg of diets: Steam corn 180.39 g, Soybean skin 55.84 g, Soybean meal 64.43 g, Extruded soybean 38.66 g, DDGS 24.48 g, Double-low rapeseed meal 25.77 g, $Ca(HCO_3)_2$ 2.58 g, $CaCO_3$ 2.58 g, NaCl 3.44 g, $NaHCO_3$ 6.01 g.

On the first day of each period, all fermenters were inoculated with ruminal fluid obtained from three rumen-fistulated cows fed the same TMR diet as used in the *in vitro* study. Animals involved in this study were cared for according to the principles of the Chinese Academy of Agricultural Sciences Animal Care and Use Committee (Beijing, China). Ruminal fluid was strained through four layers of cheesecloth and transferred to the

laboratory in a sealed container. A total 500 mL of the strained ruminal fluid was added to each of the eight fermenters, which also contained 500 mL of artificial saliva. Anaerobic conditions were established by flushing the headspace of the fermenters with N_2 at a rate of 20 mL min^{-1}. The artificial saliva was continuously infused into the flasks. The temperature of the fermenters was maintained at 39 ℃ by circulating water, and the fermenter content was stirred continuously at 25 rpm.

3.2 Rumen fluid sampling and DNA extraction

During the last three days of each period, 3 mL of fermenter liquid was collected from each fermenter at 0, 2, 4, 6, 8, and 10 h after morning feeding. Collected samples were stored at –80 ℃ for detection of ammonia nitrogen (NH_3-N) and urea nitrogen (urea-N) concentrations. The NH_3-N concentration was determined using the method based on the Berthelot (phenol–hypochlorite) reaction (Broderick and Kang, 1980). Urea nitrogen (urea-N) concentration was determined using the diacetyl monoxime method with a commercial kit (Nanjing Jiancheng Co., Nanjing, China). Rumen fluid collected at 2 h was used to extract microbial DNA with a cetyl trimethylammonium bromide (CTAB) plus bead beating method (Minas et al., 2011). Extracted DNA was assessed by agarose gel (1%) electrophoresis and quantified using a Nanodrop™ spectrometer (Thermo Scientific, Waltham, MA, USA).

3.3 Quantitative PCR of urease and 16S rRNA genes

The urease alpha subunit encoding gene (*ureC)* primers UreC-F (5'-TGGGCCTTAAAATHCAYGARGAYTGGG-3') and UreC-R (5'-SGGTGGTGGCACACCATNANCATRTC-3) were used to quantify the *ureC* gene copies (Reed, 2001). 16S rRNA genes of total bacteria were quantified using 338-F (5'-ACTCCTACGGGAGGCAGCAG-3') and 533-R (5'-TTACCGCGGCTGCTGGCAC -3') as primers (Huse et al., 2008). The assays were performed in an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SYBR® Premix Ex Taq™ II (Takara, Dalian, China). Standard curves for urease gene of rumen ureolytic bacteria and 16S rRNA gene of total bacteria were established respectively. PCR were performed using UreC-F/UreC-R or 338F/533R as primers respectively and rumen microbial DNA as templates. The amplicons were ligated into the pMD18-T Easy vector (TaKaRa, Dalian, China), and the recombinant plasmids were transformed into *E. coli* JM109 cells (TaKaRa, Dalian, China). Plasmids with bacterial *ureC* gene or 16S rRNA gene were used to build standard curves. The

copy numbers of the plasmids were calculated and then the plasmids were serial diluted (1:10). The standard curves were generated using the diluted plasmids DNA (Figure 4).

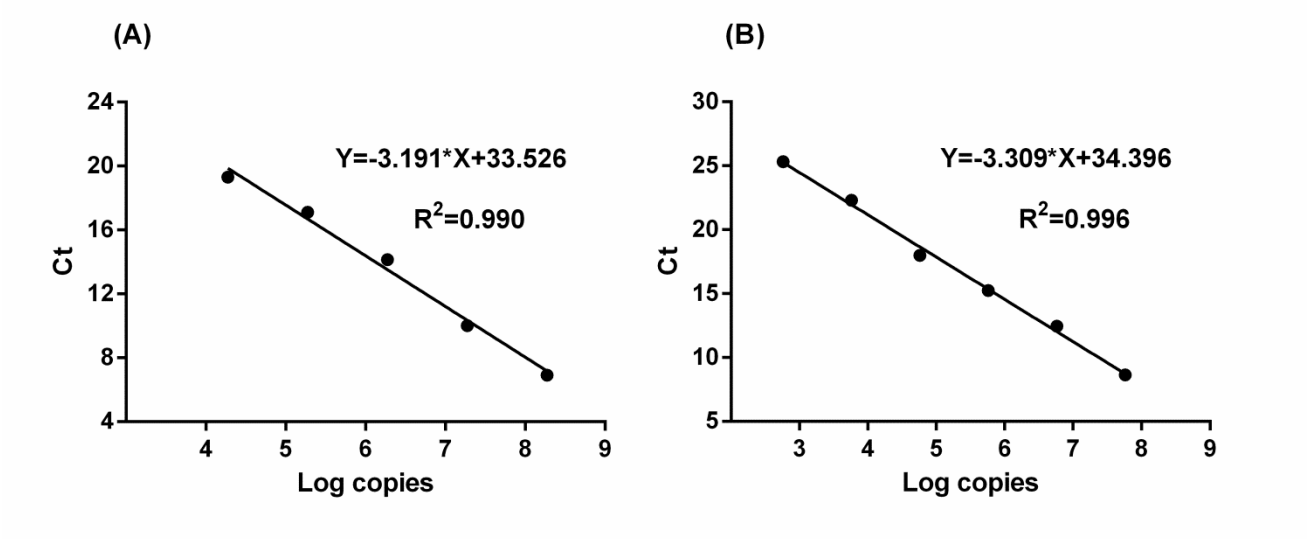

Figure 4 Standard curves generated from plasmid DNA containing *ureC* gene (A) and partial 16S rRNA gene (B)

The DNA quantification was performed in an iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) using SYBR® Premix Ex Taq™ II (Takara, Dalian). Each qPCR reaction (20 μL) included 10 μL 2 × SYBR Master Mix, 4 μL nuclease-free water, 0.8 μL each forward and reverse primer (10 μM) and 2 μL DNA template. PCR cycle parameters for *ureC* gene detection were as follows: 95 ℃ for 3 min, followed by 40 cycles at 95 ℃ for 15 s, 52 ℃ for 30 s and 72 ℃ for 30 s. PCR cycle parameters for detecting 16S rRNA genes were as follows: 95 ℃ for 3 min, followed by 40 cycles at 95 ℃ for 15 s, 60 ℃ for 30 s and 72 ℃ for 30 s. Melting curves were prepared for each PCR reaction by collecting fluorescence signal at every 0.5 ℃ increment when the temperature ramped from 60 ℃ to 95 ℃. Each sample was run in triplicate, and both standards and samples were assayed on the same qPCR plate. The qPCR reaction efficiencies with the degenerate primers ureC-F/R and 338F/533R were 106.5% and 100.7% respectively. Copy number of *ureC* gene or 16S rRNA gene in per ng of DNA was determined by relating the CT value to the standard curves. The proportion of *ureC* gene copies was calculated as the ratio of *ureC* gene copies to total 16S rRNA gene copies. The detailed qPCR protocols were provided in the Supplementary Material. The proportion of *ureC* gene copies in each treatment were shown in a boxplot constructed using R (R Core Team, 2014).

3.4 Bacterial 16S rRNA genes amplification and Illumina sequencing

Microbial DNA was used as a template for amplification of partial 16S rDNA sequence using the universal bacterial primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Nelson et al., 2014) with both primers tagged with unique barcode sequences for each sample. All polymerase chain reactions (PCRs) were carried out in 50 μL reactions with 0.5 μL of PrimeSTAR® HS DNA Polymerase (TaKaRa, Dalian, China), 10 μL 5×PrimeSTAR Buffer (plus Mg^{2+}) (TaKaRa), 0.2 μM of the forward and reverse primers, 200 μM dNTP (TaKaRa), and 100 ng microbial DNA. Thermal cycling consisted of initial denaturation at 98℃ for 1 min, followed by 30 cycles of denaturation at 98℃ for 10 s, annealing at 50℃ for 30 s, and elongation at 72℃ for 60 s, and a final elongation at 72℃ for 5 min. Unique bands were identified using agarose gel (2%) electrophoresis of PCR amplicons (Figure 5). The bands were cut and purified with a QIAGEN MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA). Amplicon libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) following the manufacturer's recommendations, with the addition of index codes. Library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina MiSeq platform (2×250 bp).

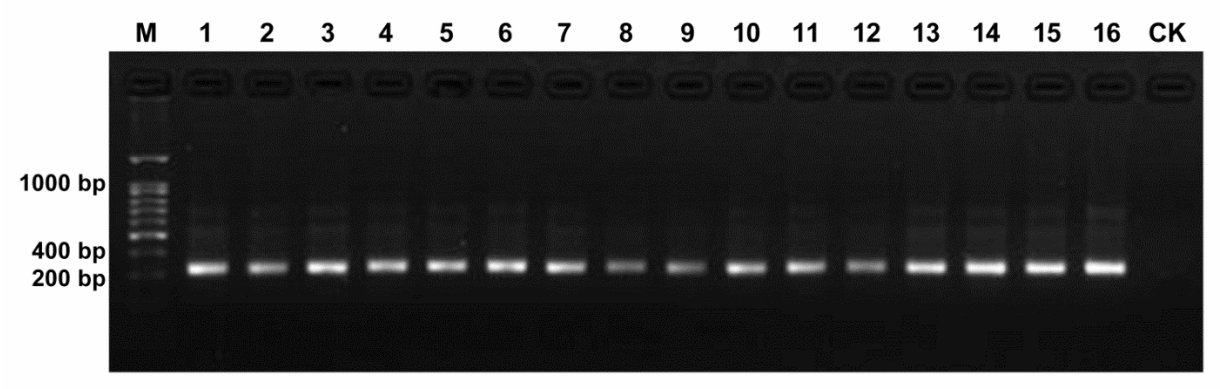

Figure 5 PCR products amplified using the universal bacterial primers 515F and 806R. M, 100bp ladder; 1-16, PCR products amplified using DNA samples as templates (U0_A0: 1, 2, 9 and 10; U0_A0.45: 3, 4, 11 and 12; U5_A0: 5, 6, 13 and 14; U5_A0.45: 7, 8, 15 and 16); CK, PCR product amplified using sterile water as template.

3.5 Sequencing data processing and analysis

Paired-end reads were merged using FLASH (Magoč and Salzberg, 2011). Merged reads were assigned to each sample based on the unique barcode, after which the barcodes and

primers were removed. The quality of raw reads was checked, and reads were truncated at any site of >3 sequential bases receiving a quality score of <Q20, and reads with <75 % (of total read length) consecutive high quality base calls were removed (Caporaso et al., 2010; Bokulich et al., 2013). Chimeric sequences were detected and removed using UCHIME (Haas et al., 2011). Operational taxonomic units (OTU) were generated by aligning the reads to the GreenGenes database released in May 2013 (DeSantis et al., 2006) and clustered at 97% sequence identity using the PyNAST tool (Caporaso et al., 2010) and the UCLUST algorithm (Edgar et al., 2011). The OTUs were filtered based on the total observation count of an OTU <10 and the number of samples in an OTU <2 in QIIME (Caporaso et al., 2010). The OTUs were further assigned to taxa using the RDP classifier (Wang et al., 2007). The OTU table was rarified for alpha diversity analysis. Simpson, Shannon, Chao1, and the PD_whole_tree index were calculated for each sample. Good's coverage was used to estimate the percentage of the total species that were sequenced in each sample (Caporaso et al., 2010). QIIME was used to calculate the weighted UniFrac distances, which are phylogenetic measures of beta diversity. The weighted UniFrac distance was used for Principal Coordinate Analysis (PCoA) (Lozupone et al., 2007). The significance of grouping in the PCoA plot was tested by analysis of similarity (ANOSIM) in QIIME with 999 permutations (R Core Team, 2014; Mahnert et al., 2015). The relative abundance of bacteria was expressed as the percentage. The potential ureolytic bacteria were selected using the criterion that their abundance increased with urea treatment and decreased with AHA treatment. The urease alpha subunit sequences of representative species from potential ureolytic bacteria were checked against the NCBI protein database and the urease activities of these bacteria were verified by published studies.

3.6 Statistical analysis

Urea-N, ammonia, proportion of *ureC* gene copies, bacterial abundance and diversity index were statistical analyzed using the SAS MIXED procedure (SAS Institute, Inc, Cary, NC) as shown in the following model: $Y_{ijk} = \mu + a_i + b_j + ab_{ij} + e_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, a_i is the effect of urea treatment i, b_j is the effect of AHA treatment j, ab_{ij} is the interaction between a_i and b_j (Both factors and their interaction are considered fixed effects),, and e_{ijk} is the residual, assumed to be normally distributed. Data of bacterial abundance were transformed to $\log_{10}$ (n+1) if necessary to ensure normal distribution. Mean separation was conducted by using Fisher's least significant difference test. Differences were

declared significant at P <0.05. Tukey's test was used to determine where the differences occurred.

3.7 Nucleotide sequence accession number

All the raw sequences after assembling and filtering were submitted to the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/Traces/sra/), under accession number SRP074113.

4. Results

4.1 Changes of urea, ammonia concentrations and proportion of ureC genes

The urea-N concentrations in the two urea treated groups were higher (P <0.01) than the other two groups at 2 h after morning feeding (Figure 6). In the two urea treated groups, Group U5_A0.45 exhibited a higher (P <0.01) urea concentration than group U5_A0, indicating a decreased urea hydrolysis rate with AHA inhibition (Figure 6). The NH_3-N concentrations of all four treatments showed a peak value after fermentation for 2 h. Urea supplementation significantly increased (P <0.01) NH_3-N concentration during whole sampling period, while in the two urea-treated groups, AHA addition also decreased NH_3-N concentration significantly (P <0.01). Two hours after the morning feeding, the proportion of *ureC* genes was higher (P <0.05) in urea-treated groups than in non-urea treated groups. The addition of AHA did not have a significant effect on the proportion of *ureC* genes (Figure 7).

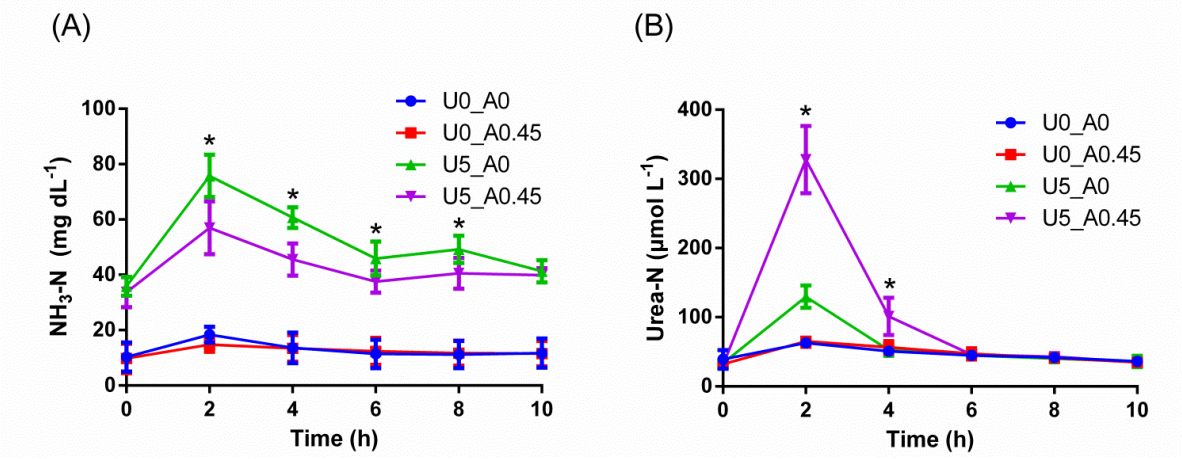

Figure 6 Changes of NH_3-N and urea-N concentrations induced by urea and AHA supplementation. (N=4)

(A) Changes of NH_3-N concentration. (B) Changes of urea-N concentration.U0_A0: basic diet only, U0_A0.45: basic diet plus AHA of 0.45 g/kg DM, U5_A0: basic diet plus urea of 5

g/kg DM, U5_A0.45: basic diet plus urea of 5 g/kg DM and AHA of 0.45 g/kg DM. *Means values in group U5_A0 was significantly different from that in group U5_A0.45 (P <0.05).

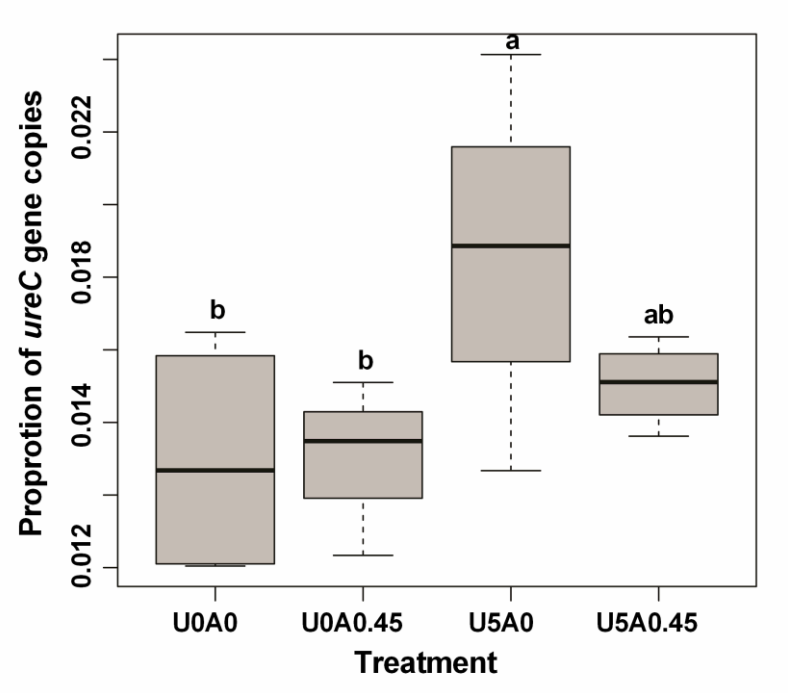

Figure 7 Changes in the proportion of *ureC* gene copies induced by urea and AHA supplementation.

The proportion of *ureC* gene copies was calculated as the ratio of *ureC* gene copies to total 16S rRNA gene copies. U0_A0: basic diet only, U0_A0.45: basic diet plus AHA of 0.45 g/kg DM, U5_A0: basic diet plus urea of 5 g/kg DM, U5_A0.45: basic diet plus urea of 5 g/kg DM and AHA of 0.45 g/kg DM. [a, b] Different letters for different treatments indicate statistically significant differences (P <0.05). (N=4)

4.2 Changes of ureolytic bacterial diversity

A total of 2,105,448 merged sequences were acquired from 16 samples, and 1,672,529 high-quality sequences, with an average read length of 253 bases were obtained. After removing chimeric sequences, the remaining 1,603,997 sequences were used to generate OTUs with 97% sequence similarity across all samples. The OTU table was filtered, leaving 5,075 OTUs for subsequent analysis. Collectively, 24 bacterial phyla were identified. *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were the three predominant phyla, representing 35%, 28%, and 23% of all sequences, respectively (Figure 8). Genera that were each represented by ≥0.1% of the total sequences in at least 1 of the 16 samples were selected for further analysis. The ten predominant genera were *Prevotella*, *Treponema*, *YRC22*, *Succinivibri*o,

Porphyromonas, *Oscillospira*, *Roseburia*, *Bacteroides*, *Butyrivibrio*, and *Coprococcus* (Figure 9).

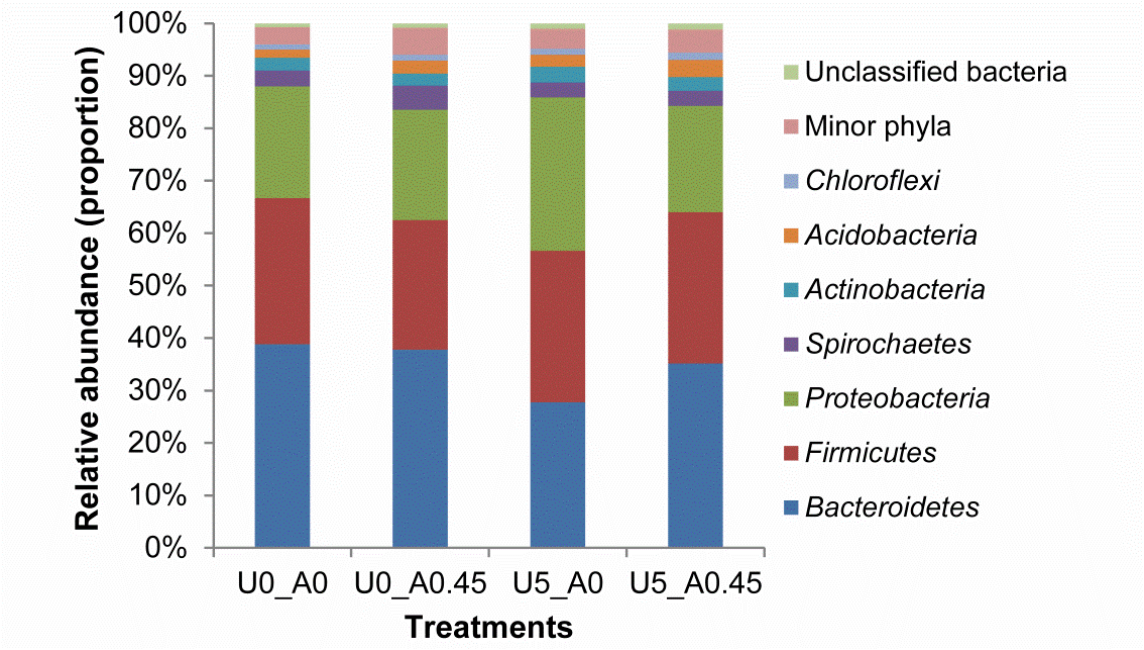

Figure 8 Composition of the most predominant bacterial phyla in the rumen.
U0_A0: basic diet only, U0_A0.45: basic diet plus AHA of 0.45 g/kg DM, U5_A0: basic diet plus urea of 5 g/kg DM, U5_A0.45: basic diet plus urea of 5 g/kg DM and AHA of 0.45 g/kg DM.

