Corso di dottorato di ricerca in Scienze e Biotecnologie Agrarie

Ciclo XXX

Titolo della tesi

"IN VIVO AND IN VITRO EVALUATIONS OF UREA-BASED PRODUCTS IN CATTLE FEEDING"

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ABSTRACT

Protein is an essential and expensive constituent of animal diets. Due to the peculiarity of the digestive system and, mainly, due to the microorganisms inhabiting it, ruminants are able to consume non-protein nitrogenous (**NPN**) compounds to fulfill their needs in the dietary protein required for maintenance, growth, and lactation, thereby producing highly-quality products form low- nutritious feedstuffs. The use of the NPN, such as urea in the ruminant nutrition has long been known. Nevertheless, actuality and interest of research over urea utilization are quite high, as due to increasing shortage of vegetable protein resources to supply demands for humans and animals as well due to its often inefficient utilization causing loss of nitrogen (**N**), water and air pollution.

The main purpose of the present thesis was to improve the knowledge concerning the aspects of urea utilization in cattle diets with respect to rumen fermentation characteristics and animal performance. A fundamental knowledge together with the recent scientific experiences regarding the concepts of rumen N utilization was presented it the theoretical part of the thesis. The experimental section is composed of three experimental parts.

The first experimental part of the thesis was composed by two trials: a feeding trial with Italian Simmental fattening bulls (Exp. 1) and *in vitro* trial with continuous culture fermenters (Exp. 2). The aim was to study the effects of partial and total dietary substitution of soybean meal (**SBM**) with urea (**U**) within two levels of intestinal digestible protein (**PDI**, 85 and 72 g/kg dry matter (**DM**), medium (**M**) and low (**L**) diets, respectively) on animal performance and on *in vitro* rumen fermentation characteristics. In Exp. 1, eighty Italian Simmental finishing bulls (495 ± 58 kg of body weight (**BW**), 14-months old) were fed the experimental diets for 120 d and slaughtered at BW of 656 ± 56 kg at 18 months of age. The average daily gain (**ADG**) was satisfactory (1.32 kg/d), however, both ADG and feed efficiency tended ($P<0.10$) to be higher in M than L diets (1.37 vs. 1.28 kg/d and 0.147 vs. 0.137 kg BW gain/ kg feed DM, respectively). Slaughter traits were unaffected by dietary treatments. In Exp. 2, continuous culture fermenters were inoculated with rumen fluid from bulls in Exp. 1 and given the same diets. Large differences in the ammonia concentrations were found *in vitro* between M and L diets over different sampling times before (at T0, $P<0.01$), and each consequent hour after feed administration (at T1, $P<0.01$), at T2 and T3, $P<0.05$). The drop in pH at T1 and T2 was less

intense (P<0.05) in U diets, likely due to the buffering capacity of urea. *In vitro* DM digestibility tended to be higher for U diets (P<0.10). Molar proportions of butyrate were higher in M diets after feed administration (P <0.01) and in U diets both before and after feed administration (P<0.01).

Additionally, rumen inoculum (**RI**) samples were analysed for volatile fatty acids (**VFA**) concentration and together with fermentation liquid (**FL**) samples were sent to a laboratory at Aberystwyth University for molecular analysis. As expected, total VFA concentration was lower in FL compared to RI (P<0.01). In the FL molar proportions of propionate, butyrate and isovalerate were higher (P<0.01), with acetate being lower (P<0.01). Diversity (Shannons and Simpson) and richness (Chao1) indexes of bacteria population were lower in FL than in RI (P<0.01). In percentage, some bacteria at the Phylum level stayed unchanged (Firmicutes and Fibrobacteres), decreased (Bacteroidetes, P<0.01), or increased (Proteobacteria, P<0.05; Tenericutes, P<0.05; Spirochaetes, P<0.01) in the FL compared to RI. Although the *in vitro* environment affected the differences in VFA concentrations and microbial community between RI and FL, it allowed the growth of the major bacterial phyla found in RI.

The second experimental part consisted of three *in vitro* trials (Exp. 1, 2, 3). The aim was to evaluate the capacity of urea-treated (14 g/kg) cereals (high-moisture barley and corn) for slow release ammonia properties and their effects on rumen fermentation characteristics *in vitro*. In Exp. 1, untreated (**CTR**), urea-treated (**UT**) and urea-added (before the experiment, **UA**) samples of milled barley and corn were incubated in the diluted rumen liquid in a batch system and sampled for the ammonia at 0, 0.5, 2, 4, 6, 8 and 24 h. Ammonia N concentration peaked at 4 h of fermentation (10.24 vs 9.01 and 7.20 mg/dl, respectively at 0 and 8 h, P<0.01), and the UT treatment released less ammonia than the UA treatment (9.76 vs 10.52 mg/dl, P<0.05), while the CTR samples showed the least ammonia N concentrations (P<0.01). In Exp.2, CTR and UT samples of barley and corn grains were incubated in distilled water (25°C) in three physical forms: whole, coarsely (to approximately 1/6 of the whole kernel, 16-20 mm), and finely milled (1mm) (report the average size of coarse and finely milled samples). At each incubation time (1, 2, 4, 6 or 8 h), filtered and dried residues were analyzed for N content. The N solubility of UT vs. CTR cereals changed according to the physical form (20.3 vs. 9.2%, 32.4 vs. 14.0 % and 43.7 vs. 15.3% for the whole, coarse and milled, respectively, treatment × physical form of grain

interaction, P<0.01). In Exp.3, CTR, UT and UA milled samples were incubated in rumen fluid first, for 96 h to measure gas production and to calculate substrate-specific halftimes (**t ½**), and second, for gas production, degradability, microbial N, VFA and ammonia measurements at t ½ (approximately 9.5 h for barley and 9 h for corn). The interaction in gas production between treatment and cereal type during the t ½ incubation was significant for all sampling times (T2, P<0.05; T4, T6, and T9, P<0.01), except T1. Thus, in barley, UT and CTR treatments produced more gas than UA during the t ½ incubation (198 vs. 179 ml/g DM, respectively), while in corn, UT and UA produced less gas than CTR (178 vs. 193 ml/g DM), Although the true substrate degradability (**TSD**) was not affected by the treatment, it had higher values for corn than for barley (87.7 vs. 79.8%, P<0.05). In both kinds of cereal the highest microbial N was found for UT samples comparing to CTR and UA (6.85 vs. 5.0 and 5.55, respectively, P<0.01). A lower butyrate concentration was detected for UT samples in barley and corn samples (P<0.01).

The third experimental part consisted of two *in vitro* gas tests (Exp. 1 and Exp. 2). The aim was to test *in vitro* rumen gas production technique in differentiating slow-release urea (**SRU**) products basing on the neutralizing effect of ammonia on VFA before they can react with the buffer and release carbon dioxide. Both trials were conducted in graduated 100 ml syringes with gas measurements at 1, 2, 4, 6, 8, 10 and 24 h of incubation (in Exp. 2 also 0.5 and 18 h) in two runs. In the Exp. 1, an amount of 500 mg of corn meal (**CM**), alone or added with four progressive urea doses (25, 50, 75 and 100 mg) were tested to study the effect of the urea addition on gas production. Rise in urea doses depressed gas production at each of measurement times (P<0.01) and increased (P<0.01) ammonia N concentration after 24 h incubation. When the urea added exceeded 50 mg, a decrease (P<0.01) of apparent substrate degradability (**ASD**, from 0.76-0.77 to 0.68-0.71) was observed. In Exp. 2 a set of 9 commercial SRU products, having from 361 to 420 g of N per kg of DM, were added to 500 mg/DM of CM in amounts equivalent to 50 mg (on N basis) of feed grade urea (**CM + SRU**), CM alone and CM added with urea (**CM + U**), were incubated following the procedure of Exp. 1. At all measurement points, gas production was highest for CM and lowest for CM + U substrates ($P < 0.01$) while for all CM + SRU mixes gas production was intermediate (e.g. from -30/40 to -70/80% of the CM gas yield, at 4 h).

In general, the results of the present thesis allow concluding the following. Despite a tendency for lower growth rates in finishing bulls fed low PDI diet, no detrimental effects neither on slaughter traits nor on *in vitro* rumen fermentation were detected. Diets containing U were expected to improve performance due to an increase of microbial protein synthesis. However, the quick urea hydrolysis in the U diets and its rumen recycling might have leveled any positive effects on animal performance. High moisture cereal grains treated with urea might be an opportunity to safety include urea in fattening diets, given their slow releasing properties and their capacity to improve microbial N synthesis found *in vitro*. Finally, a new approach to evaluate a variety of SRU products was suggested for consideration. Traditional gas-test generally used to study i*n vitro* degradability characteristics can also be useful in evaluating and ranking existing SRU products in terms of the estimated ammonia release. However, more studies are required in order to improve the susceptibility and to adjust an accuracy and precision of the method. The butyrate fluctuations in response to ammonia concentrations in fermentation fluids found throughout the experiments of this thesis suggest further research efforts to understand better a possible relationship between these two indicators.

LIST OF ABBREVIATIONS

AA Amino Acids

ADG Average Daily Gain

AIA Acid Insoluble Ash

ASD Apparent Substrate Degradability

BW Body Weight

CO_2 Carbon Dioxide

CP Crude Protein

DM Dry Matter

DMI Dry Matter Intake

DOM Digested Organic Matter

EE Ether Extract

FOM Fermentable Organic Matter

FGU Feed Grade Urea

G: F Gain: Feed ratio (Feed Efficiency)

FL Fermentation Liquid

LT Longissimus Thoracis muscle

MP Metabolizable Protein

MCP Microbial Crude Protein

MPS Microbial Protein Synthesis

N Nitrogen

NDF Neutral Detergent Fiber

NDFN Truly Undegraded Nitrogen

NH_3 Ammonia

NH_3-N Ammonia Nitrogen

NH_4^+ Ammonium

H^+ Hydrogen Ion

NPN Non-Protein Nitrogen

NSC Non-Structural Carbohydrates

OM Organic Matter

SBM Soybean Meal

SRU Slow-Release Urea

PDI Intestinal Digestible Protein

PDIA By-pass Digestible Protein

PDIE Rumen Fermentable Energy

PDIN Rumen Degradable Protein

RDP Rumen Degradable Protein

RUP Rumen Undegradable Protein

RI Rumen Inoculum

TDN Total Digestible Nutrients

TMR Total Mixed Ration

TSD true substrate degradability

VFA volatile fatty acids

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INTRODUCTION

Proteins are indispensable nutrients for humans and animals since they supply organisms with amino acids (AA) known for the high biological significance. About twenty different kinds of amino acids arranged in different ways compose the protein fraction of various plants, body tissues, milk, and eggs. They can be used as energy fuels and are also involved in important metabolic pathways. Some of AA are traditionally defined as nutritionally essential because they are not synthesized at all or not in adequate quantities by a human or animal organism and must be provided by the diet. Moreover, some of essential AA, such as methionine, lysine and histidine in cow, are considered as a "first-limiting" as they to a great extent limit the milk and meat protein production of the animal (Lean et al., 2016). Due to a richer content of essential AA, animal proteins are considered to be of higher biological value than vegetable proteins. An example of the superior nutritional value of proteins of animal origin is the greater proportion of sulfate AA in meats (methionine and cysteine, 1-1.6%) than vegetables (0.6%), while the ratio between methionine: cysteine, which is optimal in meats (60:40) is not adequate in vegetables (40:60; Pauselli et al., 2014).

The rapid growth in the world population expected to reach 9.6 billion by 2025 (OECD/FAO, 2016), together with the rise in the global economy, urbanization and people income level will substantially increase animal protein consumption in the next decades. Thus, a global production of meat and milk is anticipated to grow (by 16% and 23%, respectively, by 2025), compared to the base period (2013-2015) with the major increase in developing countries (OECD/FAO, 2016). As a result, a rise in demand for animal food protein will likely to affect prices for feed crops.

Livestock production system provides about 30% of protein consumed globally (Rosegrant et al., 2009; GASL, 2014), where almost all milk and more than a quarter of the meat are produced by the ruminant animals (FAO, 2012). The nutritive advantage over monogastric animals allows ruminants to produce high-quality (high-protein, energy-rich) food for humans from low-nutritional (fibrous and low-protein) feedstuffs. Thus, the ingested feed is subjected to microbial fermentation in the forestomach (rumen) before being normally digested in the true stomach and intestine. Microorganisms inhabiting rumen are capable to digest feeds inedible to humans and

monogastric animals, such as roughage and by-products from the food industry, converting them to forms available to further digestion by the host animal. Thus, a milking cow daily digesting more than 3.5 kg of pure fiber obtains high-quality nutrients that are largely transformed into milk constituents (Kendall et al., 2009). Moreover, consuming low-protein feeds or non-protein nitrogenous compounds (**NPN**), such as urea, a cow is able to synthesize a considerable amount (1.1-1.4 kg/d) of high-quality protein (Robinson, 2009). These low-protein feeds and NPN compounds can partially or completely substitute expensive true protein sources, such as soybean meal (**SBM**), traditionally given to ruminants in order to reduce the dietary expenses and animal competition with the human for the food.

Against the background of global food shortage, the problem of both a surplus diet protein and inefficient nitrogen (**N**) metabolism in livestock arises. Often it results in poor feed efficiency, low productivity, losses of N, energy and organic matter (**OM**) in a form of greenhouse gases (**GHG**; methane, nitrous oxide and carbon dioxide). Livestock is responsible for 14.5 percent of all anthropogenic GHG discharge, where the production of beef and milk is the major contributor (Gerber et al., 2013). Cattle and specifically, beef are also large sources of ammonia[1] emissions (Bussink and Oenema, 1998) which are closely associated with the dietary crude protein (**CP**) intake and metabolism. One solution to mitigate the environmental impact from ruminants is to apply precision feeding techniques where the match between ingested and required nutrients is met. (Pulina et al., 2016). Thus, lowering dietary CP concentration is a way to improve N efficiency, reduce N emission and diet costs since protein is the most expensive ingredient in ruminants' diet.

Overall, the increase in the N efficiency of livestock production systems and the environmental security are one of the main issues for the ruminant nutritionists. Therefore, this thesis attempts to shed a light on several aspects of urea utilization (urea in low CP finishing diets, urea-treated cereals, and commercial slow-release urea (SRU) products) in cattle diets.

[1]The term ammonia is utilized without distinction between NH_3 and NH_4^+, used in a case of specification.

THE OUTLINE OF THE THESIS

The first part of the thesis (Chapter 1) presents a literature review illustrating the aspects of rumen nitrogen metabolism and the concepts to improve the efficiency of rumen nitrogen utilization through urea and **SRU** products utilization. The scientific works published in the last decade are reviewed to compare the urea and SRU products use in a cattle feeding.

The second part (Chapter 2) is the experimental work over the urea utilization within the two CP levels on performance of Italian Simmental bulls and the rumen fermentation characteristics *in vitro* (Spanghero et al., 2017). Appendix 1 includes a comparison between volatile fatty acids (**VFA**) and bacteria population in the rumen liquid found *in vivo* and *in vitro*, the results were presented as a poster at 22nd Congress of Animal Science and Production Association (ASPA) held in in Perugia in June 2017 (Nikulina et al., 2017). Pictures of the Italian Simmental bulls (**Picture 1**) used in the *in vivo* and continuous-culture fermenters (**Picture 2**) employed in the *in vitro* trials of Chapter 2 are also presented in Appendix 1.

In Chapter 3 three experiments were conducted in order to explore the capacity of urea-treated high-moisture cereal grains to slow-ammonia release properties and to study the effects of treated grains on rumen metabolism *in vitro*. The scientific article is considered to be acceptable after major revision in Journal of Animal and Feed Sciences.

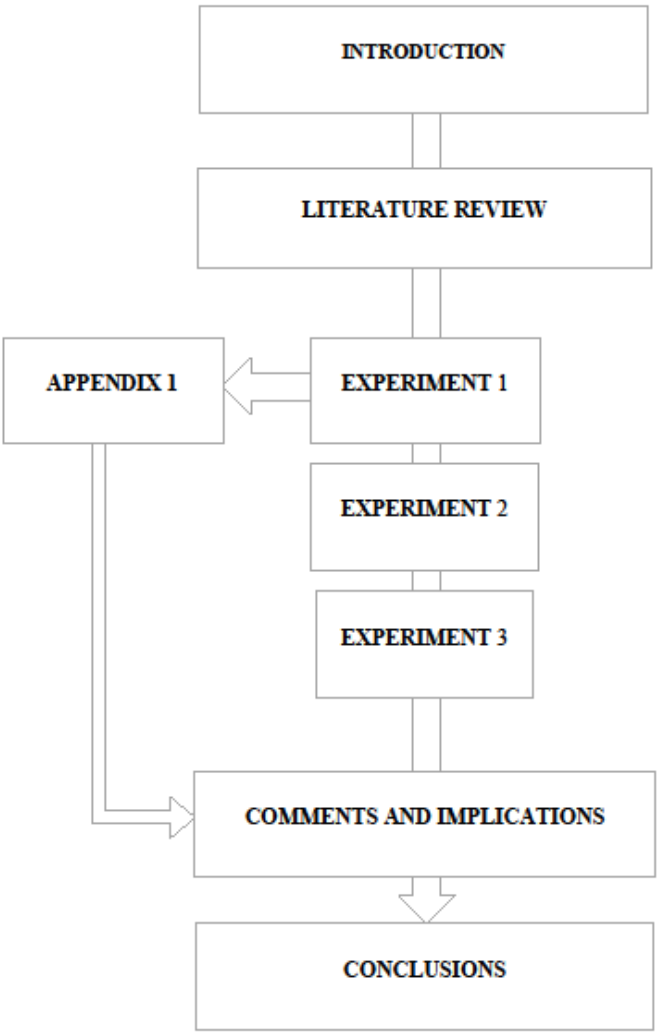
In Chapter 4, *in vitro* gas-test as a new method for evaluation of the SRU products is given for methodological consideration (Spanghero et al., 2018).

Chapter 5 reports the general conclusions of the thesis with the scientific contributions and practical applications.

Figure 1 represents schematically the contents of the present thesis.

Two of the scientific works have already been published in Livestock Science Journal in February 2017 (Chapter 2) and in Animal Feed Science and Technology in March 2018 (Chapter 4), whereas the second was currently considered to be acceptable after major revision in Journal of Animal and Feed Sciences (Chapters 3).

Figure 1. Schematic representation of the contents of the thesis.