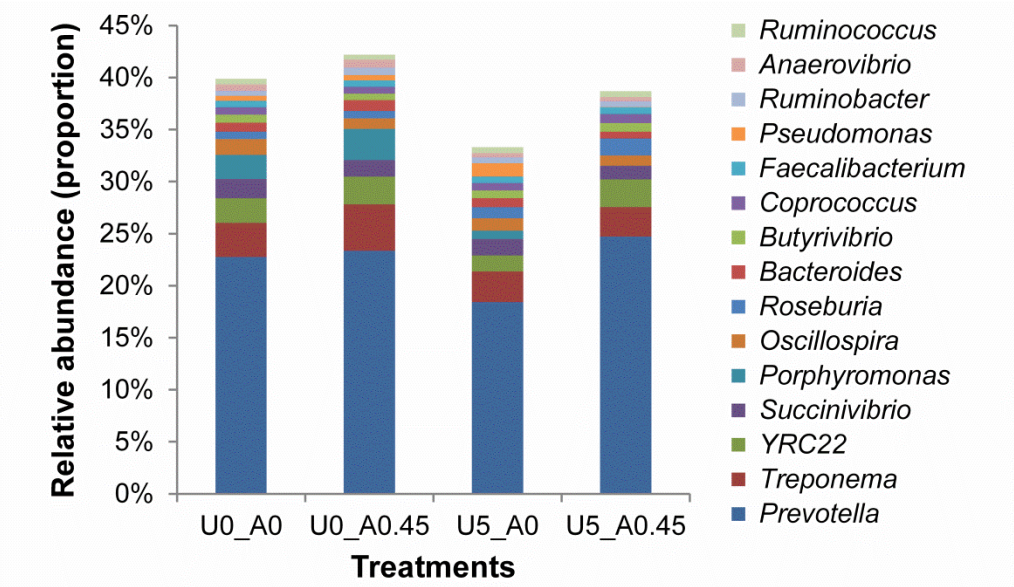

Figure 9 Composition of the most predominant bacterial genera in the rumen.
U0_A0: basic diet only, U0_A0.45: basic diet plus AHA of 0.45 g/kg DM, U5_A0: basic diet plus urea of 5 g/kg DM, U5_A0.45: basic diet plus urea of 5 g/kg DM and AHA of 0.45 g/kg

DM. The top fifteen abundant bacteria genera were shown and the others were not shown. Other genera accounted for 60.11% in group U0_A0, 57.81% in group U0_A0.45, 66.68% in group U5_A0 and 61.31% in group U5_A0.45.

After rarefaction, 9000 sequences per sample were used for diversity analysis. Alpha bacterial diversity was presented in Table 3. Group U5_A0 had the highest Chao 1 and PD_whole_tree estimates, followed by groups U5_A0.45, U0_A0.45, and U0_A0. No significant differences were observed among the four groups based on the results of the Simpson and Shannon diversity index. PCoA analysis of overall diversity based on the unweighted UniFrac metrics was performed to compare the four treatments (Figure 10). ANOSIM (cutoff =0.01) showed no significant differences in bacterial community composition between treatments U0_A0 and U0_A0.45 (R =-0.198, *P* =0.925) or between treatments U5_A0 and U5_A0.45 (R =-0.135, *P* =0.888). A tendency of difference was found between treatments U0_A0 and U5_A0 (R = 0.323, *P* = 0.091). Principal Coordinate 1 and 2 accounted for 44.19% and 25.14% of the total variation, respectively.

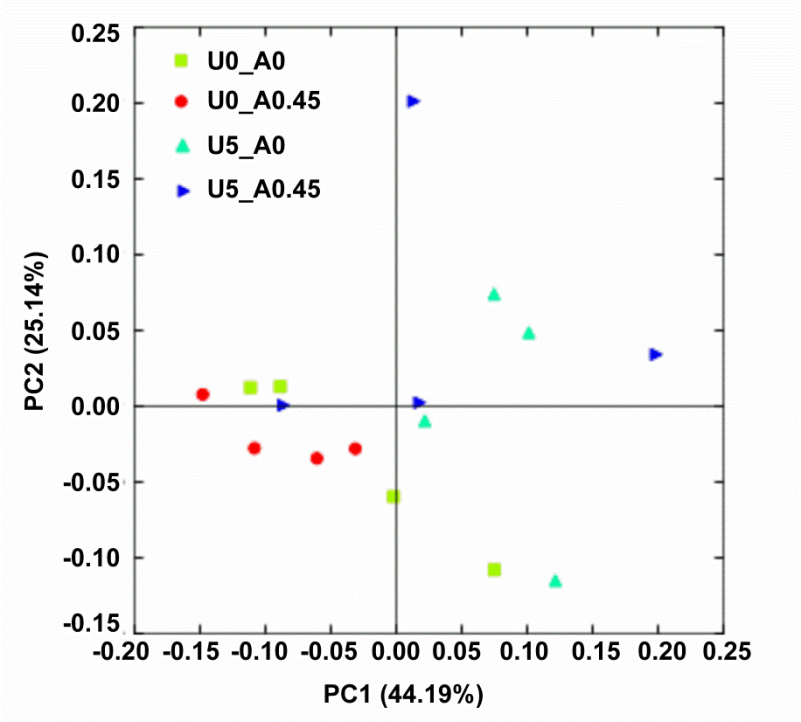

Figure 10 Principal coordinate analysis (PCoA) of the rumen bacterial community. The principal coordinate analysis is based on the weighted UniFrac distances between the microbiome profiles. U0_A0: basic diet only, U0_A0.45: basic diet plus AHA of 0.45 g/kg DM, U5_A0: basic diet plus urea of 5 g/kg DM, U5_A0.45: basic diet plus urea of 5 g/kg DM and AHA of 0.45 g/kg DM.

Table 3 Alpha diversity index of rumen bacteria among all treatments (N=4)

Indices	U0		U5		SEM	*P* value		
	A0	A0.45	A0	A0.45		Urea	AHA	Urea*AHA
Observed_species	1442	1496	1557	1563	25	0.11	0.54	0.62
Good's coverage	0.914^{a}	0.911^{ab}	0.905^{b}	0.906^{b}	0.002	0.02	0.62	0.46
PD_whole_tree	106^{b}	109^{ab}	111^{a}	109^{ab}	0.89	0.09	0.70	0.11
Chao 1	2860^{c}	2942^{bc}	3142^{a}	3043^{ab}	43	0.01	0.85	0.11
Shannon	7.59	7.73	7.77	7.59	0.08	0.92	0.92	0.46
Simpson	0.96	0.96	0.97	0.95	0.01	0.89	0.50	0.36

Note: $^{a-c}$ Mean values within a row with different letters differ significantly (P <0.05).

SEM: standard error of the mean.

U0: basic diet without urea, U5: basic diet plus urea of 5 g/kg DM, A0: basic diet without AHA, A0.45: basic diet plus AHA of 0.45 g/kg DM.

4.3 Changes of the relative abundance of ureolytic bacteria

At the phylum level, the group treated with urea only had the highest proportion of *Proteobacteria* and *Actinobacteria*, and the lowest proportion of *Bacteroidetes* compared with the other three groups (Figure 8). Both of the two urea-treated groups had relatively high proportions of *Acidobacteria* and low proportions of *Spirochaetes* compared with the other two groups. In addition, the two urea-treated groups had higher percentages of unclassified bacteria than the other two groups. At the genus level, the relative abundance represented by ≥0.1% of the total sequences in at least one of the whole samples were further analyzed (Table 4). *Pseudomonas* (1.25%) from *Proteobacteria* and *Streptococcus* (1.00%) from *Firmicutes* were more predominant in group U5_A0 compared to the other three groups (P <0.01). *Haemophilus* and *Neisseria* from *Proteobacteria*, and *Actinomyces* from *Actinobacteria* were the most abundant in the U5_A0 group compared with the other three groups (P <0.05). The relative abundance of *Bacillus* from *Firmicutes* and unclassified *Succinivibrionaceae* were higher in the two urea-treated groups compared with the other two groups (P <0.01). According to the results retrieved from the NCBI protein database and reported in previous studies, the representative species from *Pseudomonas, Haemophilus, Streptococcus, Neisseria, Bacillus, Actinomyces,* and unclassified *Succinivibrionaceae* were identified as containing urease genes and having urease activity (Table 5).

5. Discussion

In the rumen, urea is a source of nitrogen for the growth of ureolytic bacteria. AHA, an inhibitor of urease, inhibits urea usage by ureolytic bacteria and results in insufficient nitrogen source for bacterial growth. In this study, we used urea and AHA to promote or inhibit the growth of rumen ureolytic bacteria, respectively. We observed that AHA is a useful inhibitor for slowing down the hydrolysis of urea within the rumen fluid. This is consistent with previously published studies *in vivo* (Jones and Milligan, 1975; Makkar et al., 1981).

Urea supplementation significantly increased bacterial community richness and the number of bacterial species. AHA supplementation resulted in no changes of richness and diversity of bacterial community. The proportion of urease gene copies was served as a proxy to observe changes in the proportion of ureolytic bacteria. Urea supplementation significantly increased the proportion of ureolytic bacteria, which suggested that urea stimulated the growth of rumen ureolytic bacteria. In addition, ANOSIM revealed that the composition of the entire bacterial community in urea-treated groups showed a trend of difference from those in non-urea treated groups (P <0.10). Changes of the bacterial community in response to urea treatment were possibly related to urease activity and the production of ammonia. Kim et al. (2014) found that urease genes and enzyme activities were regulated by the level of ammonia in ruminal cellulytic bacteria *Ruminococcus albus* 8. The lack of a significant effect by AHA on the diversity of the rumen bacterial community may be due to microbial adaption of AHA. Previous studies found that rumen microbes could adapt to chronic AHA supplementation, while AHA was capable of short-term inhibition of urease activity in the rumen (Zhang et al., 2001).

Across the four groups, three phyla (*Bacteroidetes*, *Firmicutes* and *Proteobacteria*) were predominant. Similar to our results previously published studies have reported that the distribution of phylotypes of rumen bacterial communities fell predominantly into these three phyla (Hook et al., 2011; Wu et al., 2012; Zhang et al., 2014). The bacterial community from our *in vitro* simulation system was thus similar to the communities observed *in vivo*. The group treated with urea only had the highest proportion of *Proteobacteria* and the lowest proportion of *Bacteroidetes.* In accordance, Collier et al. (2009) investigated the diversity of ureolytic microorganisms in open ocean and estuarine planktonic communities, and found that ureolytic microorganisms were most commonly found in *Proteobacteria* and rare in *Bacteroidetes*.

Table 4 Bacterial genera that accounted for ⩾ 0.1% of the total sequences in at least one of the samples with significant variation under different treatments (abundance of the genera was expressed as %) (N=4)

Taxa (family and genus within each phylum)		U0		U5		SEM	*P* value		
		A0	A0.45	A0	A0.45		Urea	AHA	Urea*AHA
Bacteroidetes	*Porphyromonadaceae; Paludibacter*	0.13^{ab}	0.20^{a}	0.02^{b}	0.00^{b}	0.0003	0.0175	0.6091	0.3539
	Chitinophagaceae; unclassified genus	0.15^{b}	0.19^{a}	0.16^{ab}	0.11^{b}	0.0001	0.0361	0.5310	0.0080
Proteobacteria	*Succinivibrionaceae; others*	8.17^{a}	6.76^{ab}	5.06^{b}	4.04^{b}	0.0063	0.0067	0.0989	0.7615
	Succinivibrionaceae; unclassified genus	1.11^{b}	1.05^{b}	6.05^{a}	4.38^{a}	0.0083	0.0008	0.1279	0.1488
	Pseudomonadaceae; Pseudomonas	0.49^{b}	0.50^{b}	1.25^{a}	0.05^{b}	0.0020	0.2671	0.0075	0.0071
	Pasteurellaceae; Haemophilus	0.02^{b}	0.03^{b}	1.92^{a}	0.00^{b}	0.0005	<.0001	<.0001	<.0001
	Neisseriaceae; Neisseria	0.05^{b}	0.02^{b}	0.66^{a}	0.00^{b}	0.0003	0.0193	0.0111	0.0153
	Desulfobulbaceae; Desulfobulbus	0.21^{a}	0.14^{ab}	0.02^{ab}	0.01^{b}	0.0004	0.0360	0.4308	0.6472
	Campylobacteraceae; Campylobacter	0.11^{ab}	0.13^{a}	0.04^{ab}	0.00^{b}	0.0002	0.0400	0.7626	0.3891
	Moraxellaceae; Acinetobacter	0.04^{ab}	0.02^{b}	0.07^{ab}	1.10^{a}	0.0001	0.0516	0.5991	0.2099
Firmicutes	*Clostridiaceae; unclassified genus*	8.04^{ab}	6.10^{b}	8.40^{ab}	9.32^{a}	0.0051	0.0483	0.4704	0.0888
	Acidaminobacteraceae; unclassified genus	0.15^{a}	0.16^{a}	0.04^{b}	0.00^{b}	0.0003	0.0182	0.6995	0.5929
	Lachnospiraceae; Roseburia	0.72^{b}	0.73^{b}	1.08^{b}	1.64^{a}	0.0016	0.0051	0.0634	0.0731
	Lachnospiraceae; Lachnospira	0.20^{b}	0.16^{b}	0.23^{ab}	0.37^{a}	0.0003	0.0338	0.2710	0.0818
	Veillonellaceae; Anaerovibrio	0.63^{ab}	0.75^{a}	0.42^{b}	0.43^{b}	0.0006	0.0297	0.4459	0.5064
	Veillonellaceae; Veillonella	0.00^{b}	0.01^{b}	0.53^{a}	0.00^{b}	0.0026	0.0096	0.0093	0.0090
	Peptostreptococcaceae; Filifactor	0.81^{a}	0.69^{a}	0.25^{b}	0.00^{b}	0.0014	0.0041	0.1435	0.5827
	Streptococcaceae; Streptococcus	0.17^{b}	0.31^{b}	1.00^{a}	0.14^{b}	0.0002	0.0135	0.0103	0.0030
	Bacillaceae; Bacillus	0.06^{c}	0.09^{bc}	0.13^{a}	0.17^{a}	0.0051	0.0062	0.0858	0.7493
Actinobacteria	*Micrococcaceae; Arthrobacter*	0.07^{b}	0.09^{ab}	0.11^{a}	0.03^{b}	0.0001	0.5271	0.0651	0.0076
	Actinomycetaceae; Actinomyces	0.03^{b}	0.04^{b}	0.18^{a}	0.00^{b}	0.0026	0.1007	0.0286	0.0199

Note: $^{a-c}$ Means values within a row with different letters differ significantly (P <0.05).

SEM: standard error of the mean.

U0: basic diet without urea, U5: basic diet plus urea of 5 g/kg DM, A0: basic diet without AHA, A0.45: basic diet plus AHA of 0.45 g/kg DM.

Table 5 Urease gene and enzyme activity of selected genera containing ureolytic bacteria in rumen (N=4)

Genus	**Representative species**	**Urease gene (Alpha subunit accession in NCBI)**	**Urease activity (Reference)**
Unclassified *Succinivibrionaceae*	*Succinivibrionaceae* WG-1	+ (WP 010457200)	+ (Pope et al., 2011)
Pseudomonas	*Pseudomonas aeruginosa* BG	+ (KM657955)	+ (Goswami et al., 2015)
	Pseudomonas fluorescens	+ (KPU59664)	+ (Jyothi and Umamahe, 2013)
Haemophilus	*Haemophilus influenza* Rd	+ (KMZ31254)	+ (McCrea et al., 2008)
	Haemophilus haemolyticus	+ (WP 005644404)	+ (McCrea et al., 2008)
Streptococcus	*Streptococcus thermophiles*	+ (KPL38034)	+ (Zotta et al., 2008)
	Streptococcus salivarius 57.I	+ (AEJ54136)	+ (Chen et al., 2000)
Neisseria	*Neisseria sp.* KH1503	+ (KLT73764)	+ (Sakai et al., 1996)
Bacillus	*Bacillus cereus*	+ (AAS42567)	+ (Rasko et al., 2004)
	Bacillus pasteurii	+ (1S3T_C)	+ (Benini et al., 2000)
Actinomyces	*Actinomyces naeslundii*	+ (AAD13732)	+ (Morou-Bermudez and Burne, 2000)
	Actinomyces johnsonii	+ (WP 021610181)	+ (Schaal and Yassin, 2015)

+ Positive urease genes or enzyme activity.

Bacillus was in higher abundance in the two groups supplemented with urea, indicating it was more responsive to urea. *Bacillus* spp. in the rumen is able to degrade hemicellulose, and produce polysaccharidases and glycoside hydrolases to utilize polysaccharide (Williams and Withers, 1983). *B. pasteurii, B. lentus*, and *B. cereus* have proven to be ureolytic bacteria (Benini et al., 2000; Rasko et al., 2004; Sarda et al., 2009), and the urease activity of *B. pasteurii* is inhibited by AHA (Benini et al., 2000). The unclassified *Succinivibrionaceae* was also observed at a higher relative abundance in the two urea-treated groups. In the rumen, *Succinivibrionaceae* is very common and important for degradation of starch, pectin, and dextrin to succinate and propionate (Santos and Thompson, 2014). *Succinivibrionaceae* WG-1 isolated from the foregut of tammar wallaby produced urease for urea catabolism (Pope et al., 2011). Several isolates of *S. dextrinosolvens* from the rumen were also shown to have urease activity (Wozny et al., 1977).

Pseudomonas and *Streptococcus* were both relatively more abundant in the group treated with urea only, but these bacteria had lower abundance in AHA-treated groups. These results confirmed the urea stimulating and AHA inhibiting effects on the microbial community. Several species of *Pseudomonas* and *Streptococcus* are able to hydrolyze cellulose (Lynd et al., 2002; Oyeleke and Okusanmi, 2008). In the genus *Pseudomonas*, species such as *P. fluorescens* (isolated from soil) and *P. aeruginosa* (isolated from ocean) possess urease activity (Jyothi and Umamahe, 2013; Goswami et al., 2015). In addition, two *Streptococcal* species, *S. thermophiles* and *S. salivarius*, also produce urease (Chen et al., 2000; Zotta et al., 2008). Kakimoto et al. (1989) assayed about 16,000 isolates from animal feces and intestines for production of acid urease, and found 370 urease-positive strains belonging to the genus *Streptococcus*. This is consistent with the results of our study in which *Streptococcus* were found in higher abundance in response to urea supplementation.

The relative abundance of genera *Haemophilus, Neisseria,* and *Actinomyces* increased in response to urea and decrease in response to AHA supplementation. The members of *Haemophilus* ferment glucose (Kilian, 2015), and *H. haemolyticus* and *H. influenzae* Rd have urease activity (McCrea et al., 2008). The *H. somnus* strains of ruminants have varying urea hydrolysis ability (Garcia-Delgado et al., 1977). *Neisseria*, a gram-negative aerobic cocci, produces acid from different types of sugars, and some species are disease-causing (Marri et al., 2010). *N. sicca* strains SB and SC isolated from soil have proven to be urease positive (Sakai et al., 1996). *Neisseria* had a higher proportion in groups treated with urea, suggesting the potential of bacterial species in the rumen to have urea hydrolysis activity.

Actinobacteria, a group of Gram-positive bacteria, represent up to 3.00 % of the total rumen bacteria (Pandya et al., 2010; Sulak et al., 2012). Some strains of *A. meyeri, A. radicidentis*, and *A. johnsonii* are known to have urease activity (Schaal and Yassin, 2015), and *A. naeslundii* had urease gene and activity (Morou-Bermudez and Burne, 1999, 2000). However, An et al. (2006) described a novel species, *Actinomyces ruminicola* sp., from cattle rumen, was unable to hydrolyze urea. So it needs to be verified for ureolytic activity of different *Actinomyces* species.

6. Conclusion

The composition of bacterial community following urea or AHA supplementation treatment showed no significant difference compared to the groups without supplementation. In the rumen, the ureolytic bacteria were abundant in the genera including *Pseudomonas*, *Streptococcus*, *Haemophilus*, *Bacillus*, *Neisseria*, *Actinomyces* and unclassified *Succinivibrionaceae*. The insights into abundant ureolytic bacteria provide the basis for designing strategies to efficiently manipulate the bacterial community or function and improve urea utilization in ruminant production.

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CHAPTER IV

In this study, we investigated the diversity of the *ureC* genes in different rumen fractions, and revealed the predominant *ureC* gene OTUs in the rumen of dairy cows using Miseq sequencing. Animals were also fed with urea to determine if supplementation alters the growth of some populations of ureolytic bacteria or alters the ureolytic community composition.

Article 3

Differences in ureolytic bacterial composition between the rumen digesta and rumen wall based on ureC gene classification

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1. Abstract

Ureolytic bacteria are key organisms in the rumen producing urease enzymes to catalyse the breakdown of urea to ammonia for the synthesis of microbial protein. However, little is known about the diversity and distribution of rumen ureolytic microorganisms. The urease gene (*ureC*) has been the target gene of choice for analysis of the urea-degrading microorganisms in various environments. In this study, we investigated the predominant *ureC* genes of the ureolytic bacteria in the rumen of dairy cows using high-throughput sequencing. Six dairy cows with rumen fistulas were assigned to a two-period cross-over trial. A control group (n = 3) were fed a total mixed ration without urea and the treatment group (n = 3) were fed rations plus 180 g urea per cow per day at three separate times. Rumen bacterial samples from liquid and solid digesta and rumen wall fractions were collected for *ureC* gene amplification and sequencing using Miseq. The wall-adherent bacteria (WAB) had a distinct ureolytic bacterial profile compared to the solid-adherent bacteria (SAB) and liquid-associated bacteria (LAB) but more than 55% of the *ureC* sequences did not affiliate with any known taxonomically assigned urease genes. Diversity analysis of the *ureC* genes showed that the Shannon and Chao1 indices for the rumen WAB was lower than those observed for the SAB and LAB ($P < 0.01$). The most abundant *ureC* genes were affiliated with Methylococcaceae, Clostridiaceae, Paenibacillaceae, Helicobacteraceae and Methylophilaceae families. Compared with the rumen LAB and SAB, relative abundance of the OTUs affiliated with *Methylophilus* and *Marinobacter* genera were significantly higher ($P < 0.05$) in the WAB. Supplementation with urea did not alter the composition of the detected ureolytic bacteria. This study has identified significant populations of ureolytic WAB representing genera that have not been recognized or studied previously in the rumen. The taxonomic classification of rumen *ureC* genes in the dairy cow indicates that the majority of ureolytic bacteria are yet to be identified. This survey has expanded our knowledge of *ureC* gene information relating to the rumen ureolytic microbial community, and provides a basis for obtaining regulatory targets of ureolytic bacteria to moderate urea hydrolysis in the rumen.

Keywords: Rumen, ureolytic bacteria, *ureC* gene, diversity, predominant

2. Introduction

Urea is used commonly as a non-protein nitrogen source in the diet of ruminants as an economical replacement for feed proteins (Kertz, 2010). Rumen ureolytic bacteria produce ureases which catalyze the breakdown of urea to ammonia and carbon dioxide (Owens et al., 1980). The ammonia from urea can be assimilated by many rumen bacteria for synthesis of microbial protein required for animal growth and thus partially replaces feed protein as a N source in the diet of the ruminant (Milton et al., 1997). Nowadays, urea, as a highly rumen-degradable nitrogen source, has been included in the rations of ruminants to supply adequate amounts of nitrogen for microbial protein synthesis and improve ruminal fermentation (Wagner et al., 2010; Ceconi et al., 2015). However, urea hydrolysis to ammonia often exceeds the rate of ammonia utilization, which leads to poor efficiency of urea utilization in the rumen (Patra, 2015).

Following extensive research on the utilization of urea as a replacement for protein in ruminant diets, interest has focused on urea-hydrolyzing microbes for a better understanding of urea metabolism in the rumen (Cook, 1976; Wozny et al., 1977; On et al., 1998). Kakimoto et al. (1989) assayed about 16,000 isolates from animal faeces and intestines for the production of acid urease and found that most of the selected strains belonged to the genera *Streptococcus* and *Lactobacillus*. In a similar study by Laukov á and Koniarov á (1994), they tested 909 strains from the rumen of 104 domestic and wild ruminants for urease activity, and their results showed that some *Selenomonas ruminantium* strains and *lactobacilli* demonstrated medium urease activity and most of the *Enterococcus faecium* and all of the *E. faecalis* isolates expressed urease activity. In addition, *Howardella ureilytica*, a Gram-positive bacterium has been isolated from the rumen fluid of a sheep, which was strongly ureolytic and generated ATP through the hydrolysis of urea (Cook et al., 2007). All these above studies were conducted using culture based methods. However, most rumen microorganisms remain uncultured (Edwards et al., 2004), and therefore little is known about the identities and diversity of rumen organisms capable of urea hydrolysis.

Ureases synthesized by ureolytic bacteria are commonly composed of two or three subunits (*ureA*, *ureB*, and *ureC*) and require up to several accessory proteins (such as *ureD*, *ureE*, *ureF*, *ureG*, *ureH*, and *ureI*) for activation (Mobley et al., 1995). The *ureC* subunit is the largest of the genes encoding urease functional subunits and contains several highly conserved regions that are suitable as PCR priming sites. Primers for *ureC* gene have been designed and applied for analysis of the urea-degrading microorganisms in various

environments, including the open ocean (Collier et al., 2009), sponges (Su et al., 2013), and soil (Singh et al., 2009). We have previously studied the rumen ureolytic bacteria using an *ureC* gene clone library, and found that ureolytic bacterial composition in the rumen is distinct from that in other environments (Zhao et al., 2015). So it is of great interest to investigate the unknown rumen ureolytic bacteria in further detail. In this study, we investigated the diversity of the *ureC* genes in different rumen fractions, and revealed the predominant *ureC* gene OTUs in the rumen of dairy cows using Miseq sequencing. Animals were also fed with urea to determine if supplementation alters the growth of some populations of ureolytic bacteria or alters the ureolytic community composition.

3. Materials and methods

3.1. Animals and diets

Six Chinese Holstein dairy cows (550 ± 50 kg BW and 100 ± 21 days in milk) fitted with ruminal cannulas were used in a two-period cross-over trial. All cows were fed *ad libitum* the same total mixed ration (TMR) for two weeks prior to the study. Cows were divided into the following groups: Urea group received 180 g daily urea as a stimulator for ureolytic bacteria, and the control group, which did not receive urea supplementation. The experiment proceeded for a period of 21 days, followed by a 14 d washout period, after which the intervention was crossed. This cross-over was used to assess the functional diversity of the bacterial communities. Each day, the total urea was separated into three parts (70, 55 and 55 g for morning, afternoon and evening feeding respectively) and was packaged in filter paper to prevent ammonia toxicity from rapid hydrolysis. Urea was added into the rumen through a cannula during each feeding. All cows were kept in individual pens with free access to water and were fed TMR three times daily (7:00, 14:00 and 19:00). The TMR consists primarily of alfalfa hay (28.4 %), corn silage (26.7 %), corn (22.6 %) and soybean meal (11.8 %) (Dry matter (DM) basis) (Table 6). Animals involved in this study were cared for according to the principles of the Chinese Academy of Agricultural Sciences Animal Care and Use Committee (Beijing, China).

Table 6 Composition and nutrient levels of basal diets (air-dry basis)

Item	**Content (%)**
Ingredients	
Chinese wild rye	3.7
Alfalfa hay	28.4
Corn silage	26.7
Corn	22.6
Soybean meal	11.8
Cottonseed fuzzy	5.1
$CaHPO_4$	0.6
NaCl	0.5
Premix[a]	0.6
Nutrient levels, % of DM	
Crude protein (CP)	16.7
Ether Extract (EE)	2.2
Neutral detergent fiber (NDF)	44.2
Acid detergent fiber (ADF)	26.1
Ash	7.7
Ca	0.8
P	0.3

[a] One kilogram of premix DM contained the following: VA, 2,000,000 IU; VD, 600,000 IU; VE, 10,800 mg; Fe, 5,500 mg; Cu, 4,080 mg; Mn, 4,989 mg; Zn, 17,500 mg; I, 180 mg; Se, 110 mg; Co, 8,805 mg.

3.2. Rumen sampling and sample detection

For each animal, samples of rumen contents (solid and liquid phase) and rumen papilla were obtained on days 20 and 21 of the experiment shortly before morning feeding (0 h) and at 2, 4 and 6 h after morning feeding. Essentially, approximately 300 g of mixed rumen contents were taken from each cow through the rumen fistula. Rumen samples were filtered with four layers of cheesecloth, allowing the separation of rumen solids from the liquid fraction. The aliquots of the liquid fraction were dispensed into centrifuge tubes. Approximately 100 μL of hydrochloric acid (6 mol L^{-1}) was added to 10 mL of filtered rumen fluid for detection of urea nitrogen (Urea-N) and ammonia nitrogen (NH_3-N). The solid fraction was washed with 50 mL of ice-cold phosphate-buffered saline (PBS) twice and residues were kept. Rumen papillae samples were collected by scraping with a spatula from different rumen locations (the front-, middle- and post-ventral sac) via the rumen cannula and washed twice in ice-cold PBS (Petri et al., 2013). All rumen samples were snap frozen in liquid nitrogen and stored at -80 ℃ for further analysis.

Rumen fluid samples were centrifuged (13, 000 × g at 4℃ for 15 min) and supernatants were stored at -20 ℃ until analyzed. NH_3-N concentration was determined by using an adaptation of the method based on the Berthelot (phenol-hypochlorite) reaction (Broderick and Kang, 1980). Urea-N concentration was determined by the diacetyl monoxime method using a commercial kit (Nanjing Jiancheng Co., Nanjing, China). Urease activity was evaluated on total rumen microbial protein extracts by measuring the amount of ammonia released from urea according to Zhao et al. (2015). One unit of urease activity was defined as 1 μmol of ammonia released per min per mg microbial cytoplasmic protein.

3.3. Microbial DNA extraction

The rumen contents and papilla samples collected at 2 h after morning feeding were chosen for DNA extraction based on the high urea hydrolysis rates at this time. Rumen liquid fraction samples (1 ml) were centrifuged at 350 × g at 4℃ for 10 min to remove the feed residue, and the supernatant were centrifuged at 16 000 × g at 4℃ for 15 min to collect the liquid-associated bacteria (LAB). Approximate 0.5 g thawed rumen papilla and 0.5 g solid fraction was directly used for solid-associated bacteria (SAB) and wall-associated bacteria (WAB) DNA extraction, respectively. Total DNA of bacteria was extracted using cetyltrimethylammonium bromide (CTAB) plus bead beating method (Minas et al., 2011). Briefly, samples from each fraction was homogenized with 0.5 g zirconium beads (0.5 mm diameter) and 800 μL CTAB buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2 % CTAB) using a Mixer Mill MM 400 (Retsch, Haan, Germany) with vibrational frequency of 1800 min^{-1} and grinding time of 60 s. Then samples were incubated at 70℃ for 20 min and centrifuged at 13, 000 × g for 10 min, and the supernatant was mixed with 600 μL phenol-chloroform-isoamyl alcohol (volume 25:24:1). The upper layer was transferred to new tube and mixed with 0.8 times volume of isopropanol to precipitate DNA. Extracted DNA was qualitatively assessed by agarose gel electrophoresis and quantified using a Nanodrop™ spectrometer (Thermo Scientific, USA). DNA was diluted to a concentration of 50 ng μL^{-1}, and was used as templates for amplification in the following PCRs.

3.4. PCR amplification of urease genes (ureC) and Illumina sequencing

Urease (*ureC*) genes were amplified with the modified primer set, UreC-F 5'-barcode-TGGGCCTTAAAATHCAYGARGAYTGGG-3' and UreC-R 5'-GGTGGTGGCACACCATNANCATRTC-3' (Reed, 2001), where the barcode is an eight-base sequence unique to each sample. Reactions were performed in a MyCycler™ Thermal

Cycler (Bio-Rad, USA) using a 50 μL mixture containing 5 μL 10 × PCR buffer (Invitrogen, Carlsbad, CA, USA), 1.5 μL $MgCl_2$ (50 mM), 1 μL dNTP mixture (10 mM), 1.5 μL each forward and reverse primer (10 μM), 0.4 μL Platinum Taq DNA polymerase (Invitrogen), 2 μL rumen microbial DNA (100 ng μL^{-1}), and 37.1 μL sterile ddH_2O. PCR amplification began with a 5 min denaturing step at 94 ℃, followed by 30 cycles at 94 ℃ for 30 s, 50 ℃ for 30 s, and 72 ℃ for 30 s; extension was achieved at 72 ℃ for 15 min. PCR amplicons of approximately 324 bp were extracted from 2 % agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union, CA, USA) according to the manufacturer's instructions and quantified using QuantiFluor™-ST (Promega US, Madison, WI, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform according to the standard protocols.

3.5. Sequencing data processing and sequence analysis

Low-quality raw reads were eliminated using Trimmomatic (Bolger et al., 2014) based on the following criteria: a) reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp; b) 1 or more mismatch in barcode; c) > 2 nucleotide mismatch in primers. Paired-end reads were merged using FLASH (Magoč and Salzberg, 2011) with the parameter that overlap was longer than 10 bp and its mismatch rate was lower than 20%. Merged reads with length of > 200 bp were kept and assigned to each sample based on the unique barcode (Caporaso et al., 2010; Bokulich et al., 2013). Chimera sequences were detected and removed using the UCHIME *denovo* algorithm (Edgar et al., 2011). Operational taxonomic units (OTU) were clustered at a cut-off value of 0.97 similarity using USEARCH in the QIIME package (Caporaso et al., 2010; Edgar, 2010). A clustering value of 0.97 similarity was empirically confirmed by analyzing the clustering of taxonomical known *ureC* genes. Taxonomic assignment of representative sequences of OTUs was performed using GraftM (https://github.com/geronimp/graftM) with a likelihood cutoff of 0.75 when using pplacer (Matsen et al., 2010) for placement of the sequences against a compiled *ureC* gene package. The *ureC* gene package was compiled in graftM with the create command using a manually edited *ureC* alignment file. The alignment was generated from bacterial and archaeal *ureC* gene sequences with taxonomic assignment data which were downloaded from NCBI. The genes were aligned and manually edited using ARB software and then the region corresponding to the PCR amplicon was exported (Ludwig et al., 2004). Sequences containing more than 50% gaps in this region were removed with Belvue (Sonnhammer and

Hollich, 2005). A phylogenetic tree was generated using FastTree (Price et al., 2009) in QIIME for calculating UniFrac distances. Alpha and beta diversity and significant fold changes of OTU's were performed in the R packages ade4, Phyloseq, and DESeq2 (Chessel et al., 2004; McMurdie and Holmes, 2013; Love et al., 2014). The significances of grouping in the PCoA plots were tested by analysis of similarity (ANOSIM) with 999 permutations. Family level heatmap plots were generated in R using the ampvis R package (Albertsen et al., 2015), while annotated heatmaps of the top 50 OTUs were created using the NMF R package (Gaujoux and Seoighe, 2010).

3.6. Statistical analysis

The rumen NH_3-N and urea-N concentration, urease activity, and diversity indices were analyzed using the SAS mixed procedure (SAS Institute, Inc, Cary, NC, USA) as shown in the following equation: $Y_{ijkl} = \mu + t_i + b_k + c(b)_{jk} + p_l + e_{ijkl}$, where Y_{ijkl} is the observation on cow j with treatment i, order of treatment k and period l; μ is the overall mean; ti is the fixed effect of treatment i; b_k is the effect of order k of applying treatments; $c(b)_{jk}$ is the random effect of cow j within order k; pl is the effect of period l; and e_{ijkl} is the random error. Differences were declared significant at $P < 0.05$.

3.7. Nucleotide sequence accession number

All the raw sequences after assembling and filtering were submitted to the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/Traces/sra/), under accession number SRP076839.

4. Results

4.1. Urea metabolism in the rumen

Urea supplementation significantly increased ($P < 0.05$) rumen NH_3-N concentration at 2 and 4 h after morning feeding with the peak at 2 h (Table 7). No significant difference in the urease activity was observed between the control and urea groups, with both exhibiting maximum activity 2 h after feeding ($P > 0.05$). For the urea supplemented group, the increased urease activity at 2 hours also coincided with higher NH_3-N concentration.

Table 7 NH_3-N and urea nitrogen (urea-N) concentrations and urease activity in the rumen of dairy cows from different treatments (N=6)

Item	Time (h)	Treatment		SEM	*P*		
		Control	Urea		Treatment	Period	Treatment*Period
NH_3-N concentration (μmol dL^{-1})	0	18.16	21.95	1.550	0.24	0.07	0.67
	2	15.56[b]	31.05[a]	1.747	< 0.01	0.58	0.22
	4	10.65[b]	23.81[a]	2.132	0.03	0.90	0.96
	6	8.61	14.32	1.148	0.1	0.78	0.49
Urea-N concentration (mg L^{-1})	0	4.90	6.60	1.092	0.28	0.51	0.28
	2	5.16	5.33	0.195	0.54	< 0.01	0.14
	4	5.56	5.59	0.097	0.86	0.08	0.06
	6	5.52	5.58	0.156	0.76	0.05	0.45
Urease activity (nmol min^{-1} mg^{-1})	0	53.24	58.16	3.999	0.54	0.52	0.99
	2	61.37	62.32	10.397	0.97	0.75	0.80
	4	41.56	44.62	6.867	0.79	0.51	0.92
	6	33.59	31.62	6.204	0.73	0.40	0.50

[a,b] Different letters in the same row indicate statistically significant differences for treatment effect at $P < 0.05$.

4.2. Comparison of ureC gene diversity and distribution

In total, 1,059,496 quality sequence reads were obtained with an average read length of 299 bases from the 36 samples. The total number of reads from each sample varied from 20,591-39,908 and the average reads number was 29,430. The total sequences were assigned to 588 OTUs using a cut-off of 97 % sequence similarity.

Alpha diversity estimates are summarized in Figure 11 and Table 8. The total number of observed OTUs from the WAB was lower compared to the LAB and SAB fractions ($P < 0.001$). Good's coverage estimates of sampling completeness showed greater than 99 % coverage (Table 8). Similar values for estimator Chao1, Shannon and Simpson indices were obtained for bacterial samples from the control and urea groups in each rumen fraction ($P > 0.05$), demonstrating no significant difference of the diversity measure and evenness of *ureC* genes after exogenous urea was provided to dairy cows. The Shannon diversity index for the WAB was lower than for the LAB and SAB fractions ($P = 0.002$).

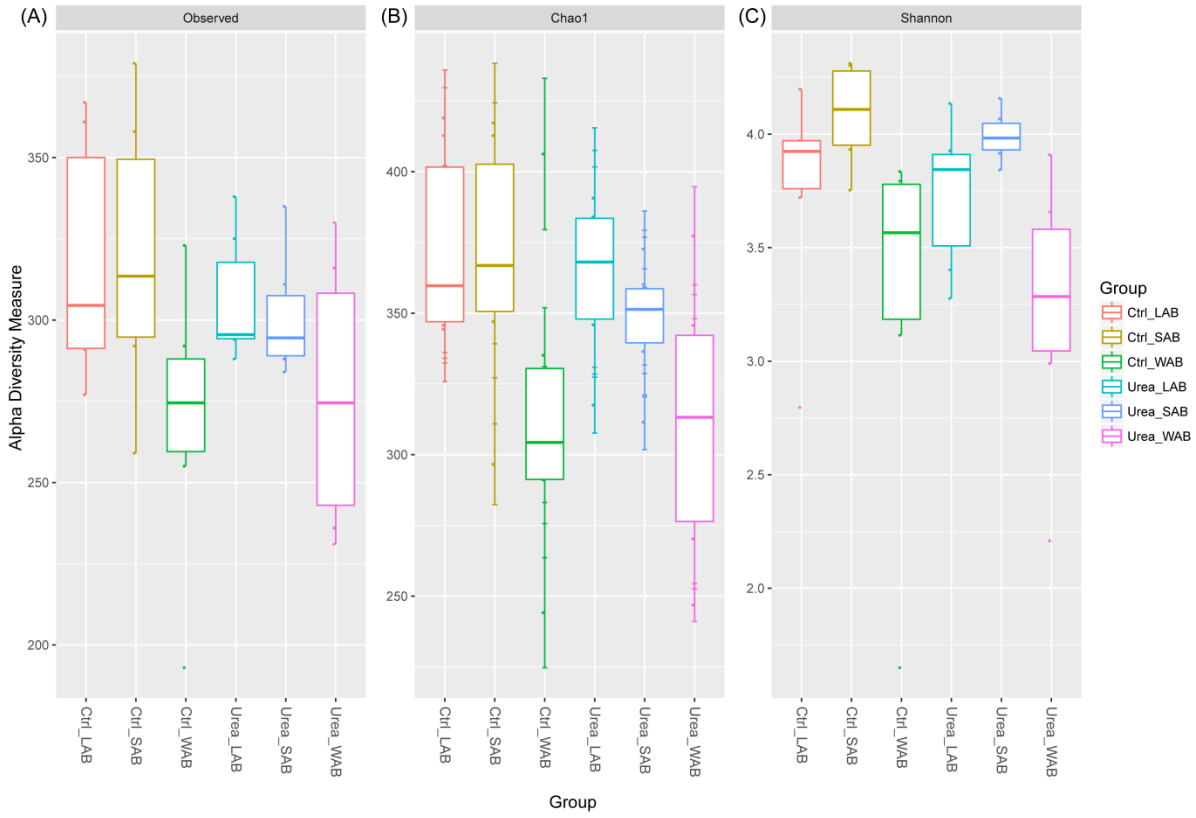

Figure 11 Alpha diversity measures for *ureC* rumen microbiomes across different treatments and fractions.