CHAPTER 1. REVIEW OF THE LITERATURE

1. Aspects of nitrogen rumen metabolism

1.1. Rumen environment

The unique function of the ruminants to produce highly nutritious animal products from low-quality feeds is due to a large number of microorganisms present in the reticulorumen (or rumen). The rumen is the major fermentation site and largest stomach compartment of the cow's stomach. It is about 200 liters comprising about 85% of the entire stomach capacity. The rumen is habitat for a large and diverse microbial population, accounting from 10^{10} to 10^{11} cell/ml of bacteria, from 10^4 to 10^6 of protozoa/ml, from 10^3 to 10^5 zoospores/ml of fungi, and from 10^{10} to 10^11/ml of bacteriophages (Kamra, 2005). Among different groups of microorganisms, the protozoa and bacteria are mainly responsible for the fermentation of ingested feedstuff (Bryant, 1959). Rumen bacteria are classified based on the type of substrate they mainly attack: fiber digesters, starch and sugar digesters, deaminating bacteria and lactate- or hydrogen-using bacteria. However, this division is quite relative, as some digestive processes, such as cellulolysis and proteolysis, are a matter of particular bacteria strains, rather than species. Living in symbiosis with each other and with the host animal allows the large diverse microbial population to sustain the delicate balance of rumen environment.

In the rumen, the ingested feed is subjected to physical and chemical breakdown. Rumination and movements of the rumen mixing digesta are physical means. The chemical action is accomplished by enzymes secreted by rumen microbiota, attaching to the substrate. Resulting fermentation products are either absorbed into the blood or used by microbiota to grow and reproduce, generating high-quality microbial biomass. Moving with digesta flow to the abomasum and small intestine microbes are digested and absorbed by the host animal providing with high-quality proteins, organic acids and bacteria synthesized vitamins for maintenance, reproduction, growth and milk production.

Rumen environment is anaerobic, where the temperature (39^{o}C) and the pH (5.7-7.3) are kept at relatively constant values, allowing a great variety of bacterial species. Rumen pH is maintained due to the rumen's buffering system, mainly provided by the constant saliva influx and specifically, due to the urea contained in the saliva. In the rumen, urea is immediately

hydrolyzed to ammonia, which due to its buffering properties, binds hydrogen ion to each molecule of NH_3 to form NH_4^+ (Aschenbach et al., 2011). Salivation is stimulated by the cud-chewing (rumination), the process when ingested food is drawn back to the mouth (up to 40-50 times), chewed and swallowed again. Fibrous feed, such as roughage, impacting the epithelium of the anterior rumen wall stimulates the rumination, and as a consequence, saliva influx and an increase in ruminal pH. Concentrate feeds which contain low or no long fiber in contrary may inhibit salivation process. Moreover, highly fermentable starch and sugars fed in large amounts result in large quantities of VFA produced which may overwhelm the ability of the buffering system to compensate, switching rumen pH to as low as 4.5-5.5. Microbes differ in susceptibility to rumen pH variations, thus while starch-digesting bacteria can tolerate low rumen pH, fiber digesters are unable to survive under pH<6, resulting in impaired fiber digestibility. Likewise, protozoa, which are important contributors to the protein, fibrous and non-fibrous carbohydrate degradation, cannot tolerate low pH (Bach et al., 2005) and might be absent in the rumen of animals fed high-concentrate diets. Therefore, the diet to a greater extent determines the prevalence of certain microbes and bacteria species in the rumen. A total number of bacteria is also highly dependent on the type of the diet provided, as the process of microbial protein synthesis (**MPS**) is subject to the adequate supply of rumen fermentable nutrients.

1.2. Rumen nitrogen degradation, recycling and microbial synthesis

Dietary protein as a source of AA and N is an essential nutrient and the most expensive ingredient in livestock diets. Basing on the rate and site of degradation it can be divided in soluble protein, which is rapidly available source of N in a form of ammonia for rumen microbiota, rumen degradable protein (**RDP**), which by definition is degraded by rumen microbes and rumen undegradable protein (**RUP**) or so-named by-pass protein comprising about one-third of total dietary CP (**Figure 2**). The latter escapes ruminal degradation and is digested in abomasum and intestine providing AA to the cow. The digestibility of the by-pass protein according to the French protein system (INRA 2007) and the nutritional tables of NRC (2001) is considered to be lower than RDP and varies considerably (from 60 to 95% and from 55 to 75%, for INRA and NRC, respectively). The soluble protein consists of soluble true proteins and NPN fraction, including urea, amides, nitrates, nitrites, uric acid and other ammonia compounds,

which are fermented to ammonia in the rumen. Soluble true protein and RDP are hydrolyzed by the action of rumen microbes to peptides and AA, and some of AA are further deaminated to ammonia. Fermentation products of soluble protein and RDP are the main contributors to the rumen available N pool and predominant forms of N for **MPS**. Amino acid composition of the microbial protein is similar to that of milk, lamb and beef (Orskov, 1992) and superior of most feed proteins used in dairy cow (Schwab, 2001). It consists of 62% of CP content where 80% is true protein (INRA and NRC systems).

Protein of microbial cells, RUP and a small amount of endogenous protein compose metabolizable protein (**MP**). Digested postruminally MP supplies AA to the ruminant animal for maintenance, reproduction, growth and milk production. The MPS comprises more than 60% of MP (NRC, 2001) and may support alone up to 25% of milk production (Van Saun, 2006). Therefore, maximizing the efficiency of MPS is the main goal for ruminant nutritionists. Moreover, improved MPS efficiency decreases the urinary N excretions (Sinclair et al., 1993), and losses of carbon dioxide and methane (Blummel et al., 1999). In order to maximize the efficiency of MPS, it is important to understand the factors influencing microbial growth.

Rumen degradable OM was considered to be a major factor affecting N utilization by rumen microbes (Shabi at al., 1998). Therefore, among feeding systems efficiency of MPS is generally defined as grams of microbial CP (**MCP**) per kg (or 100 grams) of rumen available energy, expressed as digested OM (**DOM**) or fermentable OM (**FOM)** in the rumen and is currently assumed to be constant and quite similar among existing feeding systems. Thus INRA (2007) considered MCP equal to 145 g/kg DOM, in NRC (2001) it is 130 g/kg of total digestible nutrients (**TDN**) and in the Dutch DVE/OEB MPS is 150 g/kg FOM (Tamminga et al., 1994). In the updated DVE/OEB system (Van Duinkerken et al., 2011) the rates of passage and efficiency of conversion into MCP for different fermented substrates were applied. Nonetheless, the more complexity of the latter approach has not shown an improved accuracy over the previously defined fixed values for MPS prediction (Sauvant and Nozière, 2016). However, in the recent study, Sauvant and Nozière (2016) have updated the regression between FOM and MCP, indicating that a constant conversion efficiency of FOM to MCP underestimates MPS at low and overestimates at high levels of FOM. The authors suggest a positive effect of recycled N at low

FOM values and favorable rumen pH for MPS. The same authors confirmed the fact of the importance of the nature of the fermented substrate (proteins, neutral- fiber detergent (**NDF**), starch, and other carbohydrates) to support MPS, where carbohydrate fraction is more efficient than protein. Moreover, the use of the fixed value to estimate the efficiency of MPS may be inaccurate, as together with the energy availability the efficiency of N use by rumen bacteria must be considered (Bach et al., 2005). These authors also suggested using amino acid-N rather that bacterial N as a measure of MPS due to the uneven distribution of N inside the bacteria cells among different fermentation rates.

Carbohydrate is a major energy source for rumen bacteria, and therefore the rate of its digestion has a great impact on MPS (Hoover and Stokes, 1991). Energy is required for the synthesis of microbial AA, for the direct use or through the transamination of consumed AA and peptides. Otherwise, AA are deaminated, and further fermented to VFA (Bach et al., 2005). High-degradable starch and sugars provide more energy required by rumen bacteria for N utilization than structural carbohydrates (Pathak, 2008); nonetheless, feeding high-energy concentrate may also have a depressing effect on MPS due to decreased ruminal pH and fiber digestion (NRC, 2001; Dewhurst et al., 2000). Additionally, in response to an excess carbohydrate, loss of energy occurs through the reserve carbohydrate synthesis by rumen microbes or energy spilling when ATP is dissipated as heat in futile cycles. (Hackmann and Firkins, 2015). Structural carbohydrates assure more continuous release of energy, but low-energy sources, such as low-quality forages, may limit N uptake by rumen bacteria, thereby increasing absorption of excess ammonia across rumen wall and loss of N with urine.

The N availability and degradation rate are also important drivers of rumen fermentation and thus, microbial efficiency. Despite the compensational mechanisms of the ruminants to cope with a short-term shortage in diet N, such as urea-recycling and intraruminal protein turnover, the substantial decrease in the total microbial population was found under conditions of limiting dietary CP (Belanche et al., 2012).

Overall, microbial protein synthesis largely depends on adequate energy and N supply to rumen microbes (Shabi et al., 1998), where the synchrony and the balance between these two nutrients are the main concepts to increase the efficiency of their utilization thereby maximizing

MPS. The "synchrony" refers to a simultaneous degradation of carbohydrate and protein in the rumen aiming to provide a synchronous availability of energy and N to rumen microbes. The "balance" theory excludes a condition of synchronous degradation rates focusing on the daily equilibrium between rumen available energy and N for optimal MPS. However, in practice, the division between the concepts does not appear to be definitive, as both ultimately refer to the postprandial or diurnal availability of energy and N for microbial growth and MPS (Cabrita et al., 2006). Therefore, further in the text, the term synchrony will refer to both approaches in general.

Nutrient synchrony can be achieved by manipulating various carbohydrate and protein sources with different degradability properties simultaneously or independently (NRC, 2001). This manipulation also implies the adjusting of the degradability properties by physical or chemical processing. Feeding total mixed ration (**TMR**) is a simple way to achieve a relative synchrony, providing the simultaneous consumption of various feed ingredients with multiple nutrients and different inherent degradability rates (Van Saun, 2006).

Some authors reported beneficial effects of the synchronous supply of N and energy to rumen microbes and an improved N utilization, bacterial yield and the efficiency of MPS (Sinclair et al., 1993; Kim, 1999b; Chumpawadee et al., 2006). In contrast, synchronizing the diets for energy and N among studies gave inconsistent results, giving either limited (Richardson et al., 2003; Reynolds and Kristensen, 2008) or no effect (Kim et al., 1999a; Valkeners et al., 2004; Kaswari et al., 2007) on animal performance and fermentation characteristics measured. The lack of impact might be due to recycling mechanism (Bach et al., 2005) or inherent adaptability of rumen microbes (Reynolds and Kristensen, 2008) allowing ruminants to overcome short periods of asynchrony (Valkeners et al., 2004; Cabrita et al., 2006). Additionally, Bach et al. (2005) assumed that the nutrient synchronization which favors the one microbial population may not function for the other, leaving the average MPS stable.

Faster digesta outflow is supposed to have a positive effect on MPS as it reduces maintenance costs of the microbes due to a decrease in the residence time within the rumen (Pathak, 2008; Sauvant and Nozière, 2016). Digesta passage rate varies for different feed particles that require different mastication and retention time within the rumen, both longer for forage particles comparing to concentrates. Slow passage rate may promote intraruminal microbial protein

recycling and decrease microbial protein availability (Wells and Russel, 1996). However, Oldick et al. (2000) found that the intraruminal protein recycling was not correlated with MPS efficiency. From the other hand, high passage rates decrease the OM degradation and therefore, the energy available for rumen microbes (Bach et al., 2005).

Ammonia (or urea) recycling mechanism (**Figure 2**) is another important contributor to rumen N pool providing a continuous influx of N from the blood through the saliva and the rumen wall. It represents a relevant fraction of the total dietary N, because it comprises from 49 to 178% in growing cattle and from 43 to 123% of digested N in dairy cows (Lapierre and Lobley, 2001).

A peak of rumen ammonia concentrations reaches about 20-30 mmol/L during two hours post feeding, depending on the rumen degradable N intake (Reynolds and Kristensen, 2008), where from 35 to 65% is used for MPS, about 10% passes to omasum and the rest is absorbed across the epithelium of the rumen (Aschenbach et al., 2011). The prevalence and the mode of absorption of both forms of ammonia present in the rumen (NH_3 and NH_4^+) depend on the rumen pH, thus at pH>7 uptake of ammonia is mainly in the form of NH_3 and at pH<6.5 NH_4^+ is superior (Abdoun et al., 2007).

Accumulating in the rumen, ammonia due to its buffering properties increases the pH which in turn, increases the permeability of the rumen wall, and the absorption of the ammonia into the portal vein (Remond et al., 1993; Abdoun et al., 2007). With the bloodstream, ammonia is transported to the liver where it is detoxified to urea (hepatic ornithine cycle, Bunting et al., 1987). The recycled urea returns to the rumen via ruminal epithelium and saliva (Marini and Van Amburgh, 2003) On average, 33% of synthesized urea is excreted in urine and 67% is recycled to the different sites of the gastrointestinal tract; out of it, on average, 10% is lost in feces, around 40% is reabsorbed as ammonia and 50% is used further for anabolic purposes (Lapierre and Loble, 2001), mainly via microbial protein. In the rumen, urea is again hydrolyzed by bacterial urease releasing 2 mol NH_3/mol of urea. The capacity of the liver to remove and detoxify portal ammonia may exceed the one absorbed, even at high N intakes, which helps to maintain constant arterial ammonia concentrations (Parker et al., 1995). Moreover, except for low N intakes, ammonia recycled to the rumen may exceed the required for MPS (Reynolds and Kristensen, 2008).

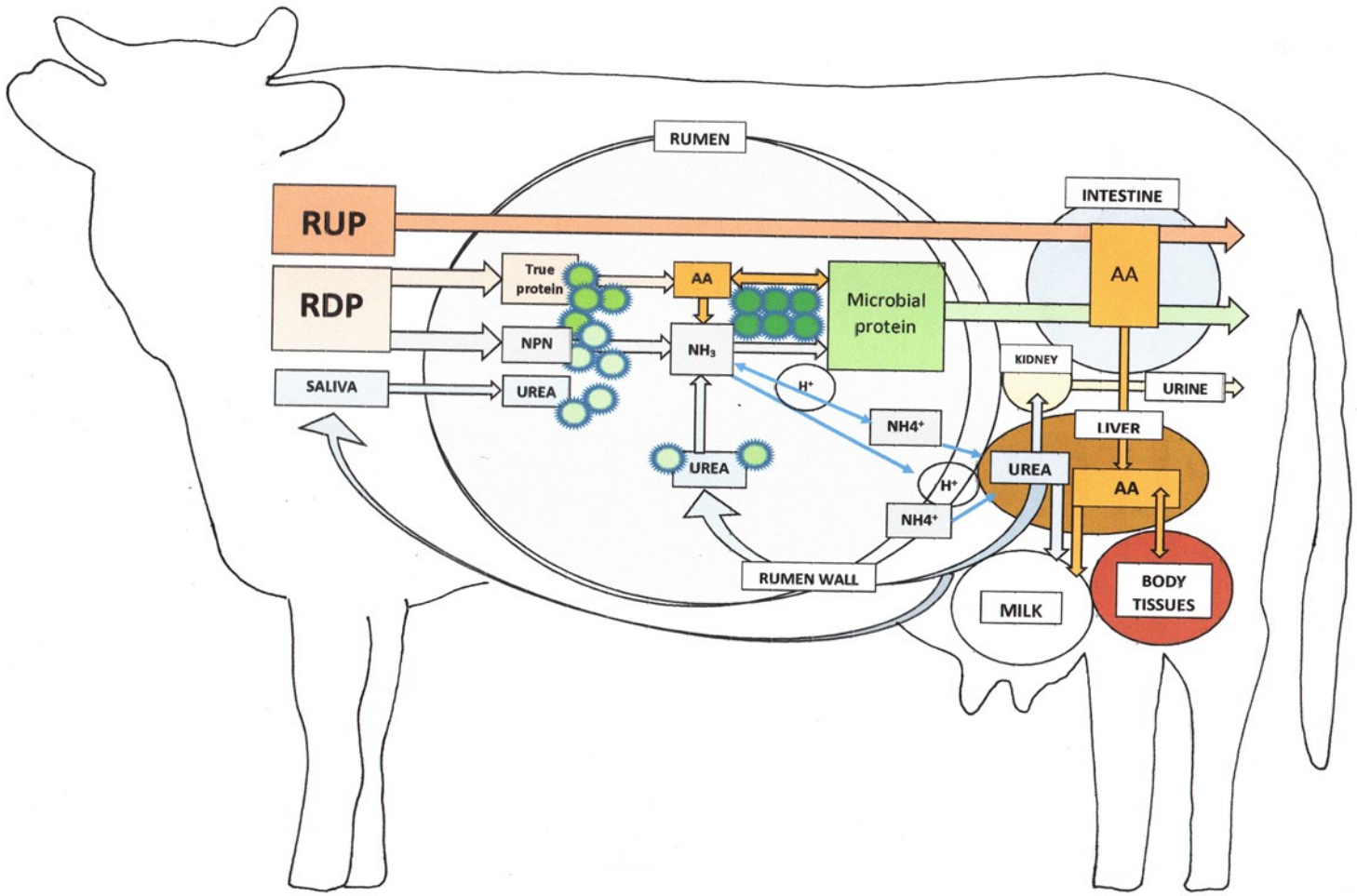
In ruminants (beef cattle, dairy cows and sheep) when dietary CP surpasses 17%, saturation and decrease in microbial N assimilation are taking place (Batista et al., 2017). An excess N promotes a greater ammonia absorption and prominent hepatic urea synthesis, which in its turn decreases the efficiency of recycled urea-N and results in greater urinary urea-N excreted, increasing air and water pollution.

Amount of urea entering the gut with saliva depends on the ration, thus, in steers, from 17% (for concentrate-based diets, Guerino et al., 1991) to 36% (for forage-based diets, Taniguchi et al., 1995) of recycled urea was reported. Similarly, assimilation of recycled urea-N into microbial N is diet-dependent, Al-Dehneh et al. (1997) reported 13 and 38% contribution of this urea to microbial N for high forage and high grain diets, respectively found in dairy cows.

Batista et al. (2017) in the extensive review resumed that the amount of urea synthesized in the liver was found to be positively correlated with N intake, composing about 72% of N intake for beef and 60.2% for dairy cattle. Nonetheless, hepatic ureagenesis was found to a greater extent depend on the origin of N absorbed (ammonia vs. AA) than on N intake in cattle fed diets adequate in N intake (Lapierre and Lobley, 2001). More diet N provided and absorbed as AA, the less N is synthesized in the liver and returned to the gut.