(A) Total observed taxonomic units, (B) Chao1 estimates and, (C) the Shannon diversity index. Boxplots indicate the first and third quartiles with the median value indicated as a horizontal line the whickers extend to 1.5 times the inter quartile range. LAB, liquid-associated bacteria; SAB, solid-adherent bacteria; WAB, wall-adherent bacteria. Urea, urea group; Ctrl, control group.

Table 8 Alpha diversity indices for the rumen bacteria *ureC* genes from each treatment groups and rumen fraction (N=6)

Indices	Control			Urea			SEM	*P*		
	LAB	SAB	WAB	LAB	SAB	WAB		Trt	Fraction	Trt* Fraction
Observed	317[a]	319[a]	268[b]	306[ab]	301[ab]	277[ab]	12.30	0.694	<0.001	0.394
Good's coverage	0.9963[c]	0.9968[abc]	0.9970b[a]	0.9965[bc]	0.9970[ab]	0.9971[a]	<0.001	0.355	0.012	1.000
PD	20.91	19.52	19.02	20.75	19.18	19.85	0.298	0.857	0.087	0.682
Chao1	373.52[a]	367.88[a]	314.19[bc]	362.37[ab]	347.23[ab]	311.09[c]	13.12	0.544	<0.001	0.776
Shannon	3.756[ab]	4.086[a]	3.254b[c]	3.378[ab]	3.991[a]	3.222[bc]	0.125	0.790	0.002	0.978
Simpson	0.938[ab]	0.968[a]	0.875[ab]	0.942[ab]	0.965[ab]	0.869[b]	0.014	0.939	0.023	0.989

[a, b, c] Different letters among various treatment groups and fractions indicate statistically significant differences ($P < 0.05$).
Observed, observed taxonomic units. PD, phylogenetic diversity; LAB, liquid-associated bacteria; SAB, solid-adherent bacteria; WAB, wall-adherent bacteria.

The community composition of ureolytic microbiome as assessed by beta diversity measures demonstrated that the bacterial *ureC* gene composition of the WAB was significantly different from LAB and SAB fractions, with approximately 36 and 64% of the variance explained for the Bray–Curtis and weighted UniFrac metrics, respectively (Bray-Curtis, $R^2 = 0.198$, $P = 0.001$; Weighted UniFrac, $R^2 = 0.343$, $P = 0.001$) (Figure 12). However there was no significant differences in bacterial community composition based on *ure*C genes between urea treated and control animals (Bray-Curtis, $R^2 = 0.015$, $P = 0.906$; Weighted UniFrac, $R^2 = 0.010$, $P = 0.791$).

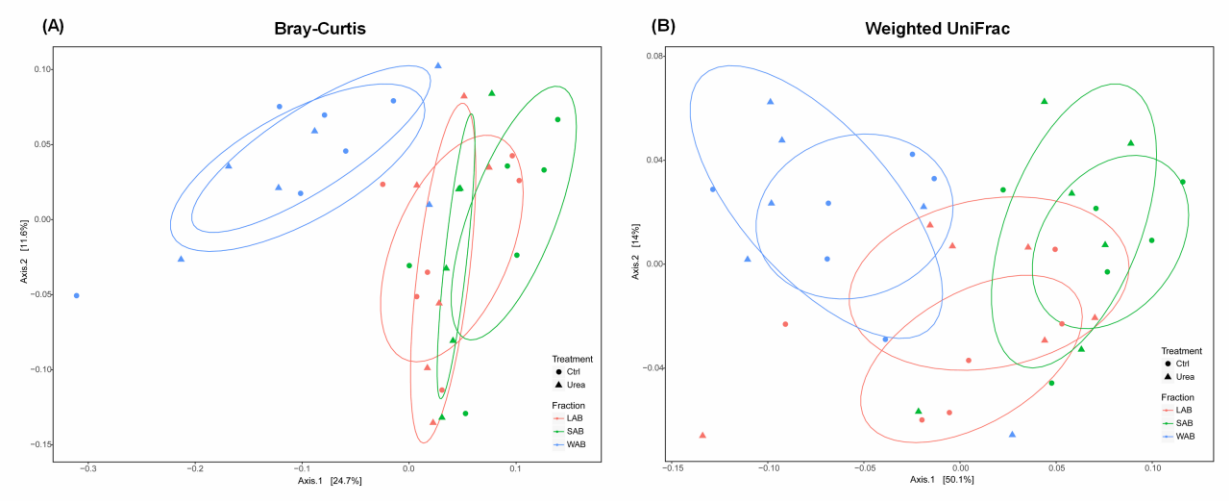

Figure 12 Principle Coordinate Analysis comparing changes in rumen *ureC* genes based on Bray–Curtis and weighted Unifrac distances.

LAB, liquid-associated bacteria (red); SAB, solid-adherent bacteria (green); WAB, wall-adherent bacteria (blue). Urea, urea group (triangle); Ctrl, control group (circle).

Approximately 55 % of the total sequences could not be confidently classified to any known phylum, while the remaining sequences were assigned to seven bacterial phyla. The majority of sequences were assigned to *Proteobacteria* (22.4–31.9%, SEM=0.015), *Firmicutes* (11.1–20.2%, SEM=0.014) and *Bacteroidetes* (0.2–0.8%, SEM=0.001) from the different treatment groups and rumen fractions (Figure 13). At the family level, the dominant classified *ureC* genes in the rumen contents were from Methylococcaceae, Clostridiaceae, Paenibacillaceae, Helicobacteraceae, and Oxalobacteraceae while Methylophilaceae and Methylococcaceae were predominant in the WAB fraction (Figure 13). Interestingly, a very small number of *ureC* genes were affiliated with archaea from the Thaumarchaeota (0.0007%).

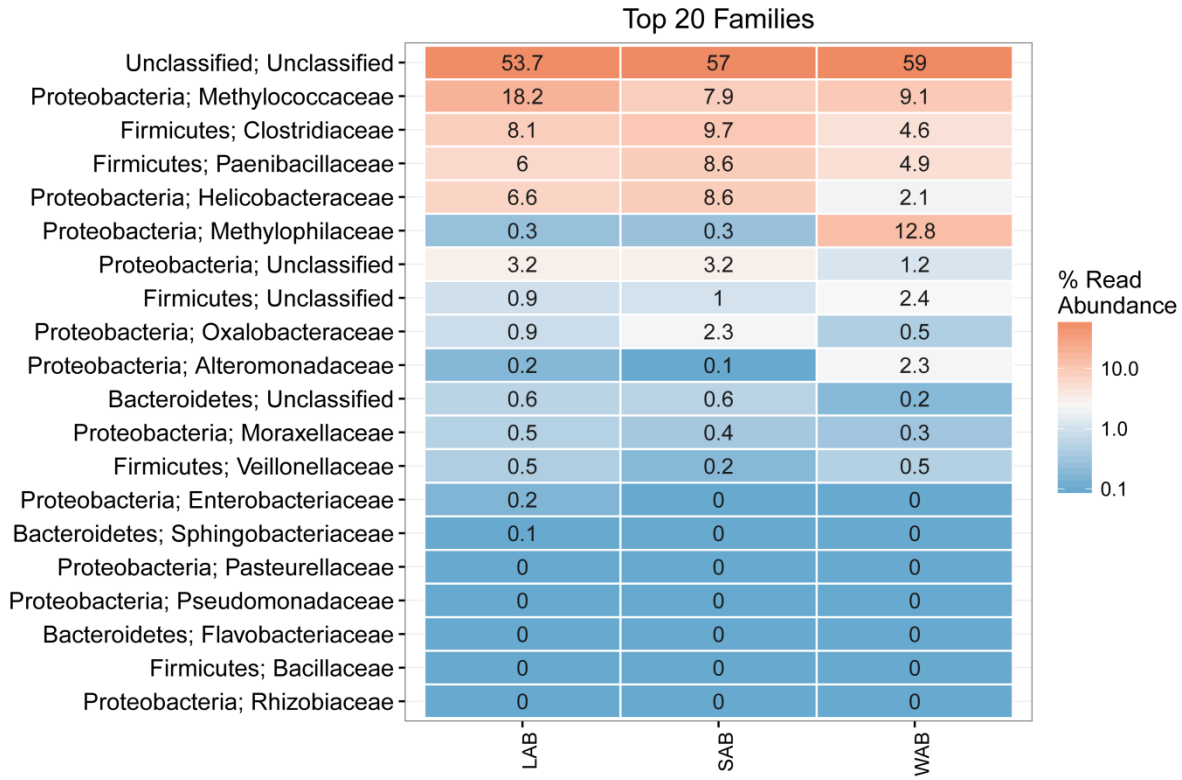

Figure 13 Heatmap of the top 20 *ureC* gene families from different rumen fractions. Taxonomic assignment shows the phylum and family level for each row. Numbers and colour scale in cells represent the relative abundance for a given family level. LAB, liquid-associated bacteria; SAB, solid-adherent bacteria; WAB, wall-adherent bacteria.

Approximately 85 % of the sequence data was attributed to the top 50 abundant *ureC* gene OTUs. A high degree of similarity was observed for the rank abundance of OTUs for LAB and SAB, which clustered together and were distinct from the WAB fraction (Figure 14). A cluster of OTUs (5, 6, 12, 15, 18, and 27) exhibited higher rank abundance in the WAB and were absent or of lower abundance in the other two fractions. All of these OTUs were found to be significantly more abundant in the WAB (adjusted $p < 0.001$) (Figure 15). Two of the most abundant WAB OTUs, 5 and 12 were unclassified. Both OTU 6 and 15 were affiliated with the *Methylophilus* genus, and OTU 18 was classified with *Marinobacter*. A moderately abundant OTU 72 was classified to the Veillonellaceae family and a low abundant Helicobacteraceae OTU was also significantly linked with the WAB. The cluster which contained OTU 0, 441, 711, 606, 1, 3, and 4 was more abundant in the LAB and SAB compared to the WAB, but was seen consistently across all samples and was not significantly different. Both OTU 1 and 4 were affiliated with *Methylomonas* genus of bacteria. The *ureC*

gene OTU 8, 30, 19, and 21 which affiliated with *Helicobacter* were most abundant in the rumen content (Figure 14), with OTUs 8 and 30 being significantly different from the WAB fraction (Figure 15).

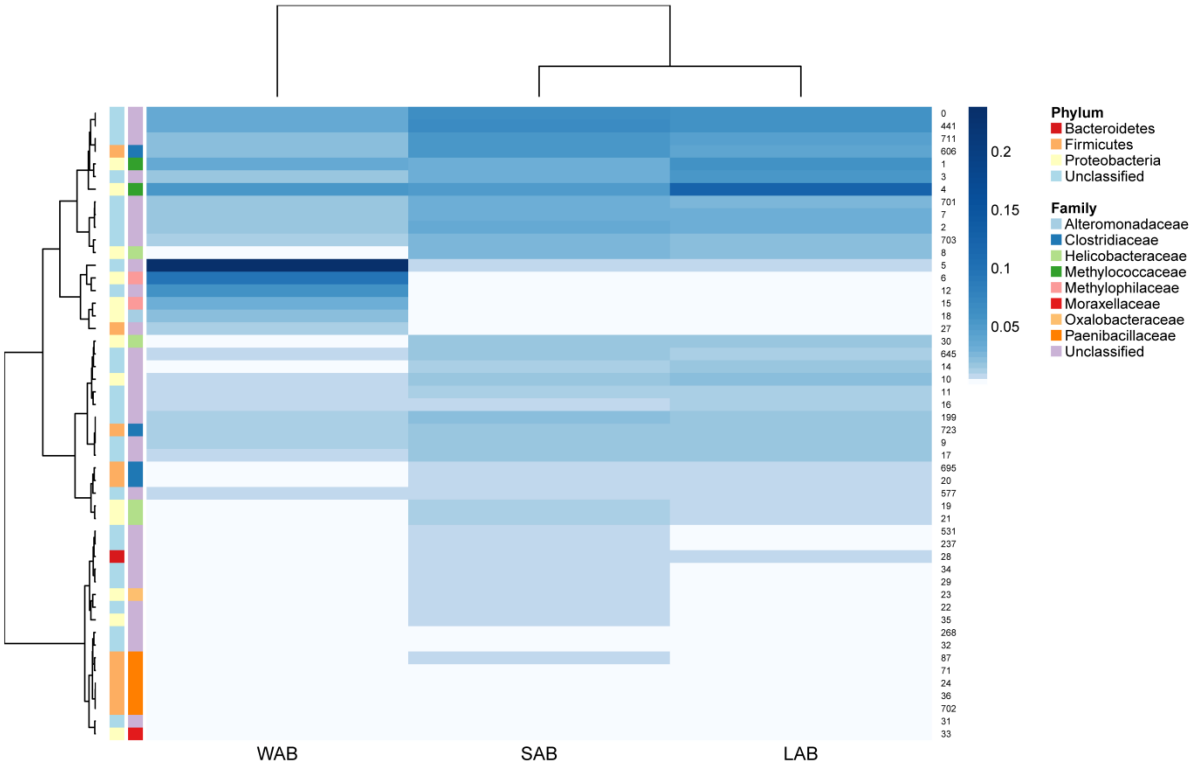

Figure 14 Rumen *ureC* gene community heat maps and clustering of the most abundant 50 OTUs from different rumen fractions.

Ward's minimum variance method was used for hierarchical clustering of the computed distance matrix for samples based on the Jaccard dissimilarity indices of the OTU data in the vegan package. LAB, liquid-associated bacteria; SAB, solid-adherent bacteria; WAB, wall-adherent bacteria.

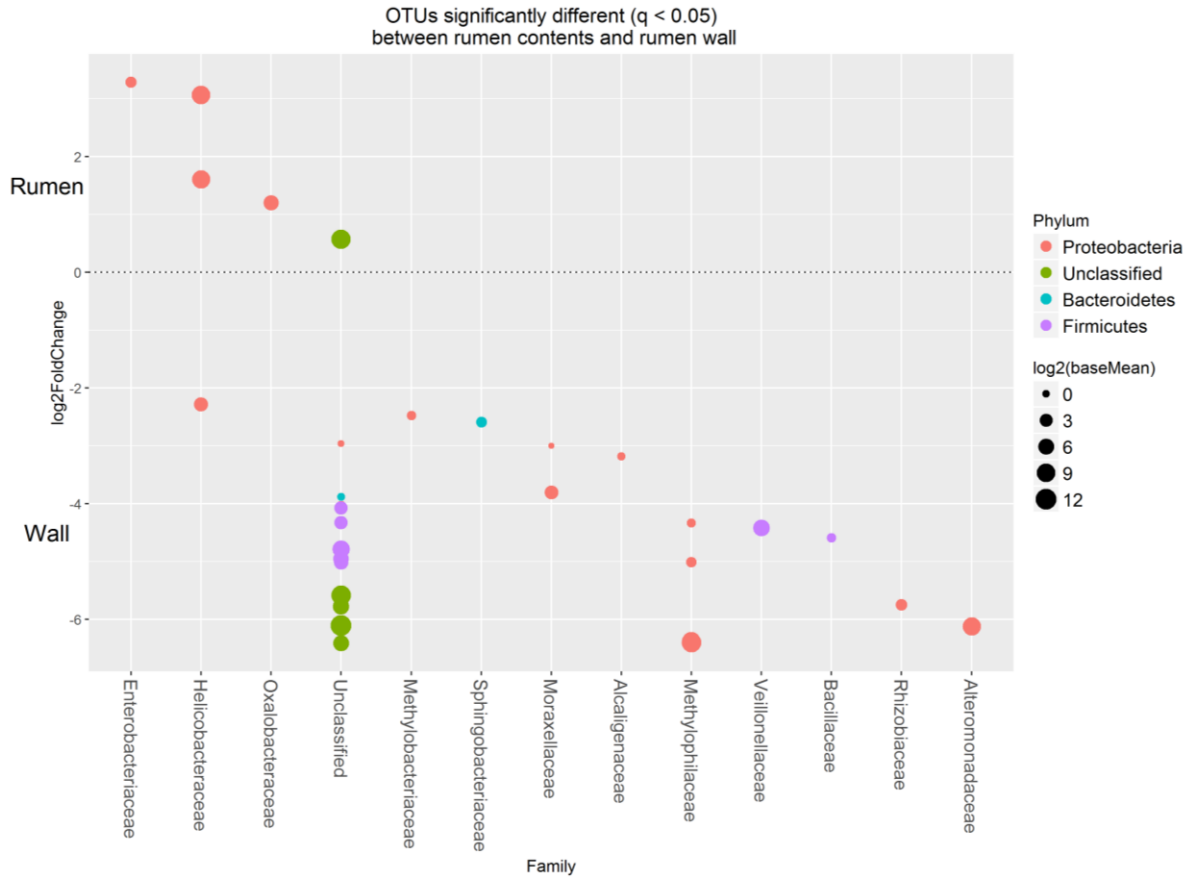

Figure 15 OTUs significantly different (q < 0.05 FDR) between the rumen contents (liquid and solid fractions) and the rumen wall.

Upper axis represents OTU's with a $\log_2$ fold positive change for rumen contents relative to the rumen wall while the lower y axis is the negative fold change of the rumen wall relative to the rumen contents. Each point represents a single OTU colored by phylum and grouped on the x axis by taxonomic family level, size of point reflects the $\log_2$ mean abundance of the sequence data.

5. Discussion

Previous studies using culture-dependent methods have revealed limited information with regard to the rumen urea-degrading bacteria (Kakimoto et al., 1989; Laukov á and Koniarov á, 1994). By using the *ureC* gene as a biomarker for phylogenetic analysis we have obtained a better estimate of the composition of the ureolytic bacteria found in the rumen. Importantly, only about 45% of the sequences obtained could be assigned to any known phylum, indicating that the rumen may contain newly undiscovered sources of urease genes. Furthermore, the reference dataset used for taxonomic assignment was predominated by sequences from the *Firmicutes* and *Proteobacteria* phyla and will produce higher likelihood

values for environmental sequences closely related to these species.

Urease genes from *Proteobacteria* constituted the highest proportion of classified sequences in all rumen samples which is in accord with studies from other environments, where urea-degrading microorganisms in open-ocean and estuarine planktonic communities were mainly affiliated with this phylum (Collier et al., 2009). In our study, the *ureC* gene OTUs which belonged to rumen wall adherent bacteria were predominately from unclassified taxa, while some were affiliated with *Methylophilus* and *Marinobacter* bacteria. Methylotrophic species of bacteria from the genus *Methylophilus* (*M. methylotrophus*, *M. quaylei* sp. nov., and *M. rhizosphaerae* sp. nov.) with urease activity have been identified in studies from sludge and river water. These groups of bacteria can use methyl compounds such as methanol and methylamines for the assimilation of ammonia into cell protein (Greenwood et al., 1998; Doronina et al., 2005; Madhaiyan et al., 2009). An active-transport system for short-chain amides and urea has been identified in *M. methylotrophus* (Mills et al., 1998). *Marinobacter* species from marine environments are efficient degraders of aliphatic and polycyclic aromatic hydrocarbons as well as acyclic isoprenoid compounds (Duran, 2010). Genomic analysis of *Marinobacter aquaeolei* indicates this bacterium has the metabolic potential to utilize oxygen and nitrate as terminal electron acceptors, iron as an electron donor, and urea, phosphonate, and various hydrocarbons as alternative N, P, and C sources, respectively (Singer et al., 2011).

Urease genes with closest affiliation to *Helicobacter spp*. and *Methylomonas spp*. were present in all rumen sample fractions but were in higher abundance in the rumen contents. Previously, Zhao et al. (2015) had attempted to examine *ureC* diversity in the rumen digesta, by cloning and sequencing *ureC* genes, and found that among the total 317 *ureC* sequences, 22% were affiliated with *H. pylori* (98-100% aa sequence identity). The data from this study indicate that greater diversity and other taxonomic groups of ureolytic bacteria are more abundant in the rumen than *Helicobacter*. *Helicobacter* spp. naturally colonize the lining of stomach and intestines in human and animals (Fox, 2002; Harper et al., 2003), and they produce urease to maintain a neutral pH in their immediate environment. Some *Helicobacter* species isolated from the gastrointestinal tracts of sheep and dolphins have tested positive for urease activity (Harper et al., 2002; Coldham et al., 2011).

Among the predominant OTUs, both OTU 4 and 1, which were dominant in the rumen liquid fraction were affiliated with the Methylococcaceae family. Previous studies in aquatic

environments have demonstrated that some *Methylomonas* spp. (*M. methanica*, *M. fodinarum* and *M. paludis*) all possess urease activity (Dianou and Adachi, 1999; Boden et al., 2011). It is known that species of *Methylomonas* are able to obtain carbon and energy from oxidation of methane or methanol and use urea as a nitrogen source (Hoefman et al., 2014; Soren et al., 2015). Our results indicate that the ureolytic bacteria from the *Helicobacter* and *Methylomonas* that inhabit the rumen likely play an important role in hydrolyzing endogenous or exogenous urea.

Urea supplementation had no significant effect on the diversity and distribution of the *ureC* genes which was unexpected. The lack of response may be due to several factors. Firstly, the crude protein (CP) content (16.67 % of DM) in the basal diet may have provided adequate ammonia, amino acid, or peptide for the synthesis of microbial protein (Agle et al., 2010;Recktenwald et al., 2014), and the bacteria may have used organic forms of nitrogen in preference to ammonia for the microbial protein synthesis (Milton et al., 1997; Lebzien, 2006). The regulation of urease synthesis in ureolytic bacteria is complex (Mobley et al., 1995), urease synthesis in some bacteria is regulated by environmental conditions, such as concentration of urea and nitrogen or pH (Collins and D'Orazio, 1993; Weeks and Sachs, 2001). However, in some organisms, urease synthesis is constitutive (Zotta et al., 2008; Carter et al., 2009; Burbank et al., 2012). Though the NH_3-N concentrations in the urea-supplemented group were higher than those in the control group, no differences in the urease activity between the two groups were observed. The conversion of urea to ammonia is rapid and not rate limiting, so on a high protein diet sufficient endogenous urea may have induced urease activity to an extent where differences did not occur between the two treatments even though urea and NH_3-N concentrations might be higher in the urea supplemented group. Besides, Greenwood et al. (1998) also found that the urease was repressed by excess amounts of its reaction product, ammonia. Collectively all these factors may have contributed to the similar urease activity between the two treatments. Thus, the rumen harbors a large diversity of ureolytic bacteria but the mechanisms controlling urease synthesis and the impact of urea hydrolysis on the growth of these bacteria need further research.

6. Conclusion

There was a predominant ureolytic bacterial community in the rumen of dairy cows but more than 55% of the *ureC* sequences did not affiliate with any known urease genes. The bacterial

urease gene profile from the rumen wall was distinctly different from the rumen contents and *ureC* genes from Methylophilus and Marinobacter were identified predominantly in this fraction. The ureolytic bacterial populations were not changed in diversity or abundance by urea supplementation. This study contributes new data to existing urease gene information relating to the predominant ureolytic microbial community in ruminants. Understanding the rumen predominant urease genes may provide basis for acquiring valid regulation targets of ureolytic bacteria to mitigate urea hydrolysis and subsequently improve urea nitrogen utilization in ruminants.

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CHAPTER V

This study aimed to identify the changes in both rumen microbial and host plasma metabolites induced by exogenous urea supplementation in dairy cows using NMR metabolomics. Multivariate data analysis was used to evaluate the differentially expressed metabolites and changed metabolic pathways.

Article 4

Urea nitrogen induces changes in rumen microbial and host metabolic profiles in dairy cows

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1. Abstract

Urea has been used in diets of cattle as a non-protein nitrogen source. It is hydrolyzed to ammonia, which can be used for microbial protein synthesis. Use of metabolomics methodologies to study the rumen microbial and host blood metabolic profiles induced by urea nitrogen has not been previously characterized. The objective of this study was to identify changes in rumen microbial and plasma metabolite profiles in dairy cows after urea supplementation using a nuclear magnetic resonance (NMR)-based untargeted quantitative metabolomic approach. Six dairy cows with rumen fistulas were randomly assigned to two groups used in a two period cross-over trial and each experimental period lasted 21 days. All the cows were fed the same total mixed rations, but were intraruminally supplemented with 180 g urea per cow daily or not during the experimental period. Rumen fluid and blood samples were collected and analyzed using nuclear magnetic resonance spectroscopy and multivariate analysis of variance. Differences in rumen and plasma metabolite concentrations in cows from the two groups were assessed using orthogonal partial least-squares discriminant analysis and identified by searching against related databases. Concentrations of valine, aspartate, glutamate, and uracil in the rumen, and urea and pyroglutamate in the plasma, were higher (1.36- to 3.17-fold, $P < 0.05$) in the urea-supplemented group than in the control group. Metabolic pathway analysis of the affected metabolites revealed that pantothenate and CoA biosynthesis, beta-alanine metabolism, valine, leucine, and isoleucine metabolism in the rumen, and urea and glutathione metabolism in the plasma were significantly increased by urea nitrogen. The levels of aspartate and glutamate in the rumen correlated strongly ($r = 0.73$ and $r=0.74$, respectively, $P < 0.01$) with the level of urea in plasma. These findings provided novel information to aid understanding of the metabolic pathways affected by urea nitrogen in dairy cows, and could potentially help to guide efforts directed at improving the efficiency of urea utilization in the rumen.

Keywords: rumen, plasma, urea, metabolites, NMR metabolomics

2. Introduction

Urea is used as a NPN in ruminant diets as a cost-efficient replacement for feed proteins (Kertz, 2010). Urea is hydrolyzed by rumen microbial urease to ammonia (NH_3) which is utilized for the synthesis of microbial protein required for the animal growth (Owens et al., 1980; Milton et al., 1997). In the rumen, hydrolysis of urea to NH_3 occurs at a greater rate than NH_3 can be utilized by rumen bacteria, therefore leading to the ruminal NH_3 accumulation and its subsequent entry into the circulation (Recavarren and Milano, 2014). Thus, the efficiency of urea nitrogen utilization in ruminants is normally low. Multiple studies were conducted for improving the efficiency of urea utilization in dairy cows (Sweeny et al., 2014; Giallongo et al., 2015). However, information on metabolic pathways involved in urea nitrogen utilization in dairy cows has not been fully characterized.

Metabolomics can provide accurate information regarding the physiological state of the microbiome or the host organism (Lindon and Nicholson, 2008). In particular, proton nuclear magnetic resonance spectroscopy (1H-NMR) is a non-invasive technique that permits objective and reproducible sample analysis (Taylor et al., 2002). NMR-based metabolomics approaches have been widely used to analyze materials including blood, urine, and tissue extracts from humans and other mammals (Benahmed et al., 2014; Duarte et al., 2014; Pinto et al., 2015). Recently, metabolomics methods have also been used to evaluate rumen microbial metabolites (Mao et al., 2016; Zhao et al., 2014), plasma (Sun et al., 2014; Li and C., 2015), milk (Sundekilde et al., 2013; Sun et al., 2015) and urine (Tang et al., 2016) from dairy cows.

In a previous study, Bertram et al. (2011) assessed the effect of dietary nitrogen content on the urine metabolite profile of dairy cows using a NMR approach and found that urea was one of the urinary metabolites that contributed to the prediction of nitrogen intake and efficiency. It is known that dietary nitrogen supplementation in the form of urea affects ruminal microbial protein synthesis and hepatic urea synthesis of dairy cows (Alves et al., 2014; Recavarren and Milano, 2014). However, most studies investigating the effect of urea nitrogen in ruminants to date have employed targeted analyses, meaning that limited numbers of metabolites are known to be influenced by dietary nitrogen levels (Sweeny et al., 2014; Zhang et al., 2014; Holder et al., 2015). Thus, we hypothesized that an untargeted metabolomics approach could provide a more complete analysis of the metabolites involved in urea nitrogen metabolism of dairy cows. This study aimed to identify the changes in both

rumen microbial and host plasma metabolites induced by exogenous urea supplementation in dairy cows using NMR spectroscopy. Multivariate data analysis was used to evaluate the differentially expressed metabolites. Our data may provide novel information to aid understanding of the metabolic pathways affected by urea nitrogen in dairy cows.

3. Materials and methods

3.1. Animals, Diets, and Sampling

Six Chinese Holstein dairy cows (550 ± 50 kg BW and 100 ± 21 days in milk) fitted with ruminal cannulas were used in a two period cross-over trial. All cows were fed the same total mixed ration (TMR) for 2 wk prior to the start of the study. Cows were divided into a Urea group (Urea) which received 180 g of urea daily, and a Control group (Ctrl) which was not urea-supplemented. Each experimental period lasted 21 d, and the first was followed by a 14 d washout period, after which the other intervention was applied. Each day, the 180 g of urea was separated into three portions (70, 55, and 55 g for each feeding, respectively) and packaged with quantitative filter paper to prevent NH_3 poisoning. Urea was added into the rumen through the fistula during each feeding period. All cows were kept in individual pens with free access to water and were fed TMR three times daily (at 0700, 1400, and 1900 h) ad libitum. The basic diet primarily consisted of alfalfa hay (28.4% DM), corn silage (26.7%), corn (22.6%), and soybean meal (11.8%) (Table 6). Experimental procedures involving the animal care and management, and sampling were approved by the Chinese Academy of Agricultural Sciences Animal Care and Use Committee (Beijing, China).

For each animal, rumen fluid samples were obtained on day 21 of each experimental period shortly before morning feeding (0 h) and at 2, 4 and 6 h after morning feeding. Approximately 300 g of mixed rumen contents was taken from each cow through its ruminal fistula and filtered through four layers of cheesecloth to obtain the rumen liquid. Aliquots were dispensed into microtubes and frozen in liquid nitrogen. Rumen fluid samples collected were used for measurements of ammonia nitrogen (NH_3-N) concentration, which was determined by using a method based on the Berthelot (phenol-hypochlorite) reaction (Broderick and Kang, 1980). The host blood samples were obtained at 2 h after the morning feeding. Blood samples (10 mL) were collected from the caudal vein into EDTA containing tubes, kept in a cooler, and transferred to the laboratory within 30 min. These samples were centrifuged at 2,000 ×*g* at 4 ℃ for 15 min, and the plasma was collected and stored at -80 ℃ until analysis.

3.2. Sample Preparations, NMR Measurements, and Data Processing

Rumen fluid samples obtained at 2 h after morning feeding were thawed at room temperature and centrifuged at 13,000 ×g at 4 ℃ for 15 min to remove particulate matter. Then, rumen fluid and blood plasma samples were sterilized by passing through a 0.22 μm ultrafiltration membrane (Millipore Corporation, Billerica, MA, USA). A 450 μL aliquot of the filtrate was transferred to a 1.5 mL Eppendorf tube, followed by the addition of 50 μL of dextran sulfate sodium (DSS, 4.088 mM) and mix for 10s. After centrifuging at 13,000 ×g at 4 ℃ for 1 min, the samples supernatants (480 μL) were then transferred to a standard NMR tube for subsequent NMR spectral analysis. Both tubes and the Millipore 3-kDa ultrafiltration filter were washed with ddH2O five times before use to remove residual glycerol.

Spectra were generated using a Bruker AV III 600 MHz spectrometer (Bruker Bio Spin Corporation, Billerica, MA, USA) equipped with an inverse cryoprobe. The first increment of a 2D-1H, 1H-nuclear Overhauser enhancement spectroscopy (NOESY) pulse sequence was utilized for the acquisition of 1H-NMR data and to suppress the solvent signal. For the rumen fluid samples, spectra were acquired with 28 K data points and 128 scans over a spectral width of 7,225.4 kHz. For the plasma samples, spectra were acquired with 32 K data points and 64 scans over a spectral width of 8,000 kHz. All 1H NMR spectra were referenced to DSS-d6 at 0.0 ppm and processed manually with Chenomx NMR suite (version 8.0, Chenomx, Inc., Edmonton, AB, Canada). Identification of metabolites was based on their chemical shifts and by reference to the Chenomx 600 MHz library. Quantification of metabolite concentrations was achieved with the described method using the Chenomx NMR suite (Weljie et al., 2006). The concentrations of the metabolites were exported in EXCEL format (Microsoft, Redmond, WA, USA) for further analysis.

3.3. Multivariate Analysis

Multivariate data analysis was performed using Simca-P software (version 14.0; Umetrics, Umea, Sweden). Data sets were scaled, using unit variance (UV) scaling to reduce noise in the models. Principal component analysis (PCA) was applied to data sets to visualize the distribution of origin data and reveal the presence of outliers beyond the 95% significance region. To reduce errors within the group and eliminate random errors, supervised orthogonal projections to latent structures-discriminant analysis (OPLS-DA) were applied (Sui et al., 2012). OPLS-DA models were generated using the first principal component (t[1]P) and the

second orthogonal component (t[2]O) (Yin et al., 2008). Scores plots combining the reliability and correlation from the OPLS-DA models were used to identify metabolites that were present at concentrations that differed between the groups. The quality of each model was evaluated using the predictive ability parameter (Q^2), which is calculated using an internal cross-validation of the data and goodness-of-fit (R^2). Based on the OPLS-DA, a loading plot was constructed that showed the contribution of variables to the differences between two groups. The variable importance in the projection (VIP) values for variables in the model calculated to indicate their contributions to the classification of the samples. Metabolites with a VIP value greater than 1.0 obtained from the OPLS-DA model were considered to be important in discriminating between groups. Further, the metabolites with a VIP value greater than 1.0 obtained from the OPLS-DA model. The rumen NH_3-N concentration were analyzed using the SAS mixed procedure (SAS Institute Inc., Cary, NC, USA) to identify any difference between the two groups. Differences were declared significant at $P < 0.05$.

3.4. Metabolic Pathway Analyses

The rumen microbial and plasma metabolites that differed in concentration between the Ctrl and Urea groups were subjected to metabolic pathway analysis using MetaboAnalyst 3.0 software (http://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml), which is based on databases including PubChem (http://pubchem.ncbi.nlm.nih.gov/), the Human Metabolome Database (HMDB; http://www.hmdb.ca) and the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.com) (Xia et al., 2015).

3.5. Correlations between the Changed Metabolites from Rumen and Plasma

To quantify correlations between the levels of altered rumen microbial and plasma metabolites, Pearson's correlation coefficients were calculated using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA) at two confidence levels ($P < 0.05$ and $P < 0.01$).

4. Results

4.1. Changes in Ruminal NH_3-N Concentrations

Compared to the Ctrl group, urea supplementation significantly increased ($P < 0.05$) rumen NH_3-N concentration at 2 and 4 h after morning feeding. In the Urea group, the concentration of NH_3-N reached a peak at 2 h after morning feeding (Figure 16).

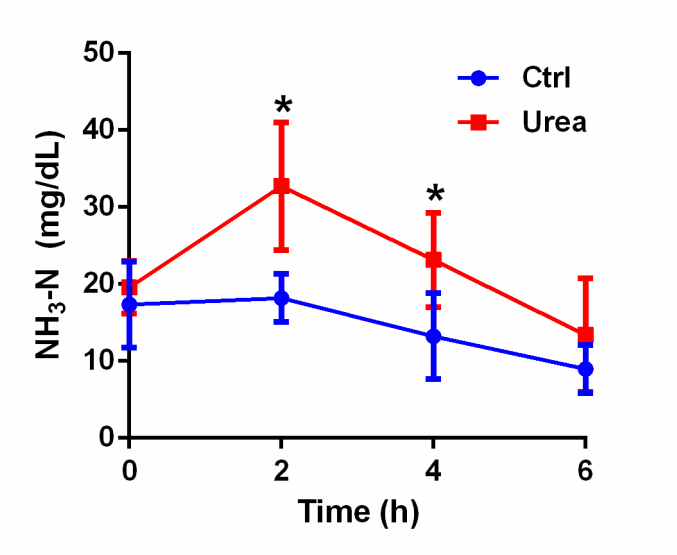

Figure 16 Changes of NH_3-N concentrations induced by urea supplementation. Urea: Group with urea supplementation, Ctrl: group without urea supplementation. *Means values in Urea group was significantly different from that in Ctrl group ($P < 0.05$).

4.2. Comparison of the Metabolic Profiles in Different Treatments

Representative ^{1}H NMR spectra for rumen fluid and plasma samples are shown in Supplementary Figure 17 and Figure 18. A total of 44 metabolites were identified in spectra from the former and 49 from the latter. PCA plots showed that both ruminal and plasma datasets had a cumulative proportion of variance of more than 50% (Figure 19), indicating that the overall composition of the fluids from the two dietary groups was different. Subsequently, the OPLS-DA model, which reduces the dimensionality of the original data, was applied to explore further the metabolic disturbances in the Urea group versus the Ctrl group. As illustrated in Figure 20 and Figure 21, both rumen and plasma samples from these two groups were separated. For the rumen samples, the values of R^2 and Q^2 were 0.909 and 0.435, respectively. For the plasma samples, the values of R^2 and Q^2 were 0.93 and 0.074,

respectively. The loading plot is complex because of the many metabolites identified, but the most important are indicated by positions far from the origin (Figure 20B and 21B).

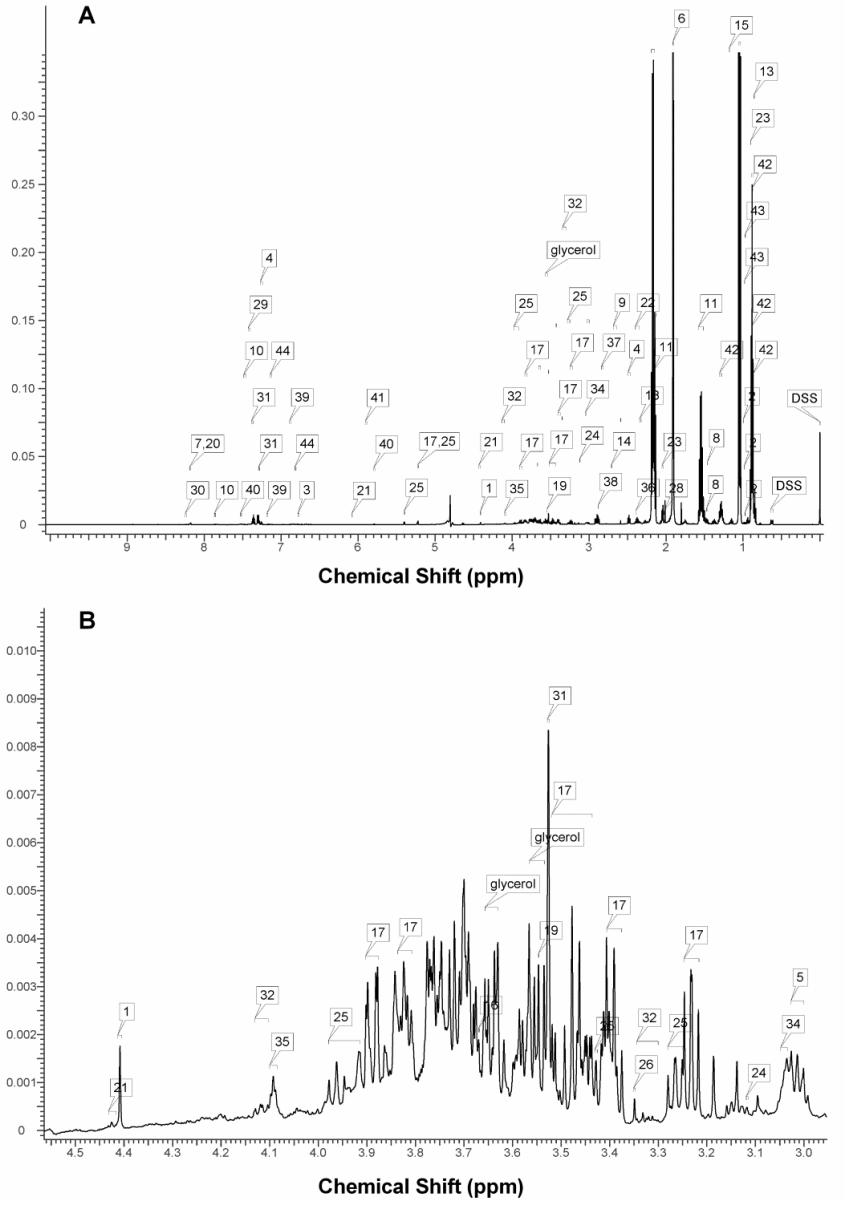

Figure 17 Representative 1H NMR spectra of rumen fluid samples.

(A) Spectra of chemical shifts from 0 to 10 ppm. (B) Spectra of chemical shifts from 3 to 5 ppm. The compounds were identified as the following: 1, 1,3-Dihydroxyacetone; 2, 2-Aminobutyrate; 3, 3-Hydroxyphenylacetate; 4, 3-Phenylpropionate; 5, 4-Aminobutyrate; 6, Acetate; 7, Adenine; 8, Alanine; 9, Aspartate; 10, Benzoate; 11, Butyrate; 12, Caprate; 13, Caprylate; 14, Dimethylamine; 15, Ethanol; 16, Ethylene glycol; 17, Glucose; 18, Glutamate; 19, Glycine; 20, Hypoxanthine; 21, Inosine; 22, Isobutyrate; 23, Isovalerate; 24, Malonate; 25, Maltose; 26, Methanol; 27, Methylamine; 28, N-Acetylglycine; 29, N-Phenylacetylglycine; 30, Nicotinate; 31, Phenylacetate; 32, Proline; 33, Propionate; 34, Putrescine; 35, Ribose; 36, Succinate; 37, Succinylacetone; 38, Trimethylamine; 39, Tyramine; 40, Uracil; 41, Uridine; 42, Valerate; 43, Valine; 44, p-Cresol.