In cases when RDP is limiting, urea recycling becomes a crucially important metabolic function, providing a substantial contribution to available N for rumen bacteria. Among studies, diets with low-level CP promoted N recycling mechanism in heifers (Marini and Van Amburgh, 2003) and steers (Wickersham et al., 2008). Partially it was due to multiple recycling of urea-N, resulted in the increase in efficiency of N uptake by microorganisms.

Figure 2. Schematic representation of ruminal N utilization and urea recycling mechanism.



Abbreviations: RUP-rumen undegradable protein; RDP-rumen degradable protein; NPN-non-protein nitrogen; AA-amino acids; NH_3-ammonia; NH_4^+-ammonium; H^+-hydrogen ion.

1.3. Urea and slow-release urea products

Rumen bacteria can derive dietary N not only from the digestion of the true protein but also from NPN compounds to produce high-quality animal protein. Feed protein sources may contain up to 30% of N in form of NPN in forages though having much lower portion in concentrates. The use of supplemental NPN partially substituting diet true protein is wide-spread and long-known practice, used by rumen nutritionists in order to decrease diet costs without compromising animal performance.

Among NPN products, such as liquid ammonium polyphosphate and ammonium sulphate, urea was found to be more satisfactory as a protein replacement due to high palatability and lower toxicity. In the rumen, urea is hydrolyzed to carbon dioxide and ammonia (Owens et al., 1980), further utilized for MPS. Additionally, owing to the buffering capacity of ammonia which elevates rumen pH (Kertz, 2010) the risk of ruminal acidosis is decreased. Due to the concentrated nature, urea is economically more profitable compared to other plant protein sources, as per N basis, as well as for storage and transportation. Thus, at a relatively similar price per ton (346.14 EUR for SBM and 346.13 EUR for the urea, Clal.it, Italy, average price for 2017), urea is about six times cheaper in respect to N concentration of urea comparing to SBM (about 46 vs. 7.3%, respectively). Despite the facts that over a century urea has been used in diet formulations and an extensive research have been performed on the effectiveness and safety of urea utilization in different diet ratios, it is often inefficiently applied worldwide in ruminant nutrition (Broderick et al., 2009) mainly due to the high rumen solubility. As much as 95% of urea is degraded in the first hour post-feeding with the remaining 5% at the rate 0.5/h (Sinclair et al., 1995).

The rate of the ammonia release from the urea hydrolysis generally exceeds the rate bacteria may utilize it (Reid, 1953). Kertz (2010) in his review reported that among studies the rumen ammonia concentration above 5mg/100 mL was not beneficial for MPS. The excess ammonia accumulates in the blood, causing the loss of N with urine, leading not only to an inefficient N metabolism but also to increase in reactive N discharge into the environment. Moreover, the rapid rise in rumen ammonia concentration increases rumen pH and permeability of rumen epithelium elevating blood ammonia concentration to the level, when it may exceed the ability of

the liver to detoxify it, a toxicity takes place (Abdoun et al., 2007). Therefore, the recommended urea feeding quantities are limited to about 1% of the total DM intake, 135 g/cow daily, not greater than 20% of total CP intake (Kertz, 2010). Moreover, it must be mixed thoroughly with other feed ingredients and requires an adaptation period. High urea supplementations may lead to depression in dry matter intake (**DMI**) and even, toxicity. Similarly, feeding urea with low-energy sources (roughages) or rapidly degradable N feeds (raw soybeans or raw soybean hulls, oilseed meals, legume forage and young grasses) can be toxic. As the efficiency of urea utilization is highly dependable on digestible energy content (Kertz, 2010), it is recommended to supply urea to the diet of finishing bulls, as these diets are low in protein and rich in rumen degradable energy, which favours efficient urea utilization (Reid, 1953) and MPS.

Ureolytic bacteria responsible for urea breakdown in the rumen are found to be a predominant community in the rumen of the cow (Jin et al., 2017). They secret enzyme urease which catalyzes urea hydrolysis to ammonia. Manipulating with the ureolytic bacteria to inhibit ureolysis is the one way to decrease the rate of urea conversion to ammonia, thereby improving utilization of urea-N and minimizing ammonia wastage. For example, immunization of the cow against urease is a promising strategy (Zhao, et al., 2015). Nonetheless, extensive studies are required as a little is known about the properties and distribution of urease-producing bacteria.

Slow-release urea products were developed to control the rate of urea degradation and the release of ammonia in the rumen over the time. This would provide the balance between rumen available N and energy thereby optimizing bacteria capacity to utilize resulting ammonia for protein synthesis. Thus, along with the increase in ammonia uptake by rumen microbes, the use of SRU would enable to increase the amount of urea in the diets without the risk of toxicity.

Among SRU products developed and applied in ruminant feeding, there are biuret, starea, urea phosphate, urea treated with formaldehyde, urea-calcium mixtures, such as urea calcium chloride and urea-calcium sulphate and coated urea, (Cherdthong and Wanapat, 2010).

An increasing interest is given to the dietary properties of the cereals treated with ammonia (gas, liquid) or urea, initially for preserving purposes. The treatnment (ammonia or urea) is applied to the high-moisture grain, due to strong affinity of ammonia to the water. In a case of urea, enzyme urease is additionally applied to catalyze the urea hydrolysis to ammonia. The rate of ammonia added varies from 0.5 to 1.5% on the grain DM. Treated grain is kept for a certain

period, depending on the treatment and the grain properties in air-tight containers to prevent ammonia volatilization to the atmosphere. During the treatment, ammonia binds to the cereals improving from 0.8 to 7.2 percentage units' value of CP (Humer and Zebeli, 2017). During the ammonia treatment, a change of colour from yellow to brown was noted, with a gradual decrease in colour intensity with the deeper levels of the kernel (Brekke et al., 1979), assuming to be a symptom of the ammonia penetration pathways. Conversely, captured inside the kernel, this ammonia may possess slow-release properties in the rumen, which was noted by Johanning et al. (1978) who incubated ammoniated corn *in vitro*. Therefore, this treatment may become a way to slow the rate of ammonia release in the rumen. Moreover, cereals high in soluble carbohydrates would provide better conditions for utilizing of ammonia released, meeting the balance between rumen available energy and N. But fewer studies are available on this matter.

Conventional coated SRU products are feed grade urea (**FGU)** granules, coated with a different rumen-degradable material, such as polymers (Galo et al., 2003) or lipids (Garrett et al., 2005). Coating dissolves in the rumen allowing urea to be gradually available to rumen microbes. Nonetheless, use of these compounds showed rather contradictory results, as inappropriate protection may let a substantial part of urea escape rumen degradation and therefore, bacteria utilization (Galo et al., 2003; Firkins et al., 2007).

A variety of the existing and emerging SRU products is needed to be evaluated in order to be introduced to the feeding practices. *In vivo* evaluation of the SRU products require live animals and therefore, being expensive, time and labour-consuming. Existing *in vitro* and *in situ* techniques to measure rumen N degradability fall in two principal groups: measuring of the ammonia or urea in the fermentation fluid over a time and gravimetrical determination of the N in the fermentation residue. Still, both approaches are considerably labour-intensive. However, the "golden" methodology, which would be quick and comprehensive in evaluating SRU products does not exist. Therefore, there is the need for rapid and robust *in vitro* method which would become a useful tool for initial assessment of SRU products for their ability to slow the release of urea.

2. Recent experiments which evaluated diets containing feed grade urea or slow release urea for cattle

This chapter is a review of studies evaluated the dietary inclusion of feed grade urea (FGU) or SRU products in terms of animal performance (feeding trials with dairy cows and fattening bulls), metabolic parameter (metabolic trials with cannulated animals) or rumen fermentation characteristics (rumen *in vitro* techniques). Main selection criteria were the year of publication (from 2007 until now) and the scientific level of the international journal, according to Scopus ranking. Out of ten top-rated Animal Science Journals,, seven containing selected s scientific papers are presented in **Table 1.** A total of eighteen studies were examined, twelve made by a single experiment each one, five made by two experiments and one by three experiments, giving a sum of twenty-five trails (**Table 2)**.

Among feeding and metabolic studies observed, four trials were with dairy cows and sixteen experiments were performed with beef cattle. Such a layout was expected, as conventional beef diets are traditionally supplied with urea. In fact, beef cattle are fed with diets rich in energy from highly rumen fermentable carbohydrates, which require fast degradable crude protein source, such as urea, to support optimal microbial growth and as a consequence, animal performance. Forage-based dairy cattle diets contain slower degradable carbohydrates and the rate of ammonia N provided by urea may overwhelm the ability of bacteria to utilize it, causing toxicity, therefore, the use of urea in these diets is limited.

The percentage of diet CP as urea products varied among studies substantially: as can be seen from **Figure 3**, there was an opposite trend of dietary CP and urea contents. Urea supplied only from 5 to 10 % CP in dairy diets, which showed high CP contents, ranging from 16.3 to 17.5 % DM. The urea inclusion ranged from 11 to 53% CP in beef feeding trials and from 20 to 71% CP in cannulated animals, and overall these diets had a CP content from 7 to 15% DM.

Throughout the feeding studies an overall positive effect of the SRU products comparing to FGU was demonstrated by the lower ruminal ammonia concentrations (Taylor-Edwards et al., 2009a,b; Xin et al., 2007 and 2010; Cherdthong et al., 2011a,b; Ribeiro et al., 2011; Sinclair et al., 2012; Holder et al., 2013), lower blood urea and blood ammonia (Taylor-Edwards et al., 2009a; Cherdthong et al., 2011a; Gardinal et al., 2017), plasma urea (Cherdthong et al., 2011c; Sinclair et al., 2012; Holder et al., 2013, 2015) and milk urea concentrations (Xin et al., 2010).

Some positive effects of SRU vs. FGU, such as improved feed efficiency (**G: F**, Taylor-Edwards et al., 2009b; Bourg et al., 2012), N retention (Holder et al., 2015) and efficiency (Sinclair et al., 2012; Holder et al., 2015), MPS (Xin et al., 2010; Cherdthong et al., 2011a; Cherdthong and Wanapat, 2013; López-Soto et al., 2014), and milk yield (Cherdthong et al., 2011a; Highstreet et al., 2010) were possibly due to better rumen synchrony between energy and N provided by slow ammonia release.

Generally, negative effects of substituting SRU products with FGU were not found, except the increase in fecal N in Holstein steers fed corn silage based diet where SRU was at 36% of total CP (Taylor-Edwards at al., 2009a). In contrary, Bourg et al. (2012) in Holstein steers reported no effect of source of N on fecal N concentration. Urinary N concentrations were either not affected (Taylor-Edwards et al., 2009a; Highstreet et al., 2010) or were decreased with the substitution of FGU with SRU (Gardinal et al., 2017).

Among feeding trials and trials with cannulated animals, DMI was not affected by the SRU treatment, except Taylor-Edwards (2009b) who reported a reduction in DMI in Angus crossbred steers over the last half of the study (from d 29 to d 56). In contrary, Xin et al. (2010) in the trial with lactating Holstein cows fed steam-flaked corn-based diet and Ribeiro et al. (2011) in cannulated beef steers found an increase in DMI in when urea was substituted with the SRU product.

Throughout the studies, nutrients' digestibility were left unaffected by SRU supplementation, except few studies where an improved apparent total tract OM and NDF digestibility in Holstein crossbred dairy cows fed rice straw (Cherdthong et al., 2011a) and NDF digestibility in beef steers fed concentrates (Cherdthong et al., 2011c) were reported. Besides, Cherdthong and Wanapat (2013) found an increased population of cellulolytic microbes (*Fibrobacter succinogenes*) when FGU was substituted with SRU in beef steer fed rice straw and concentrates.

Nevertheless, Sinclair et al. (2012) did not found the significant difference between urea and SRU supplementation in milk yield, milk true protein, nitrogen and fat with Holstein–Friesian dairy cows fed grass silage, maize silage based mixed ration where 9% of CP was SRU. Similarly, Highstreet et al., 2010 in very high producing lactating cows (around 45 kg/ of milk) were able to demonstrate only modest improvements (limited only to milk fat synthesis) by substituting urea with an SRU product.

Also in feeding trials with growing bulls the substitution of FGU with SRU did not clearly impact positively the performance. In the feeding trial of Taylor-Edwards et al. (2009b), the average daily gain (**ADG**) was reduced by SRU at 0.4 and 1.6%, but did not differ from urea at the 0.8 and 1.2% of diet DM supplementation concentrations. Similarly, Gardinal et al. (2017) also found that SRU (as well as FGU) added at 40% of CP decreased ADG in steers.

Most of studies observed investigated the effects of simple substitution of FGU with SRU (on N basis) without an increase in the amount of SRU as a percentage of dietary CP. Except for the studies of Bourg at al. (2012) and Taylor-Edwards et al. (2009b) who increased the amount of SRU in the diet, however, the results weren't quite in agreement. Thus, Bourg at al. (2012) reported that SRU fed at 53% of total dietary CP improved G: F in feedlot steers, while Taylor-Edwards et al. (2009b) concluded an impaired ADG and G: F in steers fed more than 37% of CP. Same authors reported, that this effect was similar to FGU at lower concentrations (30%).

In total, there were four *in vitro* experiments, three represented by batch fermentation systems and one by a fermentation trial in a dual-flow continuous culture apparatus. Xin et al. (2007, 2010) in two batch system experiments used a simple apparatus of gas production (e.g. 100 ml graduated syringes containing 200 mg of substrate) and incubated mixed diets containing urea (e.g. 17.0 % CP): a significantly higher ammonia concentration in fermentation liquids of FGU than SRU was measured.

In a third batch fermentation experiment (Cherdthong et al. 2011b) the measure of gas production (e.g. 50 ml bottles containing 500 mg of substrate) was used to compare the fermentation of substrates differing in urea products (FGU or different doses of two SRU types) and in the fermentable ingredient (cornmeal or cassava chips). One of the clearer results of this trial was a lower ($P<0.05$) gas production for FGU substrates than SRU (in ml gas/500 mg of the substrate after 96 h) both, cassava chips (e.g. 83.3 vs. 103-120) and cornmeal substrates (71.8 vs. 82.0-97.9). The concomitant measure of ammonia gave results higher ($P<0.05$) for FGU than SRU both for the cassava chips (e.g. 14.5 vs. 11.0-11.6 mg/100 ml) or the cornmeal substrates (15.7 vs. 13.0-13.4 mg/100 ml). This was probably a result of the buffering effect of FGU because it determines a higher production of ammonia, neutralizing the acids and reducing the indirect gas production.

The dual-flow continuous culture trial (Xin et al. 2010) compared diets containing FGU and SRU. Although, there was no appreciable difference in ammonia concentration in fermentation fluids between the treatments, a significant increase in microbial efficiency was detected in SRU diets.

Table 1. Distribution of the studies where feed grade urea was compared to slow-release urea published in 7 top-rated (Scopus) journals in a last decade (2007-2017).

N	Journal	Feeding trials		Metabolic trials	
		Cows	Beef	*In situ*	*In vitro*
1	Animal	1		1[1]	
2	Journal of Animal Science		3	4	
3	Animal Feed Science and Technology		1	3	
4	Livestock Science	2			
5	Archives of Animal Nutrition		1	1	1
6	Journal of Animal and Feed Science				1
7	Asian-Australian Journal of Animal Science	1	1	2	2
Total:	25	4	6	11 (1)[2]	4

[1] *In situ* Study with the wether sheep (Sinclair et al., 2012)

[2] *In situ* procedure was used as an analytical method to measure ruminal degradability of SRU product in feeding trial (Highstreet et al., 2010)

Table 2. Effects of supplementation with slow-release urea (SRU) vs. feed grade urea (FGU) among 18 selected studies published in selected top-rated (Scopus) in a last decade (2007-2017).

N	Reference	Animals	Base feed	CP, % DM	% NPN of CP	Effect of SRU supplementation		
						+	NO	-
1	Xin et al., 2007	*in vitro* gas	steam-flaked maize-based diet	17.4	10	ammonia concentrations	pH, 24-h gas production, total VFA and individual VFA molar proportions	
2	Taylor-Edwards et al., 2009a	cannulated Holstein steers	corn silage based diet	-	-	RAC, AUC, stable AAC, increased net hepatic ammonia uptake (not rapidly).	urinary N, Net portal ammonia release; Intake of N, DM, OM, NDF, and ADF, ATTD of DM, OM, NDF, and ADF,N retention, VFA,	fecal N, less ATTD of N, increased urea transfer to the GIT
		Holstein and Angus steers		12.9	36	AUC, less rapid peak in AUC		
3	Taylor-Edwards et al., 2009b	Angus crossbred steers	corn silage based diet	9.9, 10.7, 11.5 12.2	11; 20; 30; 40	G: F, N release, without 35d adapt.	BW, DMI over d 0 to 56	ADG and G: F at the 11 and 40% NPN of CP, DMI during d 29 to 56, limited N availability at low (11% NPN of CP)
		cannulated steers		12.5	42	RAC, ruminal urease activity (P = 0.06).	DMI, BW, VFA, pH, *in situ* rate of SRU degradation for 35d	adaptation of *in situ* rate of SRU degradation for 35d
4	Highstreet et al., 2010	multiparous early and mid-lactation cows	TMR	17.9	5	in early lactation cows: milk yield, milk fat, and protein, milk energy	DMI, mid-lactation group. Urinary N, ammonia or urea-N concentrations, fecal digestibility of CP and NDF	
		dual-flow continuous culture		13	35	microbial efficiency	CP digestibility	
5	Xin et al., 2010	*in vitro* gas	steam-flaked corn-based diet.	17.5	10	ammonia concentration		
		lactating Holstein cows		17.4	10	DMI, milk protein content, MUN and BUN concentration, less surplus ruminal N		

Table 2. (Continued) Effects of supplementation with slow-release urea (SRU) vs. feed grade urea (FGU) among 18 selected studies published in selected top-rated (Scopus) in a last decade (2007-2017).