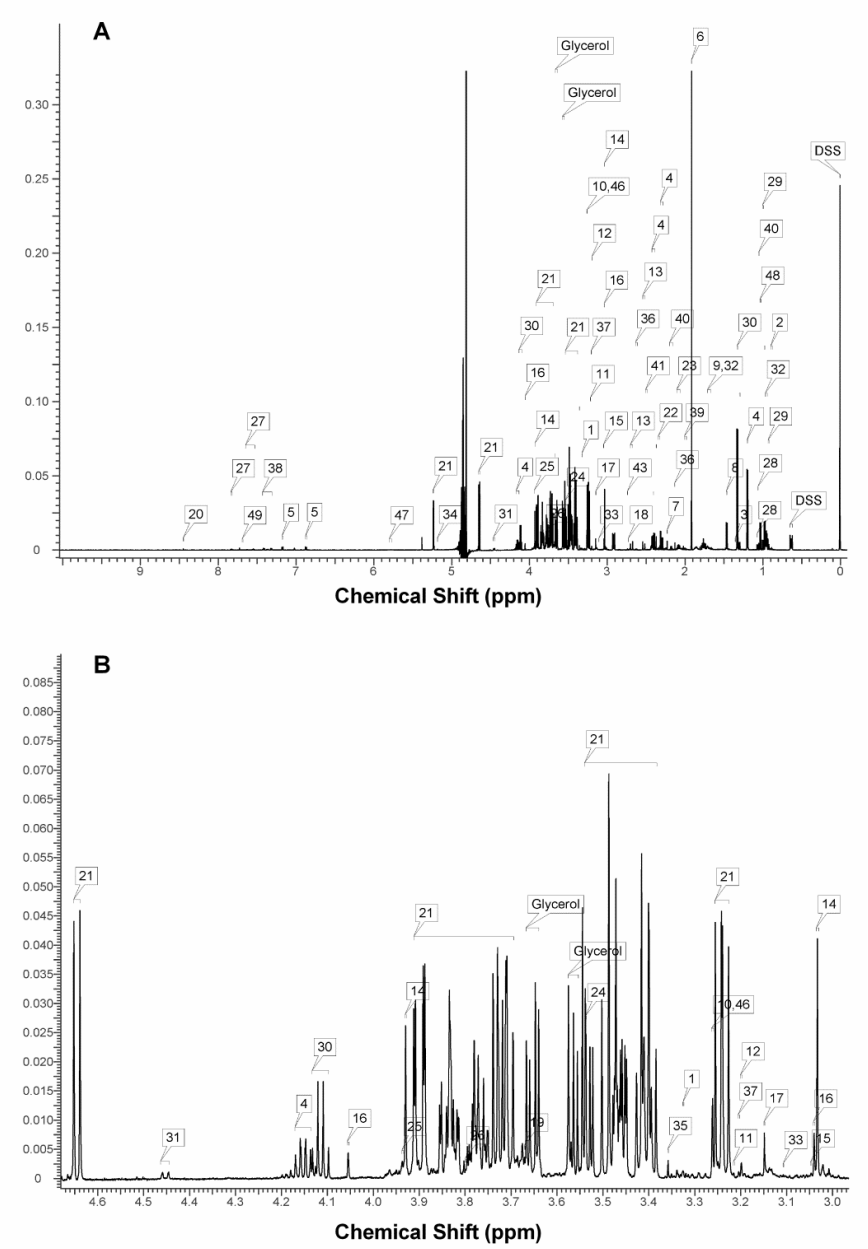

Figure 18 Representative 1H NMR spectra of plasma samples.

(A) Spectra of chemical shifts from 0 to 10 ppm. (B) Spectra of chemical shifts from 3 to 5 ppm. The compounds were identified as the following: 1, 1,3-Dimethylurate; 2, 2-Hydroxybutyrate; 3, 2-Hydroxyisobutyrate; 4, 3-Hydroxybutyrate; 5, 4-Hydroxyphenylacetate; 6, Acetate; 7, Acetone; 8, Alanine; 9, Arginine; 10, Betaine; 11, Carnitine; 12, Choline; 13, Citrate; 14, Creatine; 15, Creatine phosphate; 16, Creatinine; 17, Dimethyl sulfone; 18, Dimethylamine; 19, Ethylene glycol; 20, Formate; 21, Glucose; 22, Glutamate; 23, Glutamine; 24, Glycine; 25, Glycolate; 26, Guanidoacetate; 27, Hippurate; 28, Isobutyrate; 29, Isoleucine; 30, Lactate; 31, Lactose; 32, Leucine; 33, Malonate; 34, Mannose; 35, Methanol; 36, Methionine; 37, O-Phosphocholine; 38, Phenylalanine; 39, Proline; 40, Propionate; 41, Pyroglutamate; 42, Pyruvate; 43, Sarcosine; 44, Succinate; 45, Threonine; 46, Trimethylamine N-oxide; 47, Urea; 48, Valine; 49, τ-Methylhistidine.

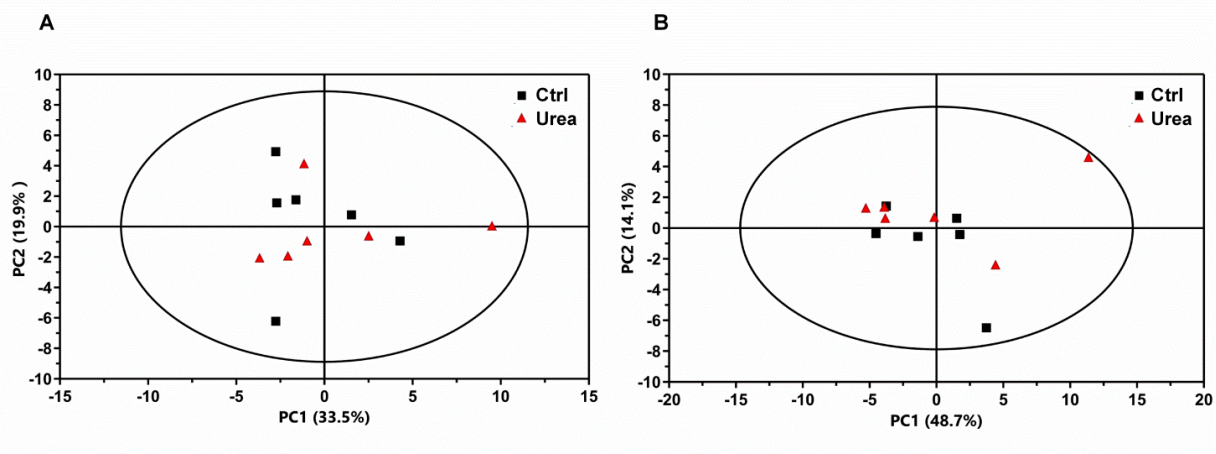

Figure 19 Principal Components Analysis (PCA) plots for rumen fluid (A) and plasma (B) metabolite profiles from Control and Urea groups.

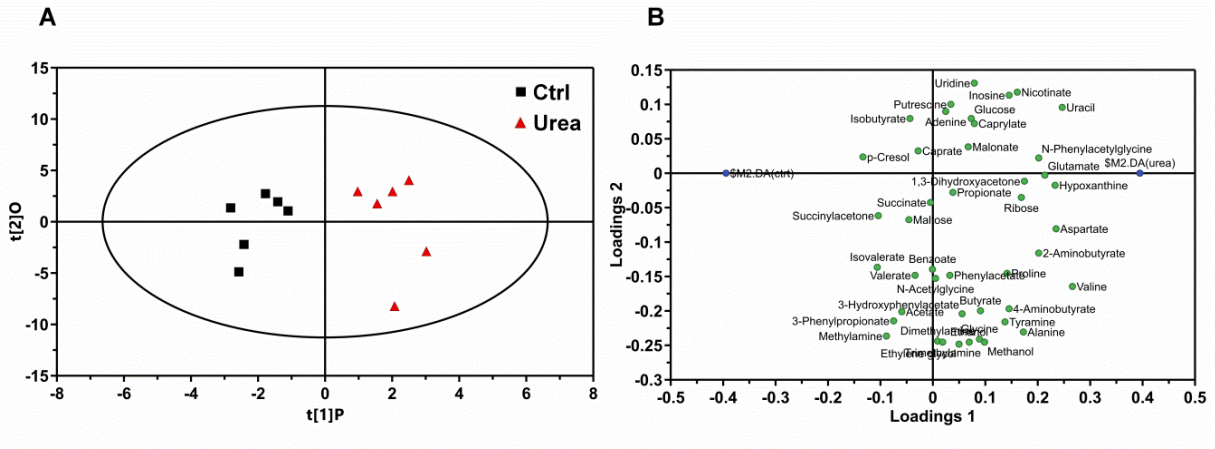

Figure 20 Score plot (A) and corresponding loading plot (B) of orthogonal partial least-squares discriminant analysis derived from NMR spectra of ruminal samples between Urea and Control groups.

Urea: Group with urea supplementation, Ctrl: group without urea supplementation.

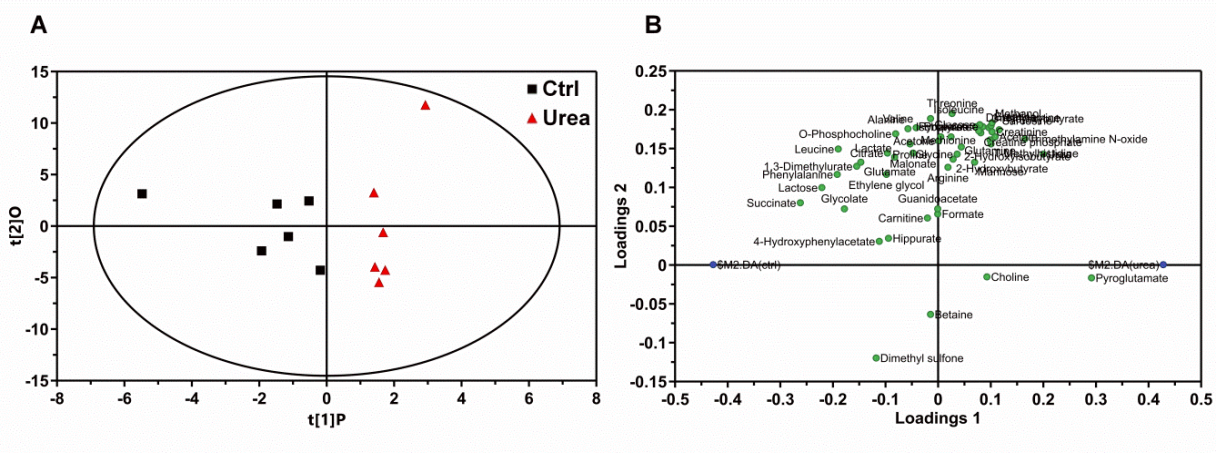

Figure 21 Score plot (A) and corresponding loading plot (B) of orthogonal partial least-squares discriminant analysis derived from NMR spectra of plasma samples between Urea and Control groups.

Urea: Group with urea supplementation, Ctrl: group without urea supplementation.

4.3. Metabolic Alterations in the Rumen and Plasma Samples

Metabolites with VIP values greater than 1.0 in OPLS-DA models (Figure 22), being the main rumen microbial and host metabolites contributing to the separation of the two groups, are shown in Table 9 and 10. In the rumen, except for the concentration of p-cresol, which was 0.87-fold lower in the Urea group than in the Ctrl group, the metabolites were all more concentrated in the Urea group than in the Ctrl group. Of these, the amino acid concentrations, including valine, aspartate, glutamate, and 2-aminobutyrate, were 1.48- to 1.69-fold higher ($P < 0.05$) in the Urea group, and the nucleic acid components, including uracil and hypoxanthine, were also more concentrated ($P < 0.05$) in the Urea group. In the plasma, the concentrations of five metabolites were increased, while those of seven metabolites were decreased, in the Urea group. Of note, the concentrations of urea and pyroglutamate were 2.62- and 3.17-fold higher ($P < 0.05$), respectively, in the Urea group than in the Ctrl group.

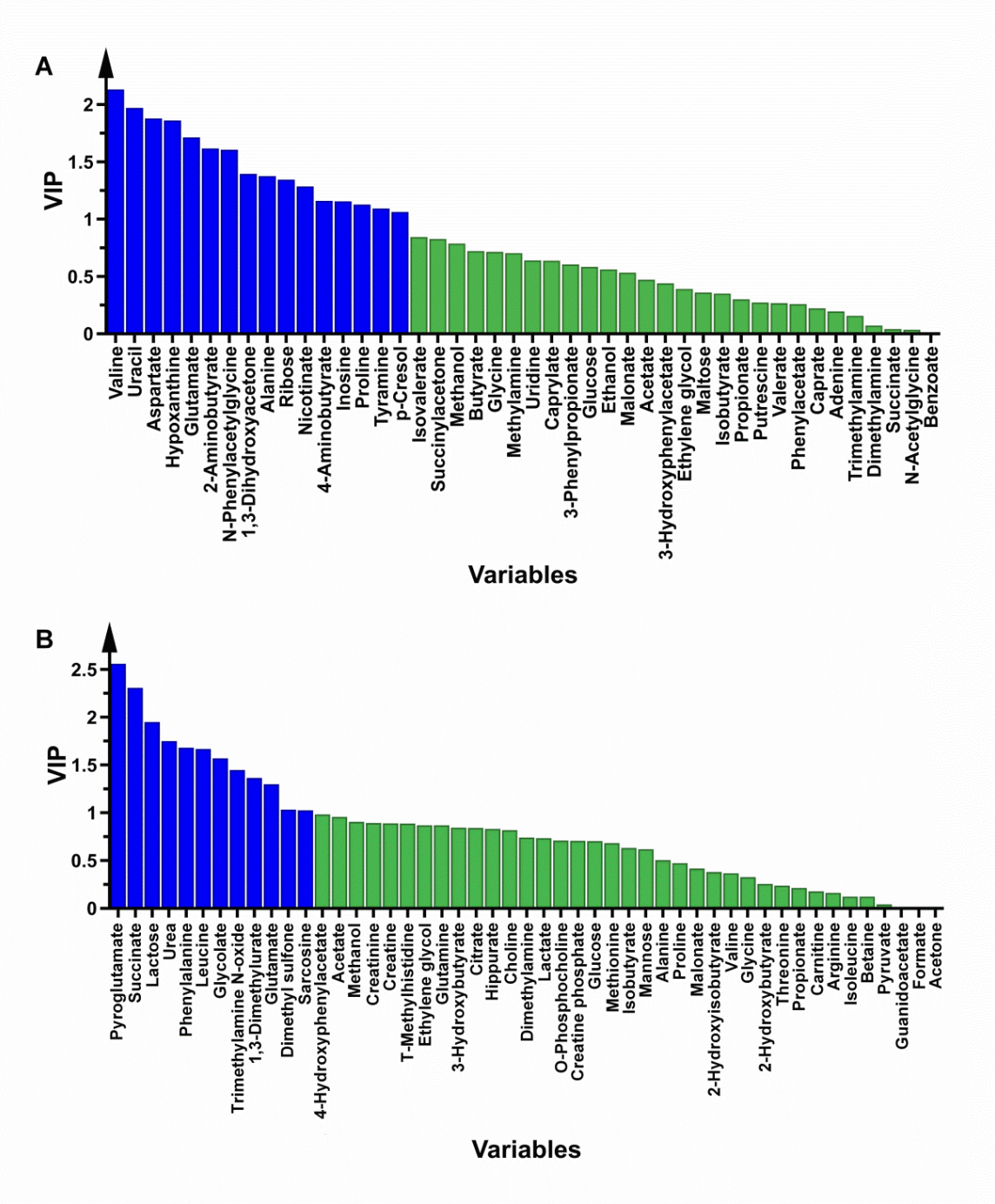

Figure 22 Variable Importance in the Projection (VIP) plots of orthogonal partial least-squared discriminant analysis of Control and Urea groups.

(A) Rumen fluid metabolites; (B) Plasma metabolites.

Table 9 Rumen microbial metabolites present in differing concentrations in cows fed a control diet (Ctrl) versus those that were urea-supplemented (Urea) (N=6)

Metabolite	Classification of metabolites	VIP[1]	Direction[2]	FD[3]
Valine	Amino acids and derivatives	2.13	↑*	1.69
Aspartate	Amino acids and derivatives	1.88	↑*	1.65
Glutamate	Amino acids and derivatives	1.71	↑*	1.57
2-Aminobutyrate	Amino acids and derivatives	1.62	↑*	1.48
Alanine	Amino acids and derivatives	1.36	↑	1.98
4-Aminobutyrate	Amino acids and derivatives	1.16	↑	1.63
Proline	Amino acids and derivatives	1.13	↑	1.25
Tyramine	Amino acids and derivatives	1.09	↑	1.59
Uracil	Nucleic acid components	1.97	↑*	1.36
Hypoxanthine	Nucleic acid components	1.86	↑*	1.72
Inosine	Nucleic acid components	1.16	↑	1.93
1,3-Dihydroxyacetone	Sugars	1.39	↑	1.15
Ribose	Sugars	1.34	↑	1.32
p-Cresol	Organic acids	1.06	↓	0.87
N-Phenylacetylglycine	Others	1.61	↑	1.18
Nicotinate	Others	1.27	↑	1.50

[1]VIP, variable importance in the projection.

[2]↑ indicates a higher concentration in the Urea group. ↓ indicates a lower concentration in the Urea group. * $P < 0.05$ versus Control.

[3]Fold difference in metabolite concentration (Urea/Ctrl).

Table 10 Host plasma metabolites present in different concentrations in cows fed a control diet (Ctrl) versus those that were urea-supplemented (Urea) (N=6)

Metabolite	Classification of metabolites	VIP[1]	Direction[2]	FD[3]
Urea	Amino acids and derivatives	1.75	↑*	2.62
Phenylalanine	Amino acids and derivatives	1.68	↓	0.91
Leucine	Amino acids and derivatives	1.67	↓	0.76
Glutamate	Amino acids and derivatives	1.30	↓	0.88
Sarcosine	Amino acids and derivatives	1.02	↑	1.28
Pyroglutamate	Amino acids and derivatives	2.57	↑*	3.17
Lactose	Sugars	1.95	↓	0.63
Succinate	Organic acids	2.31	↑	0.73
Glycolate	Organic acids	1.57	↓	0.95
Trimethylamine N-oxide	Others	1.45	↑	2.29
1,3-Dimethylurate	Others	1.36	↓	0.86
Dimethyl sulfone	Others	1.57	↓	0.83

[1]VIP, variable importance in the projection.

[2]↑ indicates a higher concentration in the Urea group. ↓ indicates a lower concentration in the Urea group. * $P < 0.05$ versus Control.

[3] Fold difference in metabolite concentration (Urea/Ctrl).

4.4. Metabolic Pathway Analysis

Metabolites that were present in different concentrations in ruminal fluid and plasma between the Ctrl and Urea groups were subjected to analysis using MetaboAnalyst 3.0 software. The varied rumen microbial metabolites between the two groups were identified to be involved in several metabolic pathways (Table 11 and 12). These pathways were filtered out by P value less than 0.05 and considered as potential target pathways. The varied rumen microbial metabolites between the two groups were identified to be involved in pantothenate and CoA biosynthesis, beta-alanine metabolism, valine, leucine, and isoleucine degradation and biosynthesis, aminoacyl-tRNA biosynthesis, histidine metabolism, and purine metabolism pathways (Figure 23). The varied plasma metabolites were identified as components involved in the glutathione metabolism pathway (Figure 24).

Table 11 Association of differentially detected rumen metabolites in Control and Urea groups with metabolic pathways identified by MetaboAnalyst 3.0 software.

Pathway name	Total	Hits	p	-log(p)	Holm p	FDR	Impact
Pantothenate and CoA biosynthesis	15	2	0.00141	6.56	0.0225	0.0225	0.00
beta-Alanine metabolism	17	2	0.00908	4.70	0.136	0.0519	0.00
Valine, leucine, and isoleucine biosynthesis	11	1	0.0130	4.34	0.182	0.0519	0.33
Valine, leucine, and isoleucine degradation	38	1	0.0130	4.34	0.182	0.0519	0.00
Aminoacyl-tRNA biosynthesis	64	3	0.0258	3.66	0.310	0.0684	0.00
Histidine metabolism	14	1	0.0269	3.62	0.310	0.0684	0.00
Purine metabolism	68	2	0.0299	3.51	0.310	0.0684	0.01
Pyrimidine metabolism	37	1	0.0536	2.93	0.482	0.107	0.09
D-Glutamine and D-glutamate metabolism	5	1	0.0953	2.35	0.763	0.169	0.00
Alanine, aspartate, and glutamate metabolism	23	2	0.146	1.93	1	0.233	0.27
Arginine and proline metabolism	44	3	0.16	1.83	1	0.233	0.09
Nicotinate and nicotinamide metabolism	13	1	0.202	1.60	1	0.261	0.00
Tyrosine metabolism	42	1	0.216	1.53	1	0.261	0.03
Butanoate metabolism	20	1	0.228	1.48	1	0.261	0.03
Pentose phosphate pathway	19	1	0.299	1.21	1	0.319	0.00
Glycerolipid metabolism	18	1	0.376	0.977	1	0.376	0.00

Table 12 Association of differentially detected plasma metabolites in Control and Urea groups with metabolic pathways identified by MetaboAnalyst 3.0 software.