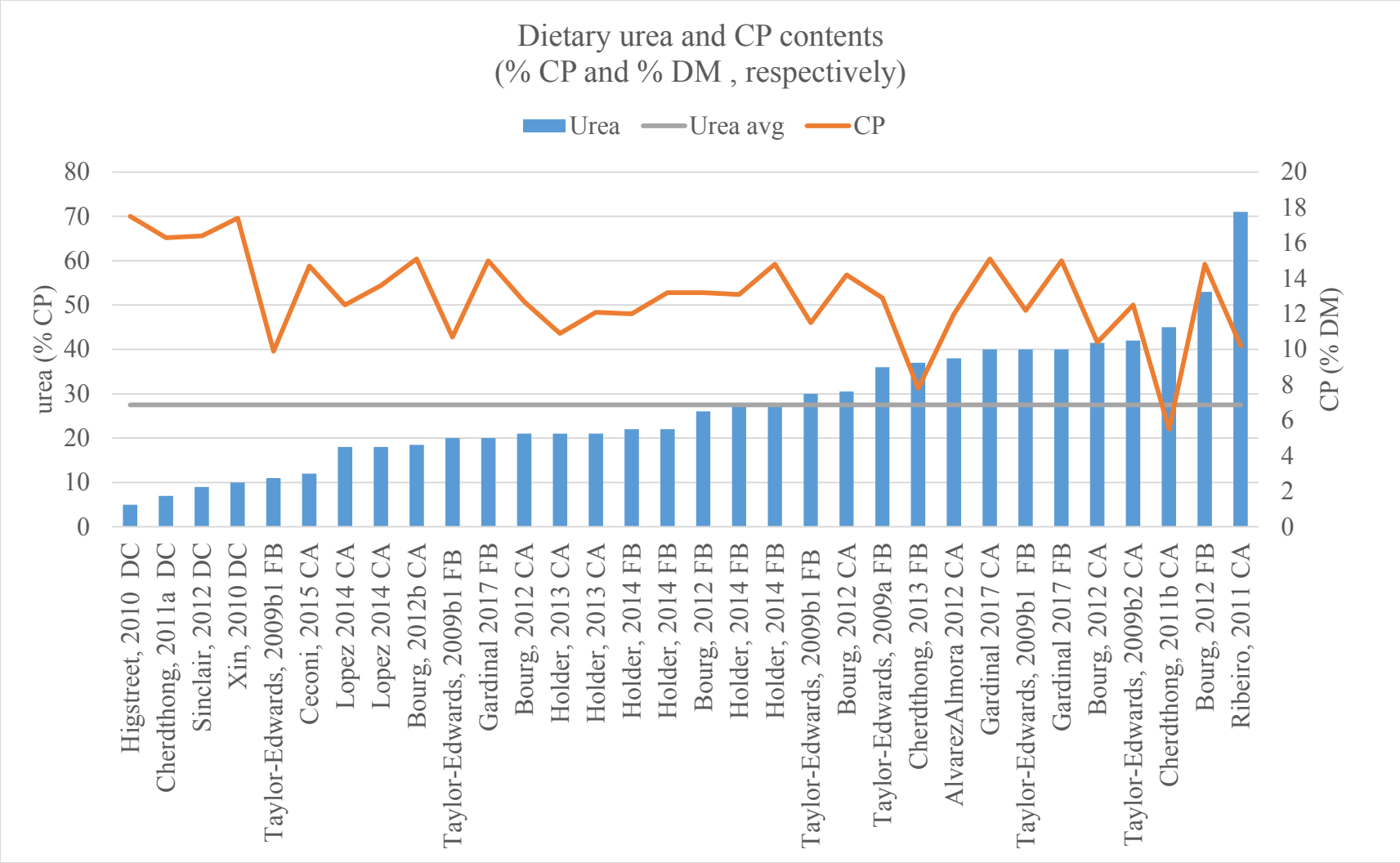
N	Reference	Animals	Base feed	CP , % DM	% NPN of CP	Effect of SRU supplementation		
						+	NO	-
6	Cherdthong et al., 2011a	Holstein crossbred dairy cows	rice straw	16.3	7	OMI, ATTD of OM and NDF, RAC and BUC, MCP, Milk yield	MUN	
7	Cherdthong et al., 2011b	*in vitro* gas	cassava chips or ground corn, rice straw	16.2	7	RAC		
8	Cherdthong et al., 2011c	cannulated crossbred (Brahman × native) beef steers	concentrates (cassava chips)	5.5	45	NDFD, RAC, PUC, total and cellulolytic bacteria, MCP synthesis	DMI, total VFA	
9	Ribeiro et al., 2011	cannulated beef steers	Brachiaria humidicola cv. Llanero hay	10.2	71	DMI, RAC, pH		
10	Sinclair et al., 2012	Holstein–Friesian dairy cows	grass silage, maize silage based mixed ration	16.4	9	LW (P <0.07), N efficiency, PUC	DMI, Milk: N, yield, fat , true protein; ADG, condition score, G: F, blood parameters, PAC, ATTD of DM, OM, NDF and N,	N intake
		cannulated wether sheep	concentrate	19.7	7	N release rate		
11	Bourg et al., 2012	finishing Angus crossbred steers	steam-flaked corn based diets	13.2 and 14.8	26 and 53	initial G: F (Period 1 and 2) for OP3.1	BW, ADG, G: F, DMI, rib section weight, lipid, protein, N content, moisture %, physical muscle, bone, subcutaneous fat, and seam fat	
		cannulated Holstein steers		12.7; 15.1; 10.4; 14.2.	21; 18.5; 41.5; 30.5	less apparent N absorption	N retention, fecal N, DMI, digestibility, performance and carcass composition	

Table 2. (Continued) Effects of supplementation with slow-release urea (SRU) vs. feed grade urea (FGU) among 18 selected studies published in selected top-rated (Scopus) in a last decade (2007-2017).

N	Reference	Animals	Base feed	CP , %DM	% NPN of CP	Effect of SRU supplementation		
						+	NO	-
12	Álvarez Almora et al., 2012	cannulated beef steers	Alamo switchgrass hay + supplements	12.0	38		DMI , DMD, N balance, RAC, VFA	
13	Holder et al., 2013	cannulated Holstein steers	fescue hay, cracked corn, molasses, SBM	10.9 and 12.1	21	RAC and PUC	N retention	
14	Cherdthong and Wanapat 2013	Thai native beef cattle	rice straw and concentrates	7.8	37	bacteria populations, counts of fungal zoospores, concentration of total bacteria, *Fibrobacter succinogenes*, MCP yield and efficiency of MN synthesis	*Ruminococcus flavefaciens* and *Ruminococcus albus*	
15	Holder et al., 2015	Holstein steers	Cracked corn, fescue hay, cottonseed hulls	12.0; 13.2; 13.1; 14.8	22 and 27	N retention at 1.14 Optigen, PUC at 1.00 DIP.		DMD
16	López-Soto et al.,2014	cannulated Holstein steers	finishing diet	12.5 and 13.6	18	MN flow and digestible energy	Ruminal pH	
17	Ceconi et al., 2015	cannulated steers	Corn earlage, mod. DG, corn silage, dry-rolled corn	14.7	12		DMI, OMI; digestibility of NDF, CP, OM, starch.	
18	Gardinal et al., 2017	cannulated steers	corn silage, ground corn	15.1	40	lower ammonia, a:p, urinary N and BUC	nutrient digestibility	
		steers		15	20 and 40			

Abbreviations: AAC-arterial ammonia concentration, ADG-average daily gain, ADF-acid detergent fiber, a:p-acetate to propionate ratio, ATTD-apparent total-tract digestibility, AUC-arterial urea concentration, BW-body weight, BUN-blood urea concentration, CP-crude protein, DM-dry matter, DMD-dry matter degradability, DMI-dry matter intake, G: F -feed efficiency, GIT –gastrointestinal tract, LW-live weight, MCP-microbial crude protein, MN-microbial nitrogen, MUN-milk urea nitrogen, N-nitrogen, NDF-neutral detergent fiber, NDFD-NDF digestibility, NPN-non-protein nitrogen, OM-organic matter, OMI-organic matter intake, PAC- plasma ammonia concentration, PUC-plasma urea concentration, RAC-rumen ammonia concentration, TMR-total mixed ratio,VFA-volatile fatty acids.

Figure 3. Dietary crude protein (CP) and urea concentrations (% DM and % CP, respectively) in recent trials selected where urea was compared with slow-release urea products (DC: lactating dairy cow trials; FB =fattening bull trials; CA = cannulated animal trials).



3. Key points from literature review and aims of the experimental work

The exam of literature allows to resume the following.

1. Despite the wide variability between trials and experiments, the overall average inclusion of urea in diets was 27.5% of dietary CP (**Figure 3**). This indicates that there is a discrete potential to increase the substitution of the dietary protein with urea in cattle diets.
2. The main part of the recent research papers concerning the use of urea products in cattle concerns the inclusion in fattening diets for bulls with respect of dairy cows (in a ratio of approx. 80:20); this reflects the fact that urea is assumed to be well used in diets having a high fermentable organic matter, such the starchy diets of fattening bulls.
3. A rational approach to the modern ruminant feeding systems to dose the dietary inclusion of N degradable source, such as urea, is based on a rumen balance between available FOM and degradable N. However, the efficiency of N capture by rumen bacteria depends on the (i) dietary degradable N level, which must be sufficient for microbial growth even considering the rumen N recycling process and (ii) on the synchrony between nutrients' degradations in the rumen. Urea protected from the fast degradation (e.g. SRU products) could be an excellent supplement of degradable N, slowly released into rumen.
4. A new perspective is to feed cattle with ammoniated or urea-treated feeds such as the cereal grains in cattle feeding, which can provide a nutrient synchrony to meet closer microbial requirements.
5. The substitution of urea with SRU did not clearly impact positively the main productive traits of the cattle (intake, digestion, milk yield, and growth rate) among studies; this might be due to the different conditions of the trials (diets and animals) but also probably due to the specific characteristics of SRU products in each trial. In fact, out of twelve trials where cannulated animals were used, only in four trials the efficiency of the urea protection in SRU products was tested by the nylon bags technique. Probably the reason of this small number of trials tested the degradability of SRU products is the technique, which requires cannulated animals and is labor expensive. Therefore, ethical and economic issues suggest to develop alternative methods based on laboratory methods.

AIMS OF THE THESIS

The experimental part of present PhD thesis is composed by three experiments, with the following aims.

1. The first *in vivo* trial with fattening bulls aimed to study the dietary substitution of SBM with urea within two levels of CP content (medium vs. low). A practical impact of the trial was the possibility to reduce CP levels in diets for Simmental bulls and yet to maintain or to improve the satisfactory growth. This would let to improve feed efficiency, reduce diet costs and environmental impact. A second aspect was to study the efficiency of urea utilization within two different conditions of predicted N degradable pools at rumen level *in vitro*.
2. The second experiment evaluated urea-treated cereals (high-moisture barley and corn) for potential slow N release with the possible beneficial effects on MPS. Our hypothesis was that N captured inside the grain matrix is released slowly in synchrony with the starchy fermentation to support an intense rumen microbial synthesis. Moreover, these treatments could have a significant impact in fattening diets, where the cereals are the principal feed ingredient.
3. Finally, the aim of the third experiment was to set up an innovative, quick and robust *in vitro* methodology to evaluate the SRU products which might become a useful tool in a screening of the protective properties of different SRU products before *in vivo* evaluation.

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Effect of diets differing in protein concentration (low vs. medium) and nitrogen source (urea vs. soybean meal) on *in vitro* rumen fermentation and on performance of finishing Italian Simmental bulls.

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CHAPTER 2. Effect of diets differing in protein concentration (low vs. medium) and nitrogen source (urea vs. soybean meal) on *in vitro* rumen fermentation and on performance of finishing Italian Simmental bulls.

ABSTRACT

This research evaluated the effect of two dietary intestinal digestible protein (**PDI**) levels and a partial substitution of soybean meal (**SBM**) with urea (**U**) on the performance of finishing Italian Simmental bulls (Exp. 1) and on *in vitro* rumen fermentation characteristics (Exp. 2). In Exp. 1, eighty Italian Simmental bulls (495 ± 58 Kg of body weight (**BW**), 14-months old) during the last 120 d of fattening were fed diets differing in terms of PDI concentration (85 and 72 g/kg dry matter (**DM**), medium (**M**) and low (**L**) diets, respectively) and nitrogen source (only SBM or SBM partly replaced by 0.5% DM of U). Animals were slaughtered at BW of 656 ± 56 kg at 18 months of age. The average daily gain (**ADG**) was satisfactory (1.32 kg/d) and the medium PDI level tended ($P<0.10$) to increase the ADG (1.37 vs. 1.28 kg/d). Apparent total tract digestibility did not differ between treatments, and the feed efficiency tended to be more favourable for the M diets (0.147 vs. 0.137 kg BW gain/ kg feed DM, $P < 0.10$). Slaughter traits were unaffected by dietary treatments. In Exp. 2, continuous culture fermenters were inoculated with rumen fluid from bulls in Exp. 1 and were given the same diets. The fermentation fluid was sampled at feeding time and 1, 2 and 3 h after (T0, T1, T2 and T3, respectively). Two PDI levels determined large differences in the ammonia concentrations ($P<0.01$ at T0, T1, and T2; $P<0.05$ at T3). The drop of pH at T1 and T2 was less intense ($P<0.05$) for diets containing U, presumably due to the buffering capacity of urea. *In vitro* DM digestibility tended to be higher for diets containing U ($P<0.10$) and was unaffected by the dietary level of PDI. Differences in volatile fatty acids concentrations were limited to butyrate, which was higher for M diets (T0:13.6 vs. 12.5 mol/100 mol, $P < 0.10$; T2: 13.8 vs. 12.5 mol/100 mol, $P <0.01$) and for diets containing U (T0: 14.3 vs. 11.9 mol/100 mol; T2: 14.3 vs. 12.1mol/100 mol, $P<0.01$).

In conclusion, L diets tended to decrease weight gain in finishing Italian Simmental bulls, but both PDI levels and SBM substitution with U had no detrimental effects on slaughter and meat quality traits. Further research efforts are required to explain the increase of butyrate in fermentation fluid of fermenters fed medium PDI diets or U diets.

Keywords: dietary protein, dietary urea, rumen continuous fermenter, Simmental bulls, carcass traits, meat quality.

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INTRODUCTION

During the fattening cycle of growing bulls, the lean accretion decreases progressively in favour of fat deposition (INRA, 2007) and finishing diets require to be fine-tuned to avoid excessive dietary protein levels and nitrogen (**N**) excretions in the environment.

However, the reduction of protein requirements during fattening is breed dependent: in particular, Continental beef specialised breeds long maintain a lean growth, in contrast with the early fat growth of dairy cattle (Gallo et al., 2014). The Simmental types are dual-purpose breeds, largely utilised in the Continental Europe (e.g., Germany, Austria, Eastern Europe, Italy, etc.) and the Italian Simmental (IS) genetic selection is oriented to maintain an equilibrium between dairy and meat traits (Luttmann, 2011). Recently Schiavon et al. (2010) successfully reduced the dietary protein levels in a beef specialised breed during the last phase of growth, but there is no related information currently available for IS bulls.

Finishing bulls are generally fed with diets rich in rumen fermentable substrates, which promote an intensive growth of rumen microbes and microbial protein synthesis if balanced by supplemental rumen degradable N (Nocek and Russell, 1988). The last fattening period of bulls is suitable to adopt sustainable diets based on low dietary protein levels and on cheap degradable N sources (e.g., urea) and a potential lack of rumen degradable N can occur on corn-based diets, given the limited degradability of corn protein (Ceconi et al., 2015b).

In the present trial, we tested corn-based diets, commonly used in intensive fattening systems of northern Italian plains (Po valley, Gallo et al., 2014), with the aim to evaluate a reduction in

the protein concentration and a partial substitution of SBM with urea. On the basis of French ruminant protein system (INRA, 2007), diets containing 82-87 g/kg DM of intestinal digestible protein (PDI) can supply 750-800 g/d of PDI to finishing bulls (450-650 kg of body weight) and would be adequate for a growth rate of about 1300 g/d. We compared such conventional diets (M) with those containing approx. 15% less PDI (L) by halving the amount of SBM. Within each PDI level, two experimental diets were created by substituting part of soya bean meal with an isonitrogenous amount of urea (U, 0.5% DM).

We hypothesized that in corn-based diets a reduction of PDI concentration and an increase in degradable N allow acceptable performance of finishing IS bulls (growth, carcass and meat traits). Moreover, an *in vitro* rumen fermentation experiment was conducted simultaneously with the *in vivo* trial to study the impact of dietary treatments (concentration and source of N) on rumen metabolism.

MATERIALS AND METHODS

This research was composed of two experiments: an *in vivo* trial (Exp. 1) was carried out at the genetic centre of the Italian National Association of Simmental Breed, (Fiume Veneto, PN, Italy) from November 2014 to July 2015, and an *in vitro* trial (Exp. 2) was performed at the University of Udine, Department of Agricultural, Food, Environmental and Animal Sciences (Udine, Italy) from May 2015 to July 2015. The *in vivo* trial followed the guidelines of animal welfare in animal research of Italian Legislative Decree 26/2014 (Italian Ministry of Health, 2014).

***In Vivo* Trial (Exp.1)**

1. Animals and diets.

Eighty Simmental purebred bulls of 495 ± 58 Kg of BW and 14-months old were housed in 16 pens (5 bulls/pen).

Animals arrived at the genetic centre at the age of about 30 d, were immediately vaccinated against the respiratory diseases (Rispoval intranasal) and were weaned using a milk replacer. After weaning, young bulls were fed a diet based on corn silage and corn meal (approximately 60%), supplemented with soya bean meal (13-14% DM of crude protein, CP), and at 12 months of age were vaccinated against *Clostridium* (Milaxan).

For the feeding trial, a basal fattening diet was composed of corn silage, corn grain, wheat bran, beet pulps and other minor ingredients and supplements (Table 1). The dietary protein values were calculated according to the French intestinal digestible protein (**PDI**) evaluating system (INRA, 2007), which sums the by-pass digestible protein (**PDIA**) with an estimated microbial protein synthesis according to the fermentable energy and rumen degradable protein (**PDIE** and **PDIN**, respectively), in order to obtain total prediction of the intestinal digestible protein. The diet was completed by 1 Kg/d mixture composed of different proportions of three ingredients (corn grain, **SBM** and **U**) in order to obtain the following four dietary treatments: medium PDI level (85 g/kg DM) without U (**M-U**) or with U (**M+U**), low PDI level (75 g/kg DM) level without U (**L-U**) or with U (**L+U**). The dietary treatments were obtained by using two inclusion rates of SBM (8 and 4% DM, for medium and low PDI levels, respectively), and within each protein level, 4% of SBM was substituted with 0.5% of U. All diets had a PDIN concentration lower than the PDIE concentration.

In practice, the total mixed ration (**TMR**) was prepared daily for all pens and contained all ingredients with the exception of corn grain, SBM or U. These last three ingredients were daily prepared for each pen and placed into separate bags; the basal TMR was delivered in front of each pen at a distance of approximately 1m from animals and aforementioned bags (containing corn grain, SBM or U) were top dressed. Then, feeds were manually mixed and pushed towards bulls. Animals were fed once a day at 0800 h, allowing for up to 10% of orts and water was always available.

All animals were initially weighed and allotted to 4 blocks (4 pens each) according to their average body weight. Within each block animals were randomly allocated to the pens, thereby, balancing pens for weight. By design, each block entered the study gradually when animals reached an initial for this study body weight range (495 ± 58 kg). It had required a period of 4 months to complete the inclusion of all 16 pens (a block per month) in the trial. Within each block, four pens (5 bulls each) were randomly assigned to one of four experimental diets. Thus for each treatment, 4 pens with 5 animals were used.

Animals were gradually adapted to the experimental diets in a period of 15 days.

2. Sample collection and slaughter measurements.

Bulls were weighed every 28 d after 12 h of feed restriction, namely, 12 h before weighing, all feed residuals were removed from the mangers. Samples of TMR were collected on weighing days for further analyzes. Faeces samples were collected to measure digestibility by using acid insoluble ash (**AIA**) as an internal marker. The procedure of faecal collection followed that adopted by Ponce et al. (2013), with few modifications. The faecal collection was performed in the middle of the growth period (on d 60 and 61) for each block of pens. Faecal samples were collected from the floor immediately after defecation twice a day (at 0800 and 1600 h) from at least two animals per pen and stored in individual plastic bags at 4°C. At the end of the second collection day, samples were composited and homogenised by pen (8 samples/pen) and then stored at –20°C for subsequent analysis.

Animals were slaughtered at the end of the fattening trial with an average BW of 656 ± 56 kg and an average age of 18-19 months. Carcasses were individually weighed and scored for conformation (6-point scale) and external fatness (5-point scale) according to the SEUROP classification system (EU Regulation No 103/2006, 1183/2006). Hot carcasses weights were recorded about 1 h post-mortem and the dressing percentage was calculated as: (hot carcass weight/slaughter weight) × 100. After chilling for 24 h, a sample joint was removed from the left side of carcass in correspondence of the 8 to the 9^{th} rib sections (approximately 5-cm thick and 2 kg of weight). The joint was manually dissected in the lean meat, fat (cover and intermuscular), bone and other tissue and the weights were recorded (Andrighetto et al., 1996). The pH, colour, drip and cooking losses and shear force were measured on the Longissimus thoracis muscle (**LT**) separated from the joint. The pH was measured using a glass piercing electrode (Crison 52-32, Crison Instruments, S.A. Barcelona, Spain) connected to a pH meter. The colour was evaluated, according to CIE L*, a*, b* colour system after 1 h of blooming period at normal refrigeration temperatures, by a Minolta CM-2600d Spectrophotometer (Minolta Camera, Osaka, Japan) with D65 as a light source. A portion of LT was used to measure the drip loss according to the method of Grau e Hamm (1952) and Sakata et al. (1991). The remaining LT portion was stored at 4° C for 7 days; cooking loss was measured at a 75°C water bath for 20 min (ASPA, 1996) and shear force (WBSF) was determined on the cooked sample, using a Warner–Bratzler device (Lloyd TA Plus; Lloyd Materials Testing, Leicester, UK) with a triangular hole in the shear blade, mounted on an Instron 4301 (Instron Ltd., High Wycombe, United Kingdom) universal testing machine.

The measurement was recorded as the peak yield force in N, required to shear, at a 100 mm/min cross-head speed, perpendicular to the direction of the fibers, three cylindrical cross-section replicates, 10 mm diameter 30 mm length, from each sample.

Continuous Culture Fermenter trial (Exp. 2)

1. Apparatus and trial organisation

Three consecutive *in vitro* continuous culture fermentations were started simultaneously with the series of bulls' slaughtering (block 2, 3 and 4 of Exp.1).

Rumen fluid for the *in vitro* trial was collected immediately after each slaughtering from 8 bulls, 2 animals per diet randomly selected within each pen. In each run, eight single-flow continuous culture fermenters were inoculated and maintained as described by Mason et al. (2015). The rumen contents collected from the 2 animals fed the same diet were mixed together and used as inoculum for 2 fermenters (as repetition/diet). The fermenters were supplied with the same diets as in the *in vivo* trial, respecting the treatment assigned through the inoculum; all ingredients were dried, milled (1-mm screen), mixed and given to each fermenter in 2 equal portions at 0900 and 1700, for a total of 20 g/d of DM. Artificial saliva (McDougall, 1948) was continuously infused into the fermenters at 1.2 ml/min, giving a dilution rate of 5% per hour. Effluent liquids, accumulated in collection tanks, were kept at 4°C by using frozen ice packs to stop the microbial activity. Each fermentation lasted 8 d (5 d of adaptation and 3 d of collection).

2. Sample collection

During the collection period for each day and fermenting bottle, the following sampling times were adopted: immediately before morning feeding (T0) and at 1000, 1100 and 1200 (T1, T2 and T3, respectively). At each sampling time, the pH of the fermentation fluid was measured with a pH meter (GLP 22, Crison Instruments, S.A. Barcelona, Spain) and samples of fermentation fluids were collected for the ammonia nitrogen (**NH_3-N**) and volatile fatty acids (**VFA**) analyses (samples acidified with H_2SO_4 1.0 and 0.1*N,* respectively).

Every morning of the collection period the effluents from each tank were removed, weighed, volume recorded and a 500-ml subsample was refrigerated. At the end of the trial all samples from each tank were pooled together, homogenised and then freeze-dried (450 ml of total effluent liquid from each fermenter) for subsequent DM and organic matter (**OM**) analysis. On

the last day, the whole content of each fermenter was removed and dried (60°C) for DM and OM analysis.

Chemical Analysis

Total mixed ration samples were dried at 60°C for 48 hours in a forced-air oven. Faecal samples from Exp. 1 (bulked and homogenized per pen) were thawed: 10g were immediately diluted with 100 ml of distilled water for the pH measurements (GLP 22), 5 g was taken for N determination and the remaining sample (about 100 g) was dried in a forced-air oven (72 h at 60°C) for DM analysis.

Predried TMR and faecal samples of the Exp.1 were milled through 1-mm screen (Pulverisette; Fritsch, Idar-Oberstein, Germany), heated at 105°C for 3 h for DM determination (method 930.15; AOAC, 2000) and then incinerated at 550°C for 2 h for ash concentration (method 942.05; AOAC, 2000). Neutral detergent fiber (**NDF**) was determined by fiber analyser (Ankom II Fiber Analyzer; Ankom Technology Corporation, Fairport, NY) following the procedure of Van Soest et al. (1991) without correction for residual ash; for ether extract (**EE**) concentration (only in TMR samples) solvent extraction method (method 954.02; AOAC, 2000) was applied. The N concentration in predried TMR and fresh faecal samples was determined by the Kjeldahl method (method 976.05; AOAC, 2000). Non-structural carbohydrates (**NSC**) were calculated as: DM - (CP + Ash + EE + NDF).

The AIA was determined according to Van Keulen and Young (1977). Samples were incinerated, simmered for 15 min in 75 ml of 3*N* HCl, the solution then was filtered through ash-free filter paper (Whatman no. 541, 20µm of porosity) with distilled water and subsequently incinerated at 550°C for 1 night. Total tract apparent digestibility coefficients for DM, CP and NDF were calculated using AIA as a marker and the following equation: 100 – [100 × (% AIA in TMR/% AIA in faeces) × (% nutrient in faeces/% nutrient in TMR)].

Samples of fermentation liquid for NH_3-N determination were centrifuged and analysed spectrophotometrically using a modified Berthelot reaction method (Krom, 1980) with the continuous flow SAN++ analyser (Skalar Analytical B.V., Breda, Netherlands). For VFA analysis the aliquots of fermentation fluid were centrifuged at 20,000 × g for 30 min at 20°C and the supernatant was then filtered using polypore 0.45 µm filters (Alltech Italia, Milan, Italy). The

filtrate was injected into a high-performance liquid chromatography instrument (Perkin-Elmer, Norwalk, CN, USA), set to 220 nm according to the method described by Martillotti and Puppo (1985). Freeze-dried effluent samples and the residues of fermentation fluid collected at the end of the trial were analysed for DM and OM (method 930.15 and 942.05, respectively; AOAC, 2000) and were used to calculate the *in vitro* digestibility of DM and OM.

Statistical Analysis

The *in vivo* data (Exp. 1) were statistically analysed as a factorial 2 × 2 randomised completely block (4 blocks) design, using the pen as experimental unit:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{ik} + \varepsilon_{ijk}$$

where:

y_{ijk} is the response of the experimental unit (pen), μ is the overall mean, α_i is the random effect (block) of the group of pens (i=1,4); β_j is the fixed effect of the dietary level of CP (j=1,2); γ_k is the fixed effect of the dietary presence/absence of U (k=1,2) and ε_{ijk} is the random error.

The *in vitro* data (Exp. 2) were analysed with factorial randomised completely block design similar to that used for Exp. 1 with the exception being the experimental unit, which was the fermentation bottle of the single-flow continuous culture fermenters, and the number of blocks, which were in total 3 (blocks 2, 3 and 4 of Exp. 1).

For all statistical analyses, trends were declared at $P \leq 0.10$, and significance at $P \leq 0.05$.

RESULTS

Experimental Diets

Diets (Table 1) were similar in proximate composition (e.g. ash, NDF and EE), apart from the variation in CP concentrations between M and L diets (128 and 110 g/kg, respectively), which were associated with opposite variations in the NSC concentrations (424 and 444 g/kg, respectively).

In all the diets the PDIN was lower than PDIE and the PDIA concentration ranged from 49 and 54 % of the PDIN. The overall dietary PDI concentration (e.g. PDIN) varied from 82 to 87 g/kg DM in M diets and was 15 % lower in L diets (from 70 to 74 g/kg DM).

***In Vivo* Digestibility, Growth Performances and Carcass Traits**

The initial BW (Table 2) had an average value of 495 ± 58 Kg. The length of the fattening trial was 120 d and bulls were between 18 and 19 months old when slaughtered. The final BW was about 656 ± 56 kg, and the bulls showed a daily gain of 1.32 kg/d.

There were no significant interactions found between protein level and the inclusion of U in the diet for *in vivo* digestibility, growth performance and carcass characteristics of bulls. The level of PDI tended ($P<0.09$) to affect the average daily gain (**ADG**), with greater growth rates for animals, fed the M diets compared to those fed with L diets (1.37 vs. 1.28 kg/d). Given the similar intake between treatments, also the feed efficiency tended to be more favourable for M diets (0.147 vs. 0.137 kg / kg feed DM, $P<0.07$) than for L diets. No differences in apparent total tract digestibility were found between treatments, except for the protein fraction, which was lower for L diets (50 vs. 55%, $P<0.01$). There was a tendency ($P=0.08$) for lower faecal pH in U diets compared to those containing SBM. No significant effects on slaughter traits attributable to CP level, U addition, or their interaction were observed (Table 3). On average, carcasses had a hot weight of 360 kg and a dressing percentage of 54.8%. According to SEUROP classification, conformation and fat covering of the carcasses were 2.9 and 2.2, respectively, and no apparent dietary effects were found. No significant differences were detected among treatments in pH, WBSF, drip and cooking losses (Table 3). Among the meat colour parameters, only the brightness (L-value) had higher values ($P<0.05$) for L diets compared to M diets.

***In Vitro* Rumen Fermentation**

There was no significant interaction found between PDI levels and U inclusions in the diet for *in vitro* rumen fermentation characteristics. After the substrate administration to the fermentation bottles, there was an increase of ammonia concentration in the fermentation fluid, which peaked at about 1 h post-feeding (Fig. 1a). The two levels of CP determined a large difference in the ammonia concentrations ($P<0.01$) in the fermentation fluid, being about 2.0 mg/dL and about 4.0 to 5.0 mg/dL for the L and M diets, respectively.

As can be seen from Fig. 1b, the drop of pH at sampling T1 and T2 was less intense ($P<0.05$) for the diets containing U in comparison to the other two.

Total VFA concentrations were not significantly different among treatments at both sampling times (before feeding and after 2 h, Table 4) and also the total daily VFA yield, calculated as

average of the 2 measures (before feeding and after 2 h) and multiplied by the volume of the daily saliva output (1549-1642 ml/d) was not affected by the treatments. However, at T0 and T2 the molar percentage of butyrate increased when SBM was substituted with U (T0:11.9 vs. 14.3mol/100mol, P< 0.01; T2:12.1 vs. 14.3mol/100mol, P<0.01) and as well as with raising of the protein concentration from L to M (T0:12.5 vs. 13.6mol/100mol, P<0.10; T2:12.5 vs. 13.8mol/100mol, P<0.01).

The *in vitro* digestibility of DM and OM (Table 4) tended to increase with substituting SBM with U (P<0.10).

DISCUSSION

Experimental Diets

Diets were based on corn silage and corn meal (approx. 600 g/kg DM) and the composition in ingredients followed the beef feeding systems adopted in the Po valley plane (Cozzi, 2007; Gallo et al., 2014). The diets had a NDF concentration of about 300 g/Kg DM, which is typical for corn silage feeding (Felix et al., 2014; Schiavon et al., 2010; Valkeners et al., 2008) and greater than those of diets based on cereal meals (Ceconi et al., 2015a).

In the Italian intensive fattening systems based on corn silage diets, the dietary crude protein concentration of 140 to 150 g/kg of DM is commonly applied (single-phase feeding) to cattle of conventional breeds (Cozzi, 2007). However, feeding trials of Piemontese (Schiavon et al., 2010), non-double-muscle Belgian Blue (Boucqué et al., 1984) and double-muscle Belgian Blue bulls (De Campeneere et al., 1999) indicated that ration CP density of 120 g/kg should be sufficient for the BW range of 460 to 680 kg.

The rumen influx of urea trough the rumen wall and the saliva (Aschenbach et al., 2011) is an amount of degradable N, which has to be taken into account to accurately balance the rumen. INRA system (INRA, 2007) proposes to formulate diets with lower values of PDIN than PDIE to avoid any excess of degradable N at rumen level. The PDIN-PDIE differences in our diets ranged were about -6 and - 12 g/kg DM in M and L diets, respectively. Such a deficits of degradable N appear not high if one considers that Schiavon et al. (2010) utilised a low protein diet having a shortage of degradable protein of -18.6 g/kg DM. Moreover, the PDIN-PDIE of a diet approximates the difference between degradable N and microbial protein estimated on the

basis of the fermentable organic matter proposed by the Dutch system (named OEB) to estimate the rumen balance (CVB, 2000). In growing bulls, Valkeners et al., (2008) demonstrated that OEB deficit of about -10 g/kg DM could be the condition to reach the maximum N efficiency and to minimize the urinary excretions.

Finally, according to the INRA system (INRA, 2007), the acceptable deficit (for fattening animals younger than 2 years old) is from 1.0 to 1.7 g/MJ of net energy (NEmg) for maintenance and growth (INRA, 2007) and the deficits of diets in present trial were 0.7-0.9 and 1.3-1.7 g/MJ of NEmg, for M and L diets respectively.

***In Vivo* Digestibility, Growth Performances and Carcass Traits**

All animals exhibited excellent health conditions during the experiment and minimal feed residuals (up to 10%) were observed in the entire study. The bulls showed a satisfactory daily gain of 1.32 kg/d, which is similar to that obtained with German Simmental (1.37 g/d, Sami et al., 2004) fed diets based on corn silage in a comparable BW range. Hungarian Simmental bulls fed diets largely based on corn silage (more than 60% of the diet) showed lower growth rates in a 300 to 550 BW interval (1.15 g/d, Hollò et al., 2009). Gallo et al. (2014) in a recent survey in Italian fattening centre measured average growth capacity of Simmental bulls imported from Eastern European countries (32 batches and approximately 2000 heads) very similar to that of this trial (1.30 vs. 1.32 kg/d).

We were not able to demonstrate an expected improvement of digestibility due to a stimulating effect of urea on rumen digestion, as suggested by the trial of Ceconi et al. (2015a). However, other trials with bulls were also not able to show digestibility improvements due to U supplementation (Ceconi et al., 2015b; Valkeners et al., 2008; Zinn et al., 2003). The tendency for a lower faecal pH in the diets containing urea could be associated to a higher ammonia circulating in the blood fed U diets, which could have passed to the lower tract of the gut and have stimulated the fermentation.

The carcasses of IS were lighter than the preferred size of the Italian retail chains (approx. 400 kg), but the conformation and fat scores of carcasses were consistent with meat market needs (Gallo et al., 2014). The meat from animals fed low protein levels showed a greater brightness in

the analysis of colour, but this result appears not associated with other measured slaughter traits, and in literature, there is no evidence that dietary protein could affect this parameter.

Overall, the impact of the PDI reduction of about 15% in diets with respect to an assumed requirement of about 85 g of PDI/kg DM (equal to approx. 780 g/d of PDI, INRA, 2007) tended to decrease growth rate (90 g/d of ADG) but did not affect carcass and meat traits. This result was partially expected considering the data from Schiavon et al. (2010), who, in a study with a meat specialised breed (double-muscle Piemontese), compared diets with protein concentration similar to those of present trial and did not find an appreciable difference in performance at a BW greater than 450 kg.

***In Vitro* Rumen Fermentation**

Diets containing urea showed a greater ammonia concentration for both PDI levels, likely, due to the fact, that urea is much more rapidly hydrolysed into ammonia than true protein sources, such as SBM (Kang et al., 2015). A pick of ammonia release 1 h post-feeding was in consistence with some the *in vivo* trials (Ceconi et al., 2015a; Valkeners et al., 2008). Due to ammonia buffering capacity (Aschenbach et al., 2011) these variations in ammonia concentration affected the pH of the fermentation fluid.

The substitution of SBM with U tended to increase DM and OM digestibility and this is in accordance with the results of Meng et al. (2000), who reported a lower digestion for isolated soy protein than urea. Our *in vitro* results were in contrast with *in vivo* digestibility data, where no effects were found. However, it is likely that in the *in vivo* ammonia was released very fast and not progressively with the fermentation of starch, and the excess was absorbed through the rumen wall. In the continuous system, there was slow removal due to a continuous flow of effluent and therefore, ammonia stayed in the flask for longer periods (as shown in **Figure 1**) and could have stimulated the bacterial digestion.