Pathway name	Total	Hits	p	-log(p)	Holm p	FDR	Impact
Glutathione metabolism	26	1	0.00134	6.61	0.0215	0.0215	0.01
Purine metabolism	68	1	0.115	2.16	1	0.25	0.00
Arginine and proline metabolism	44	1	0.115	2.16	1	0.25	0.00
Citrate cycle (TCA cycle)	20	1	0.141	1.96	1	0.25	0.03
Alanine, aspartate, and glutamate metabolism	23	1	0.141	1.96	1	0.25	0.00
Propanoate metabolism	20	1	0.141	1.96	1	0.25	0.00
Butanoate metabolism	20	1	0.141	1.96	1	0.25	0.00
Valine, leucine, and isoleucine biosynthesis	11	1	0.141	1.96	1	0.25	0.33
Valine, leucine, and isoleucine degradation	38	1	0.141	1.96	1	0.25	0.00
Aminoacyl-tRNA biosynthesis	64	2	0.168	1.78	1	0.269	0.00
Glycine, serine, and threonine metabolism	32	1	0.352	1.05	1	0.511	0.06
Galactose metabolism	26	1	0.459	0.779	1	0.534	0.02
Phenylalanine, tyrosine, and tryptophan biosynthesis	4	1	0.467	0.761	1	0.534	0.50
Phenylalanine metabolism	9	1	0.467	0.761	1	0.534	0.41
D-Glutamine and D-glutamate metabolism	5	1	0.504	0.686	1	0.537	0.00
Glyoxylate and dicarboxylate metabolism	16	1	0.875	0.133	1	0.875	0.04

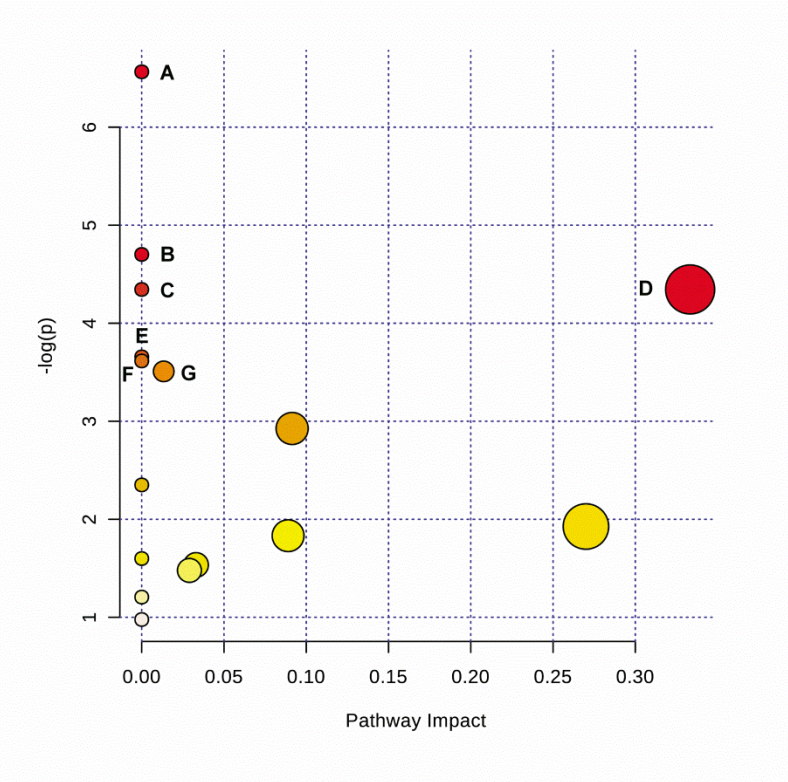

Figure 23 Pathway analysis of ruminal metabolites those were present in differing concentrations between the Urea and Control groups.

This analysis was undertaken using MetaboAnalyst. (A) Pantothenate and CoA biosynthesis; (B) beta-Alanine metabolism; (C) Valine, leucine, and isoleucine degradation; (D) Valine, leucine, and isoleucine biosynthesis; (E) Aminoacyl-tRNA biosynthesis; (F) Histidine metabolism; (G) Purine metabolism.

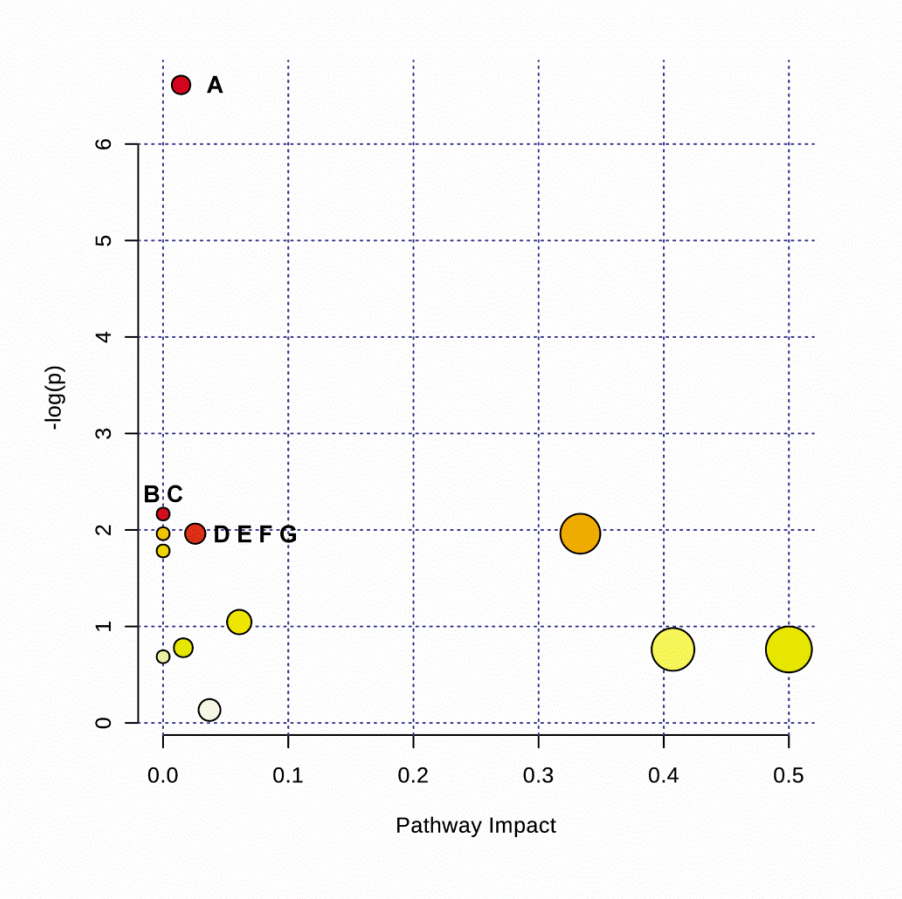

Figure 24 Pathway analysis of plasma metabolites those were present in differing concentrations between the Urea and Control groups.

This analysis was undertaken using MetaboAnalyst. (A) Glutathione metabolism; (B) Purine metabolism; (C) Arginine and proline metabolism; (D) Citrate cycle (TCA cycle); (E) Alanine, aspartate, and glutamate metabolism; (F) Propanoate metabolism; (G) Butanoate metabolism; (H) Valine, leucine, and isoleucine biosynthesis.

4.5. Correlations between Concentrations of Ruminal and Plasma Metabolites

The correlation coefficients for varied metabolites in ruminal and plasma were calculated and those with significant correlations are listed in Table 13. We found that the concentrations of aspartate and glutamate in the rumen and sarcosine in the plasma were positively correlated, while the concentrations of ruminal p-cresol and plasma sarcosine were negatively correlated ($P < 0.05$). Moreover, significant correlations were detected between aspartate and glutamate in the rumen and plasma urea ($P < 0.01$).

Table 13 Partial pearson's correlations between ruminal and plasma metabolites with significant difference.

	Blood sarcosine	Blood succinate	Blood trimethylamine N-oxide	Blood urea
Rumen aspartate	0.6615*	NS[1]	0.7562**	0.7344**
Rumen glutamate	0.7160**	NS	0.6181*	0.7437**
Rumen Valine	NS	-0.5844*	NS	NS
Rumen p-Cresol	-0.6419*	NS	NS	NS

[1]NS, no significant correlation.

*P < 0.05, **P < 0.01 versus Control.

5. Discussion

In the present study, urea was added to the rumen of cows as a source of NPN and metabolomics was used to assess the effect of this urea nitrogen on rumen microbial and host plasma metabolites. The effect of dietary nitrogen content on urine metabolites of dairy cows has been previously assessed by NMR spectrometry, and Partial least-squares (PLS) regressions confirmed a correlation between the NMR metabolite profile and both nitrogen intake and efficiency, which indicating that several metabolites may contribute to the prediction of nitrogen intake and efficiency in dairy cows, but a wide-ranging urinary metabolite profile is needed to evaluate nitrogen efficiency in ruminants (Bertram et al., 2011). In this study, metabolites in other two important biofluids, the rumen fluid and plasma, were assessed using NMR spectroscopy, and metabolites and metabolic pathways that regulated by urea nitrogen were identified.

In the rumen, NH_3 assimilation is an important process for microbial protein synthesis (Firkins et al., 2007); NH_3 release resulting from urea supplementation may affect key components of this process. In the present study, the ruminal concentrations of glutamate and aspartate were higher in the Urea group than in the Ctrl group, and the results are consisted with previous research which found that the metabolism of glutamate and aspartate is closely related to the process of NH_3 assimilation in the rumen (Wang and Tan, 2013). The NH_3

released by the hydrolysis of urea is utilized for the synthesis of the amino acids by most bacteria for growth (Patra, 2015). The glutamate dehydrogenase (GDH) and glutamine synthetase–glutamate synthase (GS–GOGAT) pathways are two classic routes for NH_3 assimilation in bacteria, leading to NH_3 molecules being incorporated into the amide group of glutamine (Purich, 1998). In some rumen bacteria, asparagine synthetase (AS) also participates in NH_3 assimilation (Ciustea et al., 2005). The rumen ammonia levels controls the pathway for ammonia uptake by lumen microorganisms (Srinivas and Gupta, 1997). In the present study, urea nitrogen provided additional substrate for NH_3 assimilation, and the higher aspartate and glutamate concentrations may be due to upregulation of these pathways. In addition, rumen bacteria may also possess effective mechanisms for alanine synthesis from NH_3 (Morrison and Mackie, 1996; Oba et al., 2005), and the enhanced beta-alanine metabolism that was observed in the Urea group is consistent with this possibility.

The rumen valine concentration was also higher in the Urea group in the present study, which could result in enhanced valine, leucine, and isoleucine metabolism of rumen microbiota. Valine, leucine, and isoleucine are all branched-chain amino acids (BCAAs). Previous studies revealed that glutamate synthesis from a-ketoglutarate could utilize BCAA-derived amino groups (Scaglia et al., 2004), and hyper-ammonia increased the activity of BCAA aminotransferase (Dam et al., 2011). These BCAAs stimulated synthesis of glutamine from glutamate and NH_3 (Holecek, 2013). Therefore, we suggest that the process of NH_3 assimilation is enhanced by urea supplementation, and that the metabolism of the related BCAAs, especially valine, may partly contribute to glutamine synthesis in the rumen. Meanwhile, Valine provides the ox-ketoisovalerate for pantothenate synthesis, and pantothenate is a constituent of CoA.(Sahm and Eggeling, 1999; Genschel, 2004) So valine also participates in the process of pantothenate and CoA biosynthesis. Thus, in the present study, the higher concentration of valine could have been responsible for enhancing pantothenate and CoA biosynthesis in the rumen.

The levels of nucleic acid components (uracil and hypoxanthine) in the rumen fluid were also higher in the Urea group than in the Ctrl group. Increases in concentrations of these bases or base derivatives (uracil and hypoxanthine) in the rumen have also been observed when feeding cows with a high-grain diet (Saleem et al., 2012). Uracil is one of the key pyrimidine metabolites, and changes in uracil can reflect perturbations in flux through the urea cycle and the formation of alternative nitrogen-carrying metabolites (Wendler et al.,

1983). Urea is an intermediate in one pathway for uracil degradation, which ultimately results in the assimilation of ammonia; (Andersen et al., 2008; Kandasamy, 2012), this process is widely distributed in fungi and in a variety of bacteria. In the present study, the pathway whereby uracil is degraded to urea may have been inhibited in the Urea group, resulting in the higher uracil concentration observed.

The level of urea was significantly increased in the plasma when the cows were supplemented with urea. Previous studies revealed that ruminants fed on diets with high NPN had higher portal blood flow, greater hepatic uptake of NH_3 and increased rates of urea synthesis (Symonds et al., 1981; De Visser et al., 1997; Holder et al., 2015). Redundant NH_3 that is transported to the liver is likely to enter the ornithine cycle (Zhou et al., 2015). The large amount of urea produced was therefore likely the result of NH_3 detoxification in the liver (Lobley and Milano, 2007). Rumen NH_3 levels generally peak 1 to 4 h after feeding in meal-fed animals (Gustafsson and Palmquist, 1993). Consistent with this, we observed peak NH_3 at 2 h after the morning feeding in the Urea group. This very rapid accumulation of NH_3 exceeds the capacity of the rumen microbes to use it, resulting in NH_3 diffusing through the rumen wall into the blood (Highstreet et al., 2010). Thus, our results indicate that the redundant NH_3 released from the supplemented urea passed into the host circulation, and its subsequent conversion to urea in the liver was the likely cause of the higher plasma urea concentration observed in the Urea group.

The plasma pyroglutamate concentration in cows supplemented with urea was also higher than in the Ctrl group. Altered plasma pyroglutamate concentration is indicative of altered glutathione metabolism, because pyroglutamate is the basic form of pyroglutamic acid, which is an intermediate in the hepatic glutathione cycle (Eckstein et al., 2008; Reed et al., 2008). Glutathione metabolism contributes to oxidative stress, which plays a key role in the pathogenesis of many diseases, including liver disease; thus appropriate regulation of glutathione metabolism is critical for human and animal health (Wu et al., 2004). In the present study, rapid NH_3 release from urea in the rumen resulted in increased diffusion of NH_3 into the blood. The main fates of blood NH_3 are to be used in the synthesis of urea, and also, to a lesser extent, glutamine (Lobley et al., 1995). Glutamine is synthesized by glutamine synthetase from glutamate and ammonia. Metabolites involved within glutathione metabolism were present in higher plasma concentrations in the Urea group, which may

contribute to protection against the negative effects of toxic NH_3 transferred from rumen to blood.

There was a strong correlation between aspartate and glutamate concentrations in the rumen and plasma urea in this study. These three metabolites are all involved in urea nitrogen metabolism in the ruminant (Wang and Tan, 2013), indicating that nitrogen metabolism in the rumen and blood is co-regulated. In the present study, rapid release of NH_3 from urea likely altered the process of ammonia assimilation, and therefore the concentrations of aspartate and glutamate, which participate in this process (Ataşoğlu and Wallace, 2002; Harper et al., 2010). Thus, the levels of rumen aspartate and glutamate and plasma urea may help predict nitrogen efficiency in ruminants.

In summary, the rumen microbial and host metabolite profiles of dairy cows supplemented or not with urea were investigated by NMR spectroscopy. Metabolites that were present in differing concentrations were selected by multivariate statistical analysis and identified as valine, aspartate, glutamate, and uracil in the rumen, and urea and pyroglutamate in plasma. Associations of these metabolites and their metabolic pathways further revealed changes in complex nutrient utilization pathways induced by the urea nitrogen supplementation. The levels of ruminal aspartate and glutamate and the level of plasma urea were closely correlated and may help predict nitrogen efficiency in ruminants. The current results should be useful to improve understanding of urea nitrogen utilization mechanisms in dairy cows.

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Appendix data not included in the paper

Milk production and milk composition analysis

Milk production was recorded and milk samples were collected on d 17, 18, and 19 of each experimental period. Two 50-mL aliquots of milk were collected during each milking, and these were pooled in a proportion of 4:3:3 (Zhu et al., 2013). To one subsample, Bronopol (milk preservative, D&F Control Systems, San Ramon, CA) was added as a preservative, and this subsample was then stored at 4℃ for future analysis of milk composition by infrared analysis (Laporte and Paquin, 1999) with a Foss-Milkoscan Minor (MilkoScan FT120, Foss Electric A/S, Hillerød, Denmark).

The milk production and composition of dairy cows were analyzed using the SAS mixed procedure (SAS Institute, Inc, Cary, NC, USA) as shown in the following equation: $Y_{ijkl} = \mu + t_i + b_k + c(b)_{jk} + p_l + e_{ijkl}$, where Y_{ijkl} is the observation on cow j with treatment i, order of treatment k and period l; μ is the overall mean; $_t$i is the fixed effect of treatment i; b_k is the effect of order k of applying treatments; $c(b)_{jk}$ is the random effect of cow j within order k; p_l is the effect of period l; and e_{ijkl} is the random error. Differences were declared significant at $P < 0.05$.

Table 14 Milk production and composition of cows fed Ctrl or Urea diet.

Item	Treatments		SEM	*P*		
	Ctrl	Urea		Trt	Period	Trt*Period
Milk yield (kg/day)	33.84	31.78	2.877	0.6171	0.9516	0.1671
Composition, %						
Protein	3.06	3.17	0.047	0.14	0.69	0.02
Fat	3.73	4.02	0.209	0.35	0.29	0.59
Lactose	4.87	4.87	0.020	0.73	0.49	<0.01
Solids	11.57	11.93	0.203	0.23	0.42	0.23
NFS	8.17	8.20	0.043	0.54	0.30	<0.01
Urea nitrogen (mg/dl)	14.84 [b]	18.80[a]	0.367	<0.01	0.29	0.72

[a,b] Different letters in the same row indicate statistically significant differences for treatment effect at $P < 0.05$.

CHAPTER VI GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

1. General discussion

1.1. Summary of the thesis

The research undertaken in this thesis investigated the rumen urea-degrading bacterial communities and urea metabolism in dairy cows. Firstly, research progresses in ruminal ureolytic bacterial community, urea utilization and regulation in ruminants have been summarized. Secondly, we found out the rumen abundant ureolytic bacterial community by using an in vitro cultivating method. Thirdly, we discovered the diversity and distribution of the ureolytic bacteria in rumen and got information about the rumen predominant ureC genes. Then, we also revealed the rumen and plasma metabolite profiles changes induced by urea nitrogen. Finally, main conclusions obtained from previous chapters as well as future perspectives of research are summarized. The findings of this current research provide foundations for proposing further new strategies to improve efficiency of urea utilization in ruminants.

1.2. Investigation of the rumen ureolytic bacterial communities

The rumen is a complex ecosystem, where microorganisms convert feedstuffs into microbial biomass and fermentation end products that can be utilized by host animals. Three taxonomic groups of microorganisms, bacteria, protozoa, and fungi, carry out this digestion process in the rumen. An improved understanding of rumen microbial ecology can give insights into the fermentation processes in the rumen and provide knowledge to increase animal feed efficiency. In the early days, culture-dependent microbiological methods have been used to isolate and characterize the functional rumen microbes. More than 200 bacterial species have been isolated and characterized physiologically from the rumen (Russell and Hespell, 1981). However, due to the difficulty in cultivating the rumen bacteria, only very limited information were known about these bacteria. In recent years, the molecular techniques and the newly available “omic” technologies, based on DNA and RNA sequence analysis, which allow for new insights into the structure and functions of these complex microbial communities (Chaucheyras-Durand and Ossa, 2014). In this research, we applied DNA sequencing methods to discover what kind of ureolytic bacteria are there in the rumen. Indeed, in order to see what kind of ureolytic bacteria were active there, we also extracted the rumen bacteria RNA and did the reverse transcription. However, we failed to amplify the *ureC* of the rumen bacteria. Previously, Dai et al. (2015) successfully used the metatranscriptomic to analyses plant cell wall polysaccharide degradation by microorganisms

in the cow rumen. The difference of their study from ours is that they did sequencing of the whole rumen bacterial community, and we just focused on the bacterial *ureC* genes. We think the main reason for our fail is that the rumen habitats millions of bacteria, in our study, we just want to amplify the *ureC* genes which only take a very small proportion of the rumen microorganism,

1.3. Analysis of ureolytic bacterial based on 16S rRNA gene sequencing

The bacterial 16S rRNA gene sequencing has been widely used to evaluate the genetic diversity and phylogenetic relationships of microorganisms in different ecosystems. In chapter III, we used the rumen fermentation system to study the rumen bacterial composition. A lot of previous studies have also investigated the rumen microbial diversity by using the DGGE or sequencing *in vitro* (Mamuad et al., 2014; Soriano et al., 2014; Kim et al., 2016; Saminathan et al., 2016), which indicating that it was an effective method to study the rumen microorganism using *in vitro* system. In our study, we first tracked the ammonia production and urea hydrolysis in the fermenters. When doing the statistical analysis, the measurements obtained from the same cow at different sampling days were treated as a repeated measure. We found the sampling days had no significant effect on any of the variables in this study, and finally removed it from the model. We found that when urea was put into the fermenters, the ammonia production increased especially in the first two hours; on the other hand, the urea hydrolysis slowed down when AHA was put into the fermenters, we think this is a good model for us to study the rumen ureolytic bacteria community.

The 16S rRNA gene sequencing was used to explore the abundant ureolytic bacteria. At the family level, the bacterial community from our *in vitro* simulation system was found to be similar to the communities observed from previous *in vivo* studies. The composition of bacterial community in urea treated groups showed a trend of difference from those in non-urea treated groups. In this study, we have four replicates for each group, and more samples would be very helpful to getter a better pattern of this tendency. While, the abundance of certain bacterial communities was affected by urea and AHA supplementation, and we paid more attention to analysis these changed bacteria communities. The potential ureolytic bacteria were selected using the criterion that their abundance increased with urea treatment and decreased with AHA treatment. Furthermore, we tested the changed bacteria induced by urea whether they had urease genes and activities according to the publication or database. We kept the bacteria with urease genes and activity as the abundant ureolytic bacteria. So the

bacteria from the selected genera not only contain the urease genes, but also the urease activity.