The main effect observed from the *in vitro* rumen fermentation was the increased butyrate yield. In other studies the administration of different sources of protein in the diet (Mota et al., 2015), the substitution of protein from soy with urea (Meng et al., 2000) and an increment of the dietary protein in yearling heifers (Chumpawadee et al., 2009) increased the butyrate production in the rumen. This is probably due to a shift of the bacterial community consequent to the

different concentration of ammonia: in fact, some bacteria known to produce butyrate from fermentations (e.g. *Butyrivibrio fibrisolvens* and *Eubacterium ruminantium*) are positively influenced by the presence of high concentration of ammonia in the fermenting fluid (Bryant and Robinson, 1962; Hungate, 1966; Sales et al., 2000).

Brooks et al. (2012) tested in a continuous fermenter substrates composed of corn, soybean and urea in different proportions: although the mixtures were not isonitrogenous, those containing urea produced fermentation fluids more rich in butyrate (11.0 to 17.0 mol/100 mol) than those based only on corn or on corn and soybean (7.0 to 9.2 mol/100 mol).

Butyrate and valerate concentrations were increased by the addition of the *Megasphera elsdeni* both *in vitro* (Leeuw et al., 2016) and in feeding trials of bulls (Henning et al., 2010). We speculate that in our experiment the greater availability of ammonia could have stimulated the activity of this ruminal bacteria strain. Furthermore, if dietary urea and/or the high concentrations of rumen ammonia promote a fermentation oriented towards butyrate, this could have a positive impact to alleviate the conditions of rumen acidity. In fact, when one mole of hexose is fermented into acetate or propionate, two moles of fatty acids are obtained, while the fermentation to butyrate produces less acidity because only 1 mole of fatty acid is obtained (Aschenbach et al., 2011).

In addition, the butyric acid has properties different from propionate and acetate in terms of lipid bilayer permeability (i.e., butyric > propionic ≥ acetic acid) and a higher rate of intracellular metabolism (Aschenbach et al., 2011). These both properties indicate a great aptitude for butyric acid to be efficiently removed from rumen content through the rumen wall. Therefore, the reason because in some *in vivo* trials (Zinn et al., 2003) the dietary urea and/or the high concentrations of rumen ammonia were not able to demonstrate an accumulation of butyric acid in the rumen could be due to its high absorption trough the rumen wall.

CONCLUSION

Our results demonstrated that as low as 70-74 g of PDIN/ kg DM in diets, not limited by the rumen fermentable energy (e.g. negative difference PDIN-PDIE), together with the substitution of SBM with an isonitrogenous amount of U (0.5 % DM), had no detrimental effects on slaughter traits and *in vitro* rumen fermentation in finishing Italian Simmental bulls.

Nevertheless, a tendency to decrease in daily weight gain was observed for the lower protein concentration. The increase of butyrate in the *in vitro* fermentation fluid of fermenters having high ammonia levels requires further research efforts to identify a specific shift of the rumen bacterial community.

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Table 1. Ingredients, chemical composition and nutritional value of the experimental diets of finishing Italian Simmental bulls.

	Diet protein concentration (C) [1]			
	M		L	
	- U	+ U	- U	+ U
Ingredient composition, g/kg DM				
Corn grain, ground	269	307	312	350
Corn silage	308	308	308	308
Dried sugar beet pulp	89	89	89	89
Soybean meal	87	43	43	0
Wheat bran	77	77	77	77
Wheat straw	89	89	89	89
Hay	18	18	18	18
Vitamin and mineral mix[2]	53	53	53	53
Hydrogenated soybean oil	10	10	10	10
Urea	0	5	0	5
Chemical composition, g/kg of DM				
Crude protein	128	127	110	109
Ash	65	62	62	60
Neutral detergent fiber	339	336	337	336
Ether extract	33	35	35	35
Non-structural carbohydrates	422	426	442	446
Nutritional value [3]				
NEmg[4], MJ/kg DM	7.63	7.63	7.68	7.69
PDIA, g/kg DM	46	40	40	34
PDIN, g/kg DM	87	82	74	70
PDIE, g/kg DM	94	87	87	80

[1] Medium-protein (M) and low-protein (L) diets with urea (+U) or without urea (-U).
[2] Provided vitamins and mineral per 500 g/animal/day: vitamin A, 200,000 IU; vitamin D_3, 12,000 IU; vitamin E, 450 mg; choline chloride, 1000 mg; $FeCO_3$, 1076 mg; KI, 39 mg; $Ca(IO_3)_2$, 21.6 mg; Mn_2O_3, 1161 mg; $CuSO_4 \cdot 5H_2O$, 275 mg; ZnO, 620 mg; $ZnSO_4$, 2055 mg; Na_2SeO_3, 3.1 mg; Sacch. Cerevisiae MUCL 39885, 120 $*10^9$ CFU (Beef Supplement 0.5%; Consorzio Agrario, Udine, Italy.
[3] Values computed according to the INRA, 2007.
[4] Net energy for maintenance and growth.

Table 2. Effect of diets differing in protein concentration (low vs. medium) and nitrogen source (urea vs. soybean meal) on BW, growth performance and apparent digestibility of finishing Italian Simmental bulls.

	Diet protein concentration (C) [1]								
	M		L		P-value				RMSE[2]
	-U	+U	-U	+U	Block	C	U	C x U	
Number of pens, n	4	4	4	4					
Initial BW, kg	483	496	493	507	0.50	0.50	0.40	0.98	31
Final BW, kg	649	664	653	660	0.44	0.97	0.48	0.79	31
DMI[3], kg/d	9.32	9.28	9.35	9.32	0.02	0.17	0.17	0.99	0.05
ADG, kg/d	1.36	1.37	1.31	1.25	0.01	0.09	0.61	0.46	0.09
G:F, kg/kg of DM	0.146	0.148	0.140	0.134	0.01	0.07	0.68	0.43	0.010
Apparent total tract digestion, %									
DM	67.6	65.6	63.7	65.7	0.09	0.19	0.99	0.16	2.63
CP[4]	53.9	56.6	49.0	50.0	0.52	0.01	0.30	0.65	3.41
NDF[5]	35.9	30.4	27.2	32.6	0.02	0.34	0.99	0.12	6.49
Feces pH	6.81	6.69	6.81	6.74	0.01	0.63	0.08	0.66	0.10

[1]Medium-protein (M) and low-protein (L) diets with urea (+U) or without urea (-U).
[2] Root mean square error.
[3] Dry Matter Intake.
[4] Crude Protein.
[5] Neutral detergent fiber.

Table 3. Effect of diets differing in protein concentration (low vs. medium) and nitrogen source (urea vs. soybean meal) on slaughter traits and meat characteristics of finishing Italian Simmental bulls.

	Diet protein concentration (C) [1]								
	M		L		P-value				RMSE[2]
	-U	+U	-U	+U	Block	C	U	C x U	
Carcass									
Hot weight, kg	354	366	356	362	0.70	0.93	0.39	0.76	19.7
Dressing percent	54.5	55.1	54.6	54.8	0.01	0.82	0.35	0.75	0.9
SEUROP									
-conformation [3]	2.8	3.0	2.9	2.8	0.95	0.99	0.20	1.84	0.2
-fat score [4]	2.3	2.2	2.2	2.2	0.01	0.23	0.23	0.99	0.1
8^{th}-9^{th} rib section									
Lean meat, %	65.4	62.6	63.3	65.3	0.20	0.77	0.74	0.08	2.4
Separable fat, %	13.6	16.2	15.3	13.1	0.73	0.56	0.86	0.06	2.2
Bone, %	18.3	17.9	18.7	18.4	0.02	0.46	0.59	0.99	1.3
Other, %	1.7	1.9	1.3	2.4	0.37	0.98	0.03	0.11	0.5
Longissimus thoracis muscle									
pH	5.4	5.4	5.4	5.4	0.40	0.99	0.22	0.89	0.1
Color									
L^*	35.1	34.7	36.3	36.2	0.93	0.05	0.67	0.77	1.2
a^*	11.4	10.7	11.1	11.3	0.01	0.75	0.58	0.42	1.1
b^*	14.8	14.1	14.5	14.7	0.05	0.64	0.46	0.18	0.7
WBSF[5], N/cm^2	40.5	36.2	32.9	31.0	0.73	0.16	0.48	0.79	8.3
Drip loss, %	2.9	2.9	3.0	2.9	0.01	0.37	0.57	0.88	0.2
Cooking loss, %	23.8	23.6	25.1	24.8	0.01	0.18	0.77	0.99	1.7

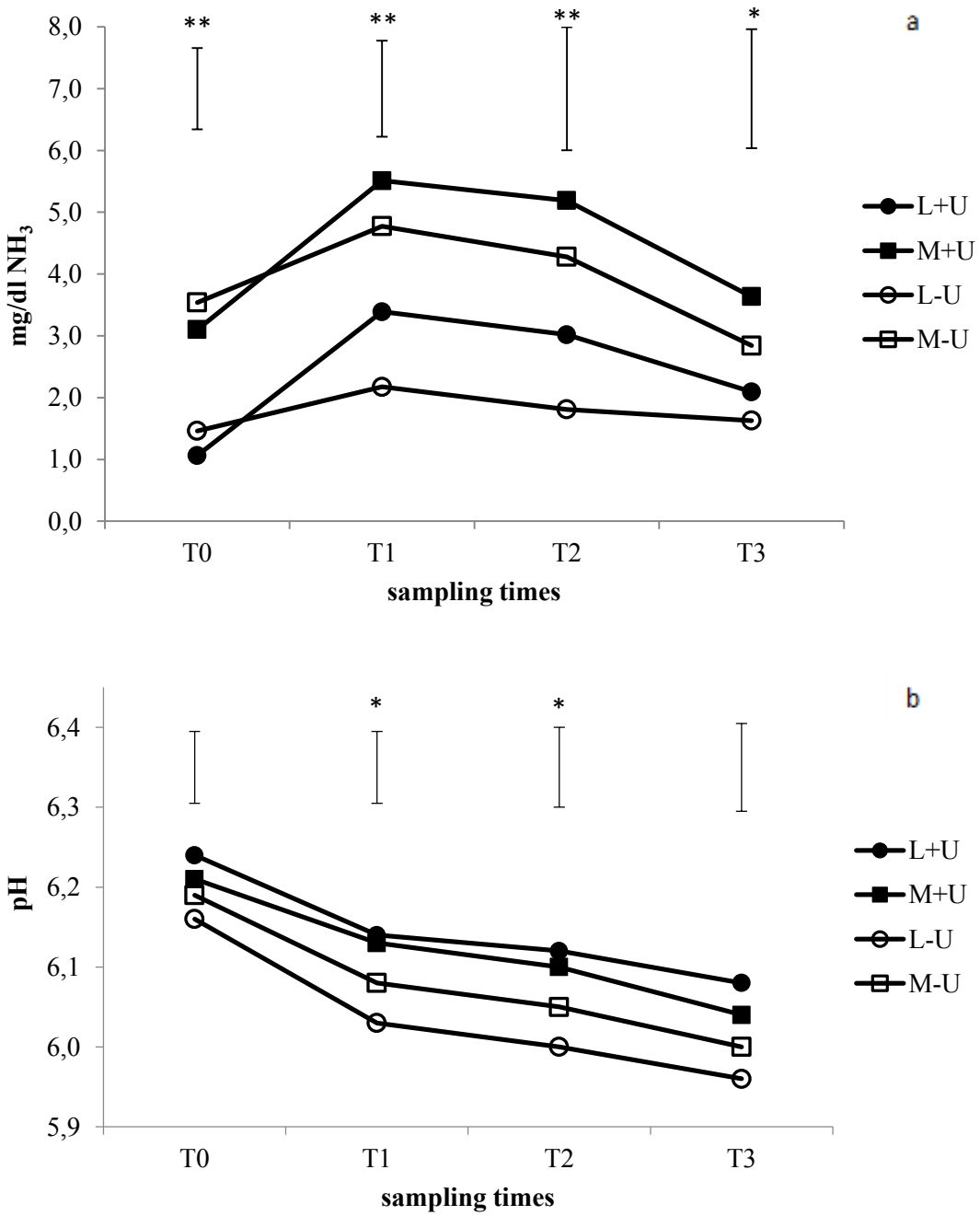
[1] Medium-protein (M) and low-protein (L) diets with urea (+U) or without urea (-U).
[2] Root mean square error.
[3] S=5 (superior), E=4, U=3,…, P=0 (poor).
[4] Class 5=5 (very fat), …, class 1=1 (very lean).
[5] Warner-Bratzler shear force.

Table 4. Effect of diets differing in protein concentration (low vs. medium) and nitrogen source (urea vs. soybean meal) on *in vitro* rumen fermentation characteristics of a continuous fermenter inoculated with rumen collected at slaughter from finishing Italian Simmental bulls.

	Diet protein concentration (C) [1]								
	M		L		P-value				RMSE[2]
	-U	+U	-U	+U	Block	C	U	C x U	
saliva flow, ml/d	1549	1618	1576	1642	0.29	0.66	0.25	0.98	138
VFA, mM/d	94.2	97.2	92.3	91.0	0.29	0.32	0.83	0.60	9.7
before feeding (T0)									
Total VFA, mM	57.9	57.7	58.4	52.5	0.48	0.29	0.17	0.20	5.3
Individual, mol/100mol									
acetate (a)	54.9	53.9	55.8	56.4	0.61	0.18	0.87	0.53	3.1
propionate (p)	27.0	25.5	28.7	25.3	0.11	0.61	0.11	0.52	3.5
butyrate (b)	12.5	14.7	11.2	13.8	0.30	0.08	0.01	0.72	1.4
isobutyrate	0.94	0.90	0.98	0.95	0.02	0.55	0.60	0.95	0.17
isovalerate	3.55	3.84	2.97	3.44	0.02	0.09	0.17	0.75	0.66
valerate	1.12	1.13	0.33	0.00	0.04	0.15	0.80	0.79	1.57
a:p	2.08	2.18	1.99	2.23	0.20	0.89	0.25	0.61	0.35
(a+b)/p	2.56	2.77	2.39	2.78	0.23	0.68	0.11	0.62	0.43
after feeding (T2)									
Total VFA, mM	63.5	62.8	59.0	58.8	0.03	0.09	0.84	0.92	5.8
Individual, mol/100mol									
acetate (a)	54.7	54.4	56.4	55.2	0.54	0.31	0.52	0.72	2.9
propionate (p)	27.5	25.9	28.6	26.1	0.40	0.66	0.16	0.74	3.4
butyrate (b)	12.8	14.9	11.4	13.6	0.79	0.01	0.01	0.84	1.1
isobutyrate	1.15	0.95	0.84	0.99	0.01	0.17	0.80	0.09	0.23
isovalerate	3.64	3.81	2.81	3.81	0.01	0.15	0.05	0.15	0.67
valerate	0.18	0.00	0.00	0.28	0.15	0.76	0.76	0.16	0.38
a:p	2.04	2.14	2.01	2.12	0.44	0.83	0.46	0.98	0.34
(a+b)/p	2.52	2.73	2.42	2.64	0.49	0.59	0.23	0.96	0.42
Ruminal digestion, %									
DM	45.9	47.5	46.4	50.2	0.01	0.26	0.06	0.41	3.3
OM	49.6	51.2	50.1	53.8	0.01	0.28	0.07	0.43	3.4

[1]Medium-protein (M) and low-protein (L) diets with urea (+U) or without urea (-U).
[2] Root mean square error.

Figure 1. Values of ammonia concentrations and pH in the fermentation liquid of the fermenter (graph a and b, respectively) measured immediately before the feed administration (T=0) and after 1-hour intervals for 3 hours (T1, T2 and T3). Dietary treatments are coded as L+U: low protein diet with urea (●); M+U: medium protein diet with urea (■); L-U: low protein diet without urea (○); M-U: medium protein diet without urea (□). Data were statistically analysed with the model described in material and methods (vertical bars in both graphs are the residual mean square errors) and there was significant effects (*: P<0.05; ** P<0.01) of the protein level on ammonia concentrations (graph a) and of the presence/absence of urea on pH (graph b).



APPENDIX 1. Microbial community structure and VFA profile in the rumen liquids from a continuous culture fermenter and *in vivo*

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Continuous culture fermenters (**CCF**) are often used to study rumen fermentation *in vitro* as an alternative to *in vivo* trials. Here we have compared the characteristics of the fermentation liquid (**FL**) from a CCF with the correspondent rumen inoculum (**RI**). The RI was collected at the slaughterhouse from 24 bulls (8 animals in each of three separate sessions) and used as inoculum for the CCF. The diet fed to bulls was used as a substrate for the fermenters and FL samples were collected on day 6, 7 and 8 of fermentation. RI and FL samples were analyzed for VFA concentration and 16S rDNA amplicon sequencing was used to characterize the bacterial population. Total VFA concentration was lower in FL compared to RI (56.6 vs. 114.6 mM, $P<0.01$). Propionate, butyrate and isovalerate were higher ($P<0.01$, 0.05 and 0.01, respectively) in the FL compared to the RI (26.6 vs. 19.2, 13.1 vs. 11.3 and 3.45 vs. 1.38 mol/100mol, respectively), while acetate was lower (55.3 vs. 66.6 mol/100mol, $P<0.01$). Variability in VFA content was estimated by the error (E) from a statistical analysis, which considered the type of fluid within the session. The molar percentage of propionate, isobutyric, butyric, isovaleric and total VFA in the FL had E values (13, 18, 11, 19 and 9 %, respectively) lower than for RI (17, 25, 16, 45 and 15 %, respectively), while similar values were obtained for acetate (5%). Ion Torrent 16S rDNA amplicon sequencing generated 2.57M high-quality sequences clustered into 4,918 unique OTU's with 11,199 sequences per sample after normalization. At the Phylum level, the *Firmicutes* did not vary between fluids, averaging 25.1%, *Bacteroidetes* decreased by about

20% in the FL (45.0 vs. 55.3%, P<0.01), whereas, *Proteobacteria*, *Tenericutes* and *Spirochaetes* increased in FL compared to RI (11.3 vs. 7.6%, P<0.05; 2.7 vs. 0.8 P<0.05; 11.8 vs. 5.0%, P<0.01, respectively). There was no effect on *Fibrobacteres* (averaging 2.1%). Both the Shannons and Simpson index of diversity was lower in FL than in RI (4.90 vs. 5.63, and 0.973 v 0.984, P<0.01, respectively) as was the Chao1 estimate of total species richness (1268 vs. 1965, P<0.01). Although, the differences in VFA concentrations and microbial populations between RI and FL were statistically significant, the studied CCF environment favored maintenance and growth of the major bacteria phyla found in RI.