1.4. Analysis of ureolytic bacterial based on ureC gene classification

The functional gene analysis has been used for the taxonomic classification of the functional bacteria (Xu et al., 2011; Tourna et al., 2014; Wilkins et al., 2015). Ureases synthesized by ureolytic bacteria are commonly composed of two or three subunits (*ureA, ureB,* and *ureC*) and the *ureC* subunit is the largest of the genes encoding urease functional subunits (Mobley et al., 1995). Primers for *ureC* gene have been designed and applied for analysis of the urea-degrading microorganisms in various environments, including the open ocean (Collier et al., 2009), sponges (Su et al., 2013), and soil (Singh et al., 2009). These studies were all taken using the clone libraries and only provided relatively limited information. For the rumen, we have also previously studied rumen ureolytic bacteria using a urease gene clone library (Zhao et al., 2015). In chapter IV, we also used the *ureC* gene for amplification, but some primers do exist for *ureA* and *ureB*, although most of these are designed for detecting the presence of *H. pylori* (Lopez et al., 1993). So new primers would need to be designed and tested for their specificity/broadness. Most environmental studies published to date have all used the *ureC* gene as a biomarker for detecting ureolytic species. It is likely that you would see some change in diversity if you used another marker gene, but the same is also likely if a different region of the *ureC* gene was used too, just like with the 16S rRNA gene. Although PCR methods suffer from this limitation the comparison and changes in diversity between sample sites is still valid as long as one remembers that they may have missed speices.

In this *in vivo* study, for each cow, a total of 180 g urea/daily was used. Based on the previous studies, a more reasonable recommendation for feeding urea to dairy cows is 135 g/animal daily that without ration intake reduction. Some studies also tried with urea supplementation exceeded by far the 135-g level, though urea couldn't be efficiently used or (Bartley et al., 1976; Huber and Kung, 1981). Helmer et al. (1970) have investigated feeding cows with urea versus the Starea (an intimate mixture of gelatinized starch and urea) and soybean meal diets on production of dairy cows. The urea content was 2.1% in the Starea-containing diet and was 2.8% in the urea-containing diet, resulting in 269 and 213 g of daily urea intake, respectively. Although cows consumed more urea with Starea, there were numerical declines in both intake and milk production for Starea compared with soybean

meal. For our study, we pained more attention on the ureolytic bacteria, and urea is an important substrate for the growth of these bacteria. The reason for choose this large amount of urea is that we want to see the obvious effect of urea on the rumen bacterial community and urea metabolism, and thus build a rumen effective model for study the rumen ureolytic bacteria and urea utilization. We used 180 g urea daily per cow, the total urea was separated into three parts (70, 55, and 55 g for morning, afternoon, and evening feeding, respectively) to let the cows adapt to the urea supplementation gradually and avoid poisoning.

First, we detected some parameters related to urea metabolism in the rumen. The measurements obtained from the same cow at different sampling days were treated as a repeated measure. We found the sampling days had no significant effect on any of the variables in this study. We also analyzed the animal effect on the variables and found that it's not significant. The ammonia production increased with urea supplementation at 2 and 4 hours after morning feeding. Urea concentration and urease activities showed no obvious difference between urea treated or non-urea treated groups, but both group showed the highest urease activities at 2 hours after morning feeding. The samples collected at 2 h after morning feeding were chosen for DNA extraction, because the urea hydrolysis rate is observed to be the highest at this time and is therefore likely to capture the majority of ureolytic bacteria. For our study, the peak NH_3 was observed at 2 h after morning feeding in the Urea group. Because we didn't detected NH_3-N between 0h and 2h after morning feeding, maybe the real peak NH_3 concentration occurred before 2 h after morning feeding. While in the Ctrl group, we didn't see the increase in NH_3-N after the morning feeding, one reason might be that after feeding 2h, the feed nutrients were digested by the rumen bacteria and the rumen bacteria fermentation produced adequate available nitrogen and fermentable carbohydrates for microbial protein synthesis. So, in our study, maybe the synchronization of available N and fermentable organic matter make it more efficiency for NH_3-N utilization (Henning et al., 1993).

Another important work we have done was the urease gene database generated from bacterial and archaeal *ureC* gene sequences with taxonomic assignment data which were downloaded from NCBI. The genes were aligned and manually edited using ARB software. This newly constructed urease gene database make the alignment of the acquired rumen bacterial urease gene sequences possible. But in our experiment, about 55% of the total sequences could not be confidently classified to any known phylum, one important reason is that the urease sequence information we use to construct the urease gene database are still

very limited. As more genomes from classified microbial isolates are sequenced and placed in the database, more taxonomic classification of environmental sequences will be acquired. As we have already known much more information about the 16S rRNA genes of the rumen bacteria, we may also try to do the metagenomics analysis of the whole rumen bacteria. By assembling the sequences from one single bacterial species, we may link the *ureC* genes with the 16S rRNA genes that already have the taxonomic information, and get more information about the rumen ureolytic bacteria.

1.5. Distinct ureolytic bacterial community in different rumen niches

Recent studies of the rumen epithelial microbiome using next generation sequencing have demonstrated in different ruminant species that the predominant populations (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*) of bacteria adhered to the wall are different from the luminal microorganisms (Chen et al., 2011; Petri et al., 2013; Jiao et al., 2015; Liu et al., 2016). Similar to 16S rDNA based studies, in this study, the composition of *ureC* genes from the rumen wall was distinct from the solid and liquid fractions, and the predominant classified *ureC* genes associated with the wall-adherent bacteria belonged to the *Proteobacteria* phylum. The distinct ureolytic bacterial community composition on the rumen wall may be related to its habitat and function of the rumen epithelium. Several mechanisms have been proposed that influence the movement of urea across the rumen wall and it is thought that ureolytic bacteria attached to the rumen epithelium facilitate this process (Wallace, 1979; Cheng and Costerton, 1980). Ruminal urease activity is likely a major modulator of urea transfer by producing a urea gradient into the rumen (Abdoun et al., 2006). Urea transporters also appear to facilitate movement of urea across the ruminant gastrointestinal tract (Stewart and Smith, 2005). Some bacteria attached to the rumen wall have distinctive metabolic activities such as urea metabolism, tissue recycling, and oxygen consumption (McCowan et al., 1978; Mead and Jones, 1981; Cheng and McAllister, 1997). It is likely that the tissue-adherent bacteria are more intimately associated with the metabolic activity of the host while the luminal bacteria are involved directly in fermentation of plant material (McCowan et al., 1980). The rumen epithelium adherent bacteria could have a significant impact on host health and should be included as members of core rumen microbiome.

So from our results, we could also hypothesize that ureolytic bacteria adherent with the wall of the rumen may specialized for breakdown recycled urea, and other populations

associated with the liquid and solid contents of the rumen are specialized for breakdown feed urea. In our current study, we used the diets containing 16% CP and this maybe one of the reason why urea supplementation had no significant effect on the diversity and distribution of the *ureC* genes. The rumen harbors a large diversity of ureolytic bacteria but mechanisms controlling urease synthesis and the impact of urea hydrolysis on the growth of these bacteria need further research.

1.6. Urea supplementation induced changes in rumen and host metabolic profiles

Metabolomics is the now well-established scientific field concerned with the study of naturally-occurring, low molecular weight organic metabolites within a cell, tissue or biofluid, and it may provide more accurate information regarding the physiological state of the microbiome or the host organism (Bundy et al., 2008; Lindon and Nicholson, 2008). As one of metabolomics techniques, 1H-NMR is a non-invasive technology that allows sample testing with good objectivity and reproducibility (Taylor et al., 2002). In addition, 1H NMR is also considered as a faster method than GC-MS and LC-MS (Tikunov et al., 2010). NMR-based metabolomic approach has been widely used to analyze various samples including blood, urine and tissue extracts from humans or other mammals (Benahmed et al., 2014; Duarte et al., 2014; Pinto et al., 2015). Recently, metabolomics methods are used to investigate the metabolites of rumen fluids (Mao et al., 2016; Zhao et al., 2014), plasma (Sun et al., 2014; Li and C., 2015), and milk (Sundekilde et al., 2013; Sun et al., 2015) in dairy cows.

In chapter V of this study, metabolites in the rumen fluid and plasma, were assessed using NMR spectroscopy, and metabolites and metabolic pathways changes that induced by urea nitrogen were identified. In the rumen, NH_3 assimilation is an important process for microbial protein synthesis (Firkins et al., 2007); Urea supplementation increased the ruminal concentrations of glutamate and aspartate which are important metabolites in the process of NH_3 assimilation in the rumen (Wang and Tan, 2013). The NH_3 released by the hydrolysis of urea is utilized for the synthesis of the amino acids by most bacteria for growth (Patra, 2015). In the present study, urea nitrogen provided additional substrate for NH_3 assimilation, and the higher aspartate and glutamate concentrations may be due to upregulation of the ammonia assimilation pathways. Besides, urea supplementation also increased the concentration of nucleic acid components (uracil and hypoxanthine) in the rumen fluid. Uracil is one of the key pyrimidine metabolites, and changes in uracil can reflect perturbations in flux through the

urea cycle and the formation of alternative nitrogen-carrying metabolites (Wendler et al., 1983). Related to these changed metabolites, metabolic pathways including pantothenate and CoA biosynthesis, beta-alanine metabolism, valine, leucine, and isoleucine metabolism in the rumen. So, urea supplementation increased the concentration of several amino acids and derivatives, and nucleic acid components in the rumen. This also may be explained by urea supplementation provided adequate nitrogen for rumen bacterial fermentation, the synchronization of available N and fermentable organic matter make it more efficiency for bacterial utilization of the nutrients and thus biomass accumulation. The enhancement of these metabolic pathways is a consequence of the rumen microbial metabolism changes induced by urea nitrogen.

We used blood metabolites to reflect the host metabolism. Blood profiles have frequently been used to assess nutrient status of cows (Puppel and Kuczynska, 2016). Blood is commonly either sampled as plasma or as serum. Advantages of plasma over serum are quick processing, higher yield, lower risk of haemolysis and thrombocytolysis, and virtually no interference from post centrifugal coagulation that can occur in serum. The blood plasma has been used for metabolomics analysis in the description of pathological diseases, discovery of novel biomarkers, and elucidation of metabolic regulatory pathways (Li and C., 2015; Pinto et al., 2015). In our study, we detected the blood plasma to see the host metabolite profiles changes induced by urea supplementation to dairy cows. We found that the glutathione metabolism was enhanced. Liver is the major source of glutathione synthesis and then glutathione is exported to the bloodstream for supply of other tissues, Acute and chronic hyperammonemia may lead to oxidative stress (Bionaz and Loor, 2007). Several previous studies proved that glutathione concentration was decreased under hyperammonemia situations (Bonnet et al., 2013; Connor et al., 2013). Abdoun et al. (2005) found that blood glutathione concentrations were depleted in the acute ammonium poisoned lambs, which indicating enhanced glutathione metabolism. One of the important functions for glutathione is to protect the cell against toxic compounds of endogenous and exogenous origin. So in our study, increased glutathione metabolism in the urea group may contribute to protection against the negative effects of toxic NH_3 transferred from rumen to blood.

We also analyzed the milk production and milk composition during the experimental period. We found that there is no obvious difference in milk yield or milk protein content between the urea and non-urea groups. But the milk urea-nitrogen concentration was significantly higher in the urea group than the non-urea group. The concentrations of urea in

milk and blood are closely associated in lactating cows (Broderick and Clayton, 1997). In our study, we have observed a higher urea nitrogen concentration in the urea treated group, which lead to increased urea transfer to the milk. Milk urea nitrogen has proven to be more closely associated with changes in dietary CP content and could serve as a biomarker of protein intake relative to requirements in lactating dairy cows (Nousiainen et al., 2004). In our study, the crude protein content in the basal diet is 16.67% (DM based), which may have provided adequate ammonia, amino acid, or peptide for the synthesis of microbial protein (Agle et al., 2010; Recktenwald et al., 2014). So, adding the extra non-protein nitrogen lead to redundant ammonia transfer to the blood and subsequent urea excretion to the milk.

1.7. Opportunities for regulating urea hydrolysis targeting the bacterial urease

For ruminants, reducing the rate of rumen urea hydrolysis is of great importance for improving urea utilization and minimizing ammonia wastage. Some strategys such as urea inhibitors and some new forms of urea have been developed to slow ammonia release in the rumen (Upadhyay, 2012; Cherdthong and Wanapat, 2013; Giallongo et al., 2015). However, the rumen microbe maybe adapt to chronic chemical inhibitors utilization have some potential unhealthy effects to the ruminants. Host immunization commonly offers a diverse and ecofriendly solution to the problems especially associated with animal health. Therefore, developing vaccines against bacterial urease appears to be an alternative and attractive approach to reduce urea hydrolysis. Researchers have tried immunization strategies to reduce the methane emissions, urease activities, lactic acidosis, and rumen protozoal numbers in ruminants (Glimp and Tillman, 1965; Shu et al., 1999; Wright et al., 2004; Williams et al., 2008). Immunization against urease has been postulated to reduce urease activity in the gastrointestinal tract and is associated with decreased production and re-absorption of ammonia from the gut. In the early days, Jackbean urease is the most widely used member of the urease family in biotechnology. A reduction in urease activity has been reported in the rumen and in the ileum and colon of jackbean urease immunized sheep (Sidhu et al., 1968; Sidhu et al., 1969). A reduced rumen ammonia concentration has also been reported in buffalo calves immunized against Jackbean urease and fed a diet containing urea (Sahota and Jethi, 1981). Marini et al. (2003) had tried to determine if nitrogen metabolism could be manipulated by jackbean urease immunization, but they were unable to detect any effect on urease activity of the gastrointestinal tract or nitrogen utilization using either conventional nitrogen balance or double-labeled urea infusion. Therefore, in ruminants, the effect of jackbean urease immunization for reducing urease activities is not efficient. This could be

due to a lack of homology between jackbean urease and bacterial urease. It will be more effective to reduce the rumen urease activity if we could immunize bacterial ureases which have higher identity of homology.

Previously, our team have tried to reveal the bacterial urease profiles using a *ureC* gene clong library, and found that most of the alpha subunit of urease proteins were with higher similarity to that of *Helicobacter pylori*. We further developed the vaccine based on *ureC* of *H. pylori* and immuned the cows, it proved to be a useful approach to reduce the urea hydrolysis in the rumen (Zhao et al., 2015). But using clone library, we also only got very limited information. In this study, by using high-through put sequencing, we have acquired large amount of information about the bacterial *ureC* gene which is the largest subunit of the urease functional genes. The rumen bacterial *ureC* gene OTUs with top 50 highest abundance from different rumen fractions were identified, and this is very useful for us to know more information about the bacterial urease genes and is an important step to obtain the regulatory targets to mitigate urea hydrolysis.

Genome walking is a method for determining the DNA sequence of unknown genomic regions flanking a region of known DNA sequence (Guo and Xiong, 2006). This is traditionally a PCR-based protocol. The power of genome walking is that it enables PCR amplification, and hence sequencing, of regions of DNA where only the sense or the antisense primer sequence is known. Genome Walking has been successfully utilized in a wide range of plants, animals, fungi, bacteria, and viral strains for both genomic and organellar/plastid genome analysis (Shapter and Waters, 2014). A number of PCR-based methods have been developed to define flanking sequences from known genomic loci. Thermal asymmetric interlaced (TAIL)-PCR is an effective method for isolation of unknown DNA sequences flanked by known sequences. With the advantages of simplicity and high efficiency, TAIL-PCR and its modified procedures have been widely used in a variety of biological research in various organisms (Liu and Chen, 2007). In a previous research of Yuan et al. (2012), they had identified lots of unique fragments of polygalacturonase and pectate lyase genes from microbial DNA in the rumen of a Small Tail Han sheep, and 66% of the sequences of these fragments had low identities (65%) with known sequences. Two full-length newly discovered pectate lyase genes were cloned from the microbial genomic DNA by degenerate PCR and TAIL-PCR with twelve nested insertion-specific primers and the reagents of a Genome Walking kit. Therefore, according to the rumen predominant *ureC*

genes acquired in this study, we also want to clone the full-length urease functional gene information by employing the TAIL-PCR and Genome Walking.

After we got the information about the full-length urease structure genes, we want to clone them into special vectors and we also need to do some work for expression of the urease. After that, specific and effective urease subunit DNA vaccine could be constructed based on these target rumen urease genes, and the ruminal urease activity could be reduced by immunization with these vaccines. This study provided a basis for acquiring the probable vaccine targets of urease in the rumen for regulating rumen bacterial urease activities.

2. Conclusions

The main conclusions drawn from this thesis are:

Urea and acetohydroxamic acid (AHA) were used as the stimulator or inhibitor for ureolytic bacteria respectively. Based on bacterial 16S rRNA genes sequencing and analysis, the rumen ureolytic bacteria were abundant in the genera including *Pseudomonas*, *Streptococcus*, *Haemophilus*, *Bacillus*, *Neisseria*, *Actinomyces* and unclassified *Succinivibrionaceae*.

The diversity and distribution of the rumen ureolytic bacteria were analyzed by urease gene classification. More than 55% of the rumen bacterial *ureC* sequences did not affiliate with any known urease genes and the rumen may contain newly undiscovered sources of urease genes. The bacterial urease gene profile from the rumen wall was distinctly different from the rumen contents and *ureC* genes from *Methylophilus* and *Marinobacter* were identified predominantly in the rumen wall fraction.

Urea supplementation increased concentrations of valine, aspartate, glutamate, and uracil in the rumen, and urea and pyroglutamate in the plasma. Metabolic pathways including pantothenate and CoA biosynthesis, beta-alanine metabolism, valine, leucine, and isoleucine metabolism in the rumen, and urea and glutathione metabolism in the plasma were significantly increased by urea nitrogen.

This study identified significant populations of ureolytic bacterial community that have not been recognized or studied previously in the rumen, and provides a basis for obtaining vaccine targets of urease in the rumen for regulating rumen bacterial urease activities, and then moderate urea hydrolysis and utilization. The findings also provided novel information to aid understanding of the metabolic pathways affected by urea nitrogen in dairy cows, and

could potentially help to guide efforts directed at improving the efficiency of urea utilization in the rumen.

3. Perspective

Our research achieved some new information about the rumen ureolytic bacterial community and urea metabolism in cattle, and this is a good exploration for the rumen bacterial community and its metabolism in ruminants. Though, previous research, especially the research conducted in the past two decades, has greatly advanced our knowledge of rumen microbiome and its functions and allowed some success of manipulation. Such as strategies for methane mitigation from ruminants, our increased knowledge about the methanogenic community has permitted the development of mitigation strategies to target the dominant methanogenic species successfully. However, due to the vast diversity, extreme complexity, functional redundancy of this complex system, the majority of the rumen microbes remain to be understood and their metabolism as well as functions to be elucidated. Before the rumen system is adequately understood, it will be challenging to rational and effective manipulate urea hydrolysis by targeting the ureolytic bacteria. The rapid advancement of "~omics" technologies, including metagenomics, metatranscriptomics, metaproteomics, metabolomics, and bioinformatics will provide the unprecedented opportunities to disentangle the complex relationships between feed and rumen microbiome, rumen microbiome and its function, rumen function and host metabolism. Therefore, a holistic approach incorporating nutrition, rumen microbiome, and host metabolism is needed in future research.

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Author's publications

1. Articles

Three articles published and two articles are going under review:

Di Jin, Shengguo Zhao, Nan Zheng, Dengpan Bu, Yves Beckers, Stuart E. Denman, Christopher S. McSweeney and Jiaqi Wang. (2017) Differences in Ureolytic Bacterial Composition between the Rumen Digesta and Rumen Wall Based on *ureC* Gene Classification. *Frontiers in Microbiology*. 8:385.

Di Jin, Shengguo Zhao, Pengpeng Wang, Nan Zheng, Dengpan Bu, Yves Beckers and Jiaqi Wang. (2016) Insights into Abundant Rumen Ureolytic Bacterial Community Using Rumen Simulation System. *Frontiers in Microbiology*. 7:1006.

Di Jin, Shengguo Zhao, Yangdong Zhang, Peng Sun, Dengpan Bu, Yves Beckers, Jiaqi Wang. (2016) Diversity shifts of rumen bacteria induced by dietary forages in dairy cows and quantification of the changed bacteria using a new primer design strategy. *Journal of Integrative Agriculture* .15(11): 2597-2605.

D. Jin, S. Zhao, N. Zheng, Y. Beckers and J. Wang. 2017. Urea metabolism and regulation by rumen bacterial urease. *Annals of Animal Science*. (Minor revision)

D. Jin, S. G. Zhao, N. Zheng, D. P. Bu, Y. Beckers and J. Q. Wang. 2017. Urea Nitrogen Induces Changes in Rumen Microbial and Host Metabolic Profiles in Dairy Cows. *Livestock Science* (Under review)

2. Conference

Di Jin, Pengpeng Wang, Shengguo Zhao, Dengpan Bu, Jiaqi Wang. Metagenomic census of predominant *ureC* genes of ureolytic bacteria in the rumen of dairy cows. The 4th International Symposium on Gastrointestinal Microbial Ecology and Functionality. Hangzhou, China. 23-24 May 2016. (Oral presentation)

3. Posters

D. Jin, J. Q. Wang, D. P. Bu, P. P. Wang, S. G. Zhao, and X. M. Nan. Changes of the rumen microbial profiles as affected by urea and acetohydroxamic acid addition *in vitro*. 2015. J. Anim. Sci. Vol. 93, Suppl. s3/J. Dairy Sci. Vol. 98, Suppl. 2.

D. Jin, S. Zhao, N. Zheng, D. Bu, Y. Beckers, and J. Wang. Metagenomic census of predominant *ureC* genes of ureolytic bacteria in the rumen of dairy cows. 2016. J. Anim. Sci Vol. 94, E-Suppl. 5/J. Dairy Sci. Vol. 99, E-Suppl. 1.

D. Jin, S.G. Zhao, N. Zheng, Y. Beckers, J.Q. Wang. Urea nitrogen induces changes in rumen microbial and host metabolic profiles in dairy cows. 2017 ADSA annual meeting. Pittsburgh, Pennsylvania. 25-28 June.