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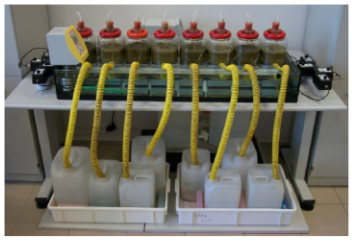
Introduction

Continuous culture fermenters (CCF) are used to study rumen fermentation *in vitro* as an alternative to *in vivo* trials. The capability to simulate well the rumen conditions is strictly connected to the maintenance of the initial rumen microbiota. According to the literature, the *in vitro* conditions affect the rumen microbial communities both in density and in structure, as well as the fermentation parameters (Muetzel et al., 2009; Soto et al., 2012; Mason et al., 2015). Here we have compared the bacteria population and VFA concentration of the fermentation liquid (FL) from a CCF with the correspondent rumen inoculum (RI).

Materials and methods

- The RI was collected at the slaughterhouse from 24 bulls (8 animals in each of three separate sessions) and used as inoculum for the CCF.
- The diet fed to bulls was used as a substrate for the fermenters and FL samples were collected on day 6, 7 and 8 of fermentation (Spanghero et al., 2017).
- RI and FL samples were analyzed for VFA concentration and 16S rDNA amplicon sequencing was used to characterize the bacterial population.
- Variability in VFA content was estimated by the residual error from a statistical analysis, which considered the type of fluid within the session.



VFA profile of the fermentation fluid and rumen inoculum

	FL	RI	Sig.[1]	RMSE[2]
n.	24	24		
Total VFA, mM	56.6	114.6	**	13.9
Individual VFA, mM/100mM				
Acetate	55.3	66.6	**	2.17
Propionate	26.6	19.2	**	3.50
Isobutyrate	0.94	0.88	ns	0.19
Butyrate	13.1	11.6	*	1.96
Isovalerate	3.45	1.38	**	0.78
Valerate	0.64	0.30	ns	1.18
A:P	2.1	3.6	**	0.52

[1] Significance (** P<0,01; * P<0,05)
[2] Root mean square error

Index of diversity and of richness of the bacteria community

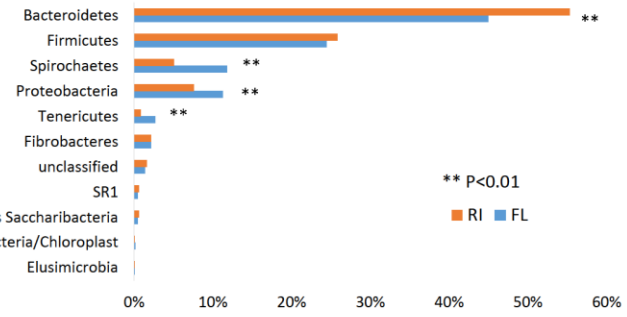
	FL	RI	Sig.[1]	RMSE[2]
Simpson	0.973	0.984	**	0.003
Shannon	4.90	5.63	**	0.137
Chao1	1268.05	1965.59	**	131.123

[1] Significance (** P<0.01; * P<0.05)
[2] Root mean square error

Results

- FL had lower VFA concentration than RI.
- Propionate, butyrate and isovalerate proportions were higher in FL compared to RI, while acetate was lower.
- Ion Torrent 16s rDNA amplicon sequencing generated 2,57M high-quality sequences clustered into 4,918 unique OUT's with 11,199 sequences per sample after normalization.
- The *in vitro* environment influenced the composition of the rumen bacteria community, with higher species diversity and richness in RI than FL and with different structure measured at Phylum level.

Bacteria community structure at Phylum level



Conclusions

Although the differences in VFA concentrations and microbial population of RI and FL were statistically significant, the *in vitro* environment of the CCF allowed the maintenance of the major bacteria phyla found in RI.

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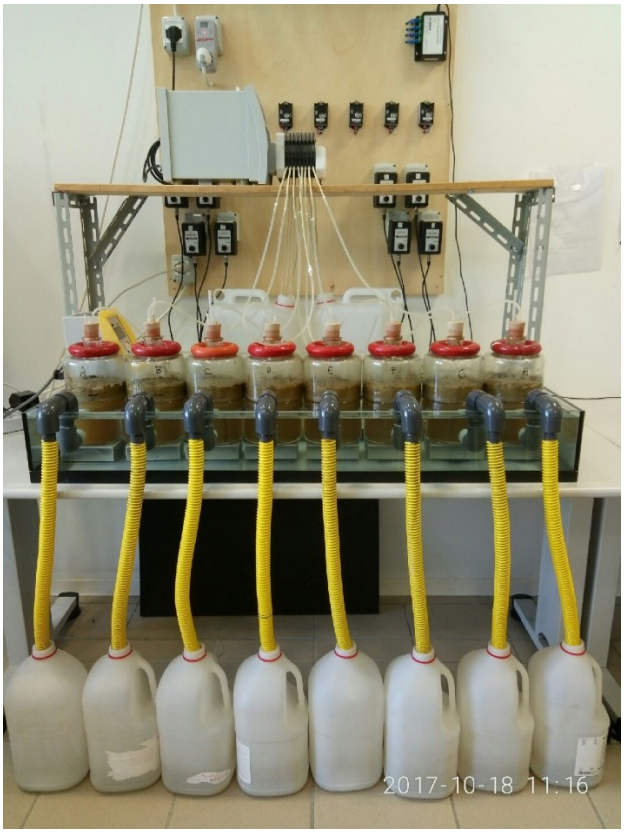
Picture 1. Italian Simmental Finishing bulls (Exp.1)

Picture 2. Continuous-culture fermenters (**Chapter 2**).



In vitro ammonia release and rumen fermentation characteristics of urea-treated high moisture barley and corn grain.

(Considered to be acceptable after major revision in Journal of Animal and Feed Sciences)

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CHAPTER 3. *In vitro* ammonia release and rumen fermentation characteristics of urea-treated high moisture barley and corn grain.

ABSTRACT

The aim of the present study was to evaluate high-moisture barley and corn kernels treated with urea (**UT**) in terms of rumen *in vitro* nitrogen (**N**) release and fermentability. Untreated samples (**CTR**) of barley and corn grains were compared with UT and with the CTR samples added with urea just before the experiment (**UA**). In Exp. 1, ground CTR, UT and UA samples were incubated during two fermentation runs in fermentation flasks containing 200 ml of diluted rumen fluid, which was sampled at 0, 2, 4, 6 and 8 h for ammonia analysis. Ammonia N concentration peaked at 4 h of fermentation (10.24 vs 9.01 and 7.20 mg/dl, respectively at 0 and 8 h, $P<0.01$), and the UT treatment released less ammonia than the UA treatment (9.76 vs 10.52 mg/dl, $P<0.05$), while the CTR samples showed the least ammonia N concentrations ($P<0.01$). A second Exp. examined the N release from CTR and UT samples prepared in three physical forms (whole grain, coarse ground, and meal). During two incubation runs, samples were weighed into fermentation flasks (100 ml) with 25 ml of distilled water and placed in a shaking water bath (25 °C) for five incubation periods (1, 2, 4, 6 or 8 h). At the end of each incubation period, the N was measured in the filtered residue to calculate N solubility. The UT samples have solubilized more N compared to the CTR in the milled form (43.7 vs 15.3%) than in the whole form (20.3 vs 9.2%), with intermediate values for the coarse form (32.4 vs 14.0 %, treatment × physical form of grain interaction, $P<0.01$). The third experiment was composed of two gas production trials: initial 96 h gas test and a halftime gas test. In initial 96 h gas-test (2 runs) meal samples (500 mg) of CTR, UT and UA grains were incubated, in triplicate in 100 ml calibrated glass syringes and gas recorded at 0, 1, 2, 4, 6, 8, 16, 18, 20, 24, 28, 30, 48, 54, 60, 72 and 96 h of incubation. In both kinds of cereal, the extent of gas production resulted higher for corn compared with barley (389 vs 371 ml/g of incubated DM, respectively; $P<0.01$). Moreover, CTR and UT samples showed a higher ($P<0.05$) gas volume (386 and 384 ml/g of incubated DM, respectively) than the UA samples (371 ml/g of incubated DM). A significant interaction between cereal type and the feed treatment was observed for the rate of gas production, which was found to be significantly higher ($P<0.05$) for UT in barley and did not differ between corn samples. Corn had a halftime of

gas production shorter than barley (8.78 *vs* 9.39 h, respectively; P<0.01) and, between treatments, the UT samples (8.70 h on average) had the shortest halftime value (P<0.01). Halftime gas test (3 runs) trial followed the same procedure with some modifications. Samples were incubated in quadruplicates, incubations were stopped at the substrate-specific halftime and microbial N was determined. In barley, UT and CTR produced more gas than UA, while in corn, UA and UT samples produced less comparing to CTR (P<0.05 or P<0.01). In both kinds of cereal, microbial N was greater in UT than in CTR and UA samples (P<0.01). Results of this study confirmed the slow-ammonia release effect of the UT cereals, reduced by the disruption and milling of the sample. Although cereals had a different pattern of fermentation during the first fermentation phase, the urea treatment stimulated microbial N synthesis in both kinds of cereal.

Keywords: urea; grain; microbial nitrogen; *in vitro* techniques; rumen; ammonia.

INTRODUCTION

Cereal grains and meals are important dietary ingredients for high producing ruminant animals. They are rich in starch and increase the energy density of diets, but due to an insufficient protein content, they need to be supplemented with nitrogen (**N**) sources, mainly protein vegetable meals but also non-protein products, such as urea.

A conservation method of high-moisture cereal kernels is based on a treatment with urea at harvesting (e.g. 15 g per kg of kernels having approx. 20% of the moisture). In presence of urease and moisture, ammonia produced by the hydrolysis of the urea penetrates the kernels, binds with the vegetal matrix and preserves wet grains. Overall, this treatment increases the level of crude protein in treated grains up to seven percentage points (Humer and Zebeli, 2017).

The treatment is known for a long time (Ørskov et al., 1979; Ørskov and Greenhalgh, 1977) and, the preservation effect of ammonia on wet grains is well documented both by scientific papers (Mowat et al., 1981) and technical advertisements (Auerbach, 2016; Mickan, 2009). Nutrient utilization of such cereals has also been repeatedly evaluated by the researchers. A positive effect of ammonia treatment on cereal fiber and starch digestibility was demonstrated by Rode at al. (1986). Humer and Zebeli (2017) recently reviewed feeding and metabolism trials

with dairy and beef cattle concerning ammoniated cereals fed to ruminants. These authors found variable results in terms of animal performance (growth rate and milk yield) and apparent total tract digestibility but scarce information was available about the modifications in the rumen utilization due to the treatment.

In the present paper, it has been hypothesized that the rumen release of ammonia from the treated grain matrix could be slower than from urea, added to the grain before feeding. This would allow considering urea treatment as not only a simple way to preserve wet cereals but also a method to add N slowly released into the rumen, with beneficial effects on the rumen N utilization and microbial growth.

The paper is based on three *in vitro* experiments conducted to evaluate the dynamic of ammonia release and the fermentation characteristics of high moisture whole barley (*Hordeum vulgare* L.) and corn (*Zea mays subsp. mays*) grains treated with a commercial product containing urea and ureases.

MATERIALS AND METHODS

Rumen *in vitro* batch culture incubation (Exp. 1), water solubility test (Exp. 2) and two gas production tests (Exp. 3) were held at the Department of Agricultural, Food, Environmental and Animal Sciences (University of Udine, Italy) from February 2016 to April 2017.

Treatments

In this study, conventional barley grain and corn grain were used as a controls (**CTR**), while the corresponding urea-treated grains (**UT**) were produced in laboratory according to the following procedure: whole untreated grains were mixed with feed-grade urea (46% of N), a mixture of additives containing enzyme urease (activity 5000 pgN/g/min) and water (14, 5, and 56 g/kg, respectively). The UT cereals were kept for 21 days in anaerobic conditions at the room temperature (22 °C) to allow urea be transformed into ammonia and penetrate inside the whole kernels. Further, all treated grains were sealed and stored in airtight bags during the experimental period at 4 °C. Finally, urea-added (**UA**) treatment was also tested, where feed-grade urea was added to the untreated grains or meals just before the incubations in the amounts corresponding to the UT treatment. In both experiments, the same samples obtained by the same original batch were utilized.

Batch culture incubation (Exp.1) to test the release of ammonia from the grain matrix

For each cereal (barley and corn), CTR, UT and UA samples were dried and milled to 1 mm (Pulverisette; Fritsch, Idar-Oberstein, Germany) and used in three subsequent fermentation runs, following the method of Cooke et al. (2009).

During each fermentation run, samples were incubated in duplicate in 12 Erlenmeyer flasks (250 ml) containing 200 ml mix of rumen fluid and a nutrient solution (1:4 ratio, v/v) and maintained at 39 °C in a water bath (**Picture 3)** for 24 h. Nutrient solution contained bicarbonate-mineral-distilled water mixture (1:1:2). Rumen fluid for *in vitro* experiments was obtained from a slaughterhouse from Dairy cattle and was immediately delivered to the laboratory in airtight glass bottles refluxed with CO_2 and immersed in thermoses filled with 39 °C water. In the laboratory, rumen fluid was strained through 1mm wire mesh filter and added to the nutrient solution under constant infusion of CO_2.

The amount of substrate was weighed basing on same the N content resulting in 49 mg of N for barley and 53 mg of N for corn treatments on dry matter (**DM**) basis. At each sampling time (0, 0.5, 2, 4, 6 and 24 h) the pH of the fermentation fluid was measured and 10 ml samples of fermentation fluid from each flask were collected for ammonia nitrogen (**NH_3-N**) determination (samples acidified with 0.4 ml of H_2SO_4 9 *N*).

N solubility in water (Exp. 2) to test the release of ammonia from the grain matrix

The trial was conducted according to Căpriţă et al. (2010) with some modifications. For each cereal (barley and corn), CTR and UT samples were prepared in three physical forms: whole grain (**whole**), coarsely milled (**coarse**) with a coffee mill to approximately 1/6 of the whole kernel, and milled to 1 mm (**milled**), providing 12 treatments in total. Samples were weighed (500 mg of DM) in duplicates into Erlenmeyer flasks (100 ml) and incubated with 25 ml of distilled water in a shaking (120 rpm) water bath (**Picture 3)** at 25 °C for five incubation periods (1, 2, 4, 6 or 8 h). At the end of each incubation period, the contents of the flask were filtered through ash-free filters (Whatman n. 541, 20 μm of porosity), filtrates were discarded and the filters with the residues were dried at 60 °C overnight and then analysed for N content. The study was replicated in two runs.

***In vitro* gas production tests with concomitant microbial mass measurement (Exp. 3)**

Initial 96 h and a halftime gas production tests were conducted according to the procedure described by Menke et al. (1979).

Initial 96 h gas test (2 runs) was performed to determine the substrate-specific halftime (**$t_{½}$**) corresponding to the half of the asymptotic gas volume produced, which is considered to be a time close to the maximum microbial efficiency (Makkar, 2004). Samples and procedure of rumen fluid collection were those of Exp. 1. Milled samples (500 mg) of CTR, UT and UA barley and corn grains were incubated with 40 ml of incubation medium in triplicate in 100 ml calibrated glass syringes. Incubation medium contained 10 ml of the rumen fluid and a double strength buffer (30 ml of bicarbonate-mineral-distilled water mixture, 2:1:3) according to Blümmel and Becker (1997). Syringes were placed vertically in a water bath at 39 °C (**Picture 4**) and three syringes without substrate were used as blanks. Gas production was recorded at 0, 1, 2, 4, 6, 8, 16, 18, 20, 24, 28, 30, 48, 54, 60, 72 and 96 h of incubation. Volumes of gas corrected for blanks were fitted to the exponential model proposed by Ørskov and McDonald (1979) without a lag phase as:

$y = B \cdot (1 - \exp^{-k \cdot t})$,

where 'y' is the cumulative gas volume produced at time 't'[h],'B'[ml] is the asymptotic gas volume, 'k'[ml/h] is a rate constant. The halftime of gas production for each substrate incubated was calculated using the following formula:

$t_{½} = \ln 2/k$.

A halftime gas test (3 runs) followed the same procedure as described for the 96 h incubations but was stopped at $t_{½}$ (9.5 h for barley and 9 h for corn) to obtain degradability and microbial N production measures. Four syringes for each substrate and 4 blanks were used providing at the end of the fermentation two samples for apparent substrate degradability (**ASD**) and two for true substrate degradability (**TSD**) estimation. Two 40 ml samples of the incubation medium at the beginning of the incubation were collected and considered as blanks att_0.

For ASD at the $t_{½}$, the contents of 2 syringes for each substrate and of two blanks were centrifuged at 20,000 × g for 10 min at 20 C (Blümmel et al. 1997). Pellet was recovered, freeze-dried, and ASD was calculated after correction for the blanks. Further, pellet residues were analysed for N (pellet N at $t_{½}$). Two 40 ml samples of incubation medium collected at t_0 undergone the same centrifugation procedure and were also analysed for N (pellet N at t_0). The

supernatant was sampled for NH_3-N (5 ml-samples acidified with 0.5 ml of H_2SO_4 1.0 *N*) and volatile fatty acids (VFA) analyses (5 ml-samples acidified with 5 ml of H_2SO_4 0.1 *N*) at $t_{½}$.

The TSD was determined according to Goering and Van Soest (1970) with some modifications. The contents from two syringes (for each treatment) and two blanks were filtered through the Ankom bags (F57 filter bags of 25 μm porosity; ANKOM Technology Corp.), dried at 60 °C overnight, sealed, and refluxed in an Ankom fiber analyser with neutral-detergent solution prepared without sodium sulphite for 1 hour in order to detach the microbial biomass from the undegraded substrate. Then, bags were dried at the 105 °C overnight and TSD was calculated after correction for blanks. For truly undegraded N (**NDFN** at $t_{½}$), bags' residue further proceeded for N.

Microbial N was determined by N balance approach (Getachew et al. 2000) with the following equation:

microbial N production at $t_{½.}$ = pellet N at $t_{½}$ – pellet N at t_0 – NDFN at $t_{½}$.

Chemical analysis

The pH measurements were performed using a glass electrode connected to a pH meter (GPL 22, Crison Instruments, S.P.A., Barcelona, Spain). In particular, the pH of the cereals was measured according to Martillotti and Puppo (1985): 10g of substrate pre-dried (at 60 °C overnight in a forced-air oven) and milled to 1 mm (Pulverisette; Fritsch, Idar-Oberstein, Germany) were mixed with 100 ml of distilled water. The N content of the substrates, of the dry residues of Exp.2 and on the freeze-dried pellets and dried bag residues of Exp.3, was determined by the Kjeldahl method (method 976.05; AOAC, 2000). Further, DM determination was performed on cereals samples, and pellet residues of the Exp.3 drying at 105 C for 3 h for (method 930.15; AOAC, 2000); ash concentration was determined on incinerated samples at 550 °C for 2 h (method 942.05; AOAC, 2000).

Samples for NH_3-N determination were centrifuged and analysed spectrophotometrically using a modified Berthelot reaction method (Krom, 1980) with the continuous flow SAN++ analyser (Skalar Analytical B.V., Breda, Netherlands). For VFA analysis of the fermentation fluids of Exp.3, aliquots of the supernatants obtained after centrifugation were filtered using polypore 0.45 μm filters (Alltech Italia, Milan, Italy). The filtrate was injected into a high-

performance liquid chromatography instrument (Perkin-Elmer, Norwalk, CN, USA), set to 220 nm according to the method described by Martillotti and Puppo (1985).

Statistical Analysis

The analytical determinations of feeds and data from experiments were statistically analysed as factorial designs with repeated measures (Exp. 1) and as factorial designs (analytical data and Exp. 1 and 3), according to the following models:

Analytical data: $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$,

Exp. 1: $Y_{ijkm} = \mu + \alpha_i + \beta_j + \gamma_k + \zeta_m + (\alpha\beta)_{ij} + (\alpha\zeta)_{im} + (\beta\zeta)_{jm} + (\alpha\beta\zeta)_{ijm} + \varepsilon_{ijkm,}$

Exp. 2: $Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + \zeta_m + (\alpha\beta)_{ij} + (\alpha\delta)_{il} + (\alpha\zeta)_{im} + (\beta\zeta)_{jm} + (\beta\delta)_{jl} + (\delta\zeta)_{lm} + (\alpha\beta\delta)_{ijl} + (\alpha\beta\zeta)_{ijm} + (\delta\beta\zeta)_{lim} + \varepsilon_{ijklm,}$

Exp. 3: $Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + \varepsilon_{ijk,}$

where: y is the measure of the experimental unit (single analytical determination, flask or syringe), μ is the overall mean, α_i is the fixed effect of the substrate type (CTR, UT, UA, i=1,3, in Exp. 1 and 3; CTR, UT, i=1,2, in Exp. 2); β_j is the fixed effect of the cereal type (barley, corn, j=1,2); γ_k is the random effect of the fermentation run (block, k=1, 3 or 1, 2 according to the experiments); δ_l is the fixed effect of the physical form (whole, coarse, milled, l=1,3); ζ_m is the fixed effect of sampling time (0, 2, 4, 6, and 8 h or 1, 2, 4, 6, and 8 h of fermentation, in Exp. 1 and 2 respectively, m=1,5) and ε_{ijk} is the residual error.

For all statistical analyses, significance was declared at $P \leq 0.05$ and at $P \leq 0.01$.

RESULTS

Treatments

A visual effect of treatment was browning of the pericarp both in barley and corn (**Picture 5**). Chemical composition and pH of barley and corn kernel samples are shown in **Table 1**. The interaction between cereal and treatment was found to be significant ($P<0.05$) for the DM content, with a higher difference between CTR and UT in corn than in barley. Ash (21.5 *vs* 12.0 g/kg DM, for barley and corn respectively) and crude protein (**CP**) contents (117.5 *vs* 90 g/kg DM, for barley and corn respectively) were affected by the type of the cereal ($P<0.01$); furthermore, the treatment with urea increased the CP content of about three percentage points in

both kinds of cereal (P<0.01). The urea treatment increased the cereal pH of 2.6 and 2.1 in whole kernels and in the meal of barley and of 1.7 and 2.5 in whole kernels and in the meal of corn (P<0.01 for the interaction feed treatment* cereal type).

Batch culture incubation (Exp.1) to test the release of ammonia from the grain matrix

The ammonia N concentrations and the pH of the fermentation fluid at different incubation times for feed treatments and cereals are shown in **Figure 1**. For the ammonia N, there was a significant effect of incubation time and feed treatment (P<0.01) and no significant interactions. During incubation, ammonia N concentrations showed a curvilinear pattern with a peak value at 4 h, higher than that at 0 and 8 h of fermentation (10.24 vs 9.01 and 7.20 mg/dl, respectively, P<0.01). The CTR samples produced the lowest ammonia N concentrations (P<0.01), while the UT treatment determined (on average among incubation times) a lower yield of ammonia than the UA treatment (9.76 vs 10.52 mg/dl, P<0.05).

For the pH of fermentation fluid, there was a significant interaction of the incubation time with the feed treatment and type of cereal (P<0.01). As can be seen from **Figure 1**, the interactions are mainly due to a non-perfectly linear decline between levels of treatments. In fact, the pH between cereals was not statistically different at 0 and 8 h and different at 2 and 4 h, while for the feed treatments values were not different at 0 h, and for the others incubation times the CTR samples had the lowest pH (P<0.01).

N solubility in water (Exp. 2) to test the release of ammonia from the grain matrix

The statistical model of Exp. 2 considered several factors (cereal type, feed treatment, physical form, incubation time) and significant effects were obtained only for the interaction between feed treatment and physical form (P<0.01) and for sampling time (P<0.01). As can be seen from **Figure 2**, the difference between treated vs. control samples was low in the whole (20.3 vs 9.2%), intermediate in coarse (32.4 vs 14.0 %) and reached the highest value for the milled form (43.7 vs 15.3%). Solubility measured after 1 h (18.9%) increased (P<0.05) to a value of 22.1% at 2h and then further increased (P<0.01) to values between 23.6 and 24.1% at longer times.

***In vitro* gas production test with concomitant microbial mass measurement (Exp. 3)**

In **Table 2**, the mean values obtained by the combination of feed treatment and cereal type are presented because the interaction between these factors resulted statistically significant for several variables.

The extent of gas production resulted higher for corn compared with barley (389 vs. 371 ml/g of incubated DM, respectively; $P<0.01$). Moreover, CTR and UT samples showed a higher ($P<0.05$) gas volume (386 and 384 ml/g of incubated DM, respectively) than the UA samples (371 ml/g of incubated DM). A significant interaction between cereal type and the feed treatment ($P<0.05$) was observed for the rate of gas production, which was found to be significantly higher for UT in barley (0.084 vs. 0.073 ml/h, UT vs. CTR and UA, respectively) and did not differ between treatments in corn (0.081 ml/h). Corn had a halftime of gas production shorter than barley (8.78 vs. 9.39 h, respectively; $P<0.01$) and, between treatments, the UT samples (8.70 h on average) had the shortest ($P<0.01$) halftime. During the halftime incubation, the interaction between treatment and cereal type was found to be significant for gas production at all readings, except the first hour (2 h: $P<0.05$; 4, 6 and $t_{½}$; $P<0.01$). At each time point, UA barley syringes produced less gas than UT and CTR, while from 2 h to $t_{½}$ corn CTR syringes produced more gas than UA and UT. The ASD of barley was lower for UT compared to CT and UA samples, while for corn showed a lower value for CTR compared to UT and UA samples. Although the TSD was not affected by the treatment, it had higher values for corn than for barley (87.7 vs. 79.8%, $P<0.05$).The highest microbial N production was found for UT samples of both kinds of cereal ($P<0.01$).

Total VFA amount and the molar concentrations of the individual VFA produced were not affected by the treatment (**Table 3**), with the exception for the butyrate concentration, which was lower in UT samples in both kinds of cereal ($P<0.01$). Overall, barley samples produced more acetate ($P<0.05$) and less propionate ($P<0.01$) than corn, with a consequent higher acetate to propionate ratio ($P<0.01$).

Ammonia N concentration did not significantly differ between UT and UA treatments and was higher than CTR ($P<0.01$).Barley samples produced more NH_3-N than corn ($P<0.01$).

DISCUSSION

A visual darkening of the pericarp in barley and corn after urea-treatment is likely due to pigment substances obtained by non-enzymatic reactions of sugars with amines groups (Srivastava and Mowat, 1980).

The first nutritional effect of urea treatment of the cereals was the increase in CP (around of 3.2-3.3 percentage points) which was in the middle of the range reported in the review of Humer and Zebeli (2017). Calculated recovery of the urea-N added with the treatment was around 74-77%, the remaining probably being lost by volatilization.

The pH measured after solubilizing in distilled water increased more than two points, changing from sub-acid (6.3-6.6) to alkaline conditions (8.2-8.7). The measurement conducted on whole kernels gave comparable results in both kinds of cereals, but the milling determined a reduction of pH in barley and a small increase in corn, which is probably due to differences in mineral and organic matter composition of the cereals.

In Exp.1, we monitored the ammonia content in fermentation fluid to detect differences between different samples. Cooke et al. (2009) used this technique and were able to obtain a progressive accumulation of ammonia using substrates very high in N (e.g. soya bean meal and urea). Comparing to the study of Cooke et al. (2009), in our experiment, the concentration of ammonia in fermentation fluids was lower given the low N content of our substrates and was almost stable within the first hours of incubation (till 6 h, with a weak peak at 4 h). During the first hours of incubation, there was probably an equilibrium between the ammonia release and its capture for the microbial protein synthesis. In the last part of fermentation, the concentration declined due to a shortage of nitrogenous compounds with respect to the requirements of biosynthesis. Basing on the reports of some authors (Mowat et al., 1981; Srivastava and Mowat, 1980) about the ammonia interaction with the grain constituents, we've hypothesized that ammonia captured within a cellular matrix might be released slower than free ammonia added to the diet per se as urea. Our hypothesis was proven by the fermentation trial because the ammonia N concentration in UT fermentation fluids was lower than measured in UA samples.

However, these results should be considered with some caution because in samples low in N content and high in fermentable energy (such as cereals added with urea) it is possible that the different kinetics of ammonia release is masked by the microbial capture for growth. On the contrary, such a limitation could be of limited importance in samples having high N

concentrations (e.g. extracted protein meals or urea-based products) where the ammonia is in great excess with respect to microbial uptake.

Then, in Exp. 2 we proceeded to perform a solubility test to evaluate also a possible effect of the physical form of the grains on the rate of ammonia release.

It is well known that ammonia is highly water soluble, while grain proteins are mainly water-insoluble. In our solubility test (Exp.2) the difference between CTR and UT cereals was a gross measure of solubility of N added as urea. In whole kernels (Figure 1) there was a limited difference in N solubility and this indicates that during the urea treatment ammonia-N penetrated and embedded inside the wet grain kernel structure where it was retained. On the contrary, in milled cereals, the treated samples showed a very higher N solubility than controls because the matrix rupture facilitated the soluble process. The intermediate values found for coarse milled cereals confirm that the thinner is the physical form of the treated grain, the lesser is the capacity to retain NH_3-N inside the grain structure. Moreover, the solubility showed a limited increase during the incubation test (maximum of 5-6 percentage points during the 8h of incubation) and this indicates that water ammonia solubilization is very rapid and confirms that the process is mainly dependent by the surface of cereal in contact with water.

Given the slow ammonia release properties of the UT grains demonstrated in Exp.1 and 2, in final experiment of this study (Exp.3) the effects of UT on the rumen fermentation parameters, microbial mass and gas production kinetics were investigated.

The gas production kinetic parameters were similar to those previously reported for barley and corn using the same methodology (e.g. syringes) and model of data fitting (e.g. exponential). In corn samples, the extent of gas yield (392 ml/g of DM) was comparable with that tested by Getachew et al. 2004, while Gallo et al. (2016a, 2016b) in barley and corn obtained maximum gas productions similar to those in the present paper, with values higher for corn (334-367 ml/g OM) than those for barley (294-354 ml/g OM).

A clear result from the present gas production experiments is that in both kinds of cereals during the first 9 hours of fermentation the urea addition caused lower gas volumes than CTR samples (about -16% on average). This does not actually mean that the urea was reducing microbial function but simply that the urea addition increased the ammonia yield, which neutralizes the protons from VFA before they can react with the buffer to release carbon dioxide

and thus a gas yield. It is known that gas production in part originates directly from the fermentation of feed ("direct gases production", about 40% of the total), while the remaining is produced from the buffers, because for every mole of VFA produced, the used bicarbonate buffer releases roughly one mole of carbon dioxide ("indirect gas production", Makkar, 2005). Thus, approximately 231 ml/g were released from the buffer compared to 386 ml/g of DM of the total gas volume produced in given 96 h gas-test. Direct gas production originates primarily from the fermentation of carbohydrates and much less from proteins (Theodorou et al., 1998). Cone and van Gelder (1999) estimated that for every 1%-increase in CP, gas production is reduced by 2.48 ml/g of organic matter incubated. Therefore, the depression of gas production when nitrogenous compounds are fermented is mainly due to the production of ammonia, reducing the indirect gas production.

Our hypothesis was confirmed by the similar VFA concentration in the fermentation fluids and by the absence of any depression of VFA due to the addition of urea. The only effect of dietary treatments on VFA profile was observed for butyrate, which decreased for the UT cereals. A greater molar ratio of acetate to propionate for barley vs. corn was a consequence of a higher fiber content of barley.

However, in our experiment the UT substrates showed an opposite behavior between the cereals in terms of gas production: in fact, while UT barley produced high gas volumes, similar to the CTR sample, the UT corn reduced the volume of gas as the UA sample. These results suggest that in barley syringes the microbial population probably sequestered more ammonia than in corn syringes, with a consequent lower buffering effect in barley UT samples compared to those of corn.

Due to the greater rumen ammonia requirements to maximize the degradation of barley than that to maximize the degradation of maize (Odle and Schaefer, 1987), higher ammonia uptake is expected in barley syringes. Therefore, the decrease in gas production in UA barley might be explained by the reaction of the excess ammonia with the buffer. Contrarily, in UT barley, slow-released ammonia possibly assured its better utilization than in UA barley. In corn, where microbial requirements for ammonia were smaller, ammonia accumulation caused suppression in gas production in both UT and UA syringes, masking the slow-release effect of the UT corn, as it was in barley.

Slow-ammonia release in UT cereals possibly provided better-balanced energy to N ratio of the substrate fermented and therefore, more efficient microbial growth (Rode et al., 1986), which at the similar true substrate degradability and total VFA concentrations resulted in the greater microbial N estimated for UT products.

CONCLUSION

The N added to wet cereal kernels by the urea treatment was released into the rumen fermentation liquid more slowly than that simply added as urea before incubation. Moreover, based on solubility data, also the physical form of the cereal has a relevant impact on the release of N, being the larger particles more resilient in releasing. Although the effect of the urea treatment on the *in vitro* fermentation characteristics was not consistent in barley and corn, a significant increase in microbial N synthesis was detected in both kinds of cereal for the urea treated samples compared to controls or to cereals only added with urea. *In vivo* experiments should be organised to evaluate rumen N metabolism and to confirm present *in vitro* data.

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Table 1. Chemical composition and pH of the control (CTR) and urea-treated (UT) barley and corn kernels

	Cereals (C)				P-value			
	Barley		Corn		C	T[1]	C x T	
	CTR	UT	CTR	UT				RMSE[2]
DM (g/kg)	877	825	870	798	<0.01	<0.01	<0.05	4.5
Ash (g/kg DM)	22	21	12	12	<0.01	0.98	0.93	2.4
CP (g/kg DM)	101	134	74	106	<0.01	<0.01	0.42	0.9
pH								
-meal	5.75	8.32	6.47	8.16	<0.01	<0.01	<0.01	0.020
-whole kernel	6.55	8.62	6.25	8.72	<0.01	<0.01	<0.01	0.076

[1] Effect of the urea treatment (CTR: control; UT: urea-treated);
[2] Root mean square error

Table 2. Effect of cereal type (C) and urea treatment (T) on the *in vitro* kinetics parameters and halftime of the initial 96 h gas production, and gas yield at measurement time points, apparent and true substrate degradability (ASD and TSD, respectively), and microbial nitrogen (MN) of the halftime ($t_{½}$) gas test

	Barley[1]			Corn[1]			P-value			
	CTR	UT	UA	CTR	UT	UA	C	T	C x T	RMSE[2]
Kinetic parameters and halftime[3]										
V (ml/g)	376^{x}	379^{x}	359^{y}	396^{x}	389^{x}	382^{y}	<0.01	<0.05	-	10.5
k (ml/h)	0.074^{b}	0.084^{a}	0.072^{b}	0.080^{a}	0.082^{a}	0.081^{a}	<0.05	<0.05	<0.05	0.005
$t_{½}$ (h)	9.6^{x}	8.76^{y}	9.82^{x}	8.93^{x}	8.66^{y}	8.76^{x}	<0.01	<0.05	-	0.468
Measurement time points[3] (ml/g DM)										
1 h	41^{X}	40^{Y}	32^{Z}	27^{X}	21^{Y}	17^{Z}	<0.01	<0.01	-	4.6
2 h	67^{a}	68^{a}	57^{b}	47^{b}	39^{c}	35^{c}	<0.01	<0.01	<0.05	6.1
4 h	113^{A}	114^{A}	98^{B}	89^{B}	78^{C}	73^{C}	<0.01	<0.01	<0.01	7.2
6 h	159^{A}	161^{A}	142^{B}	139^{B}	126^{C}	123^{C}	<0.01	<0.01	<0.01	6.8
$t_{1/2}$	198^{A}	198^{A}	179^{B}	193^{B}	179^{C}	177^{C}	<0.01	<0.01	<0.01	4.9
Substrate degradability and MN synthesis[3]										
ASD	49.6^{a}	38.5^{b}	44.4^{a}	45.1^{a}	46.2^{a}	51.1^{a}	-	<0.05	<0.05	5.35
TSD	78.6	78.2	82.6	84.6	88.1	90.4	<0.05	-	-	7.56
MN	4.1^{Y}	7.0^{X}	5.5^{Y}	5.9^{Y}	6.7^{X}	5.6^{Y}	-	<0.01	-	0.83

[1] CTR: control; UT: urea-treated; UA: urea-added; [2] Root mean square error; [3] When the interaction "C x T" is statistically significant, means in the rows having different superscripts are statistically different (A, B, C…P<0.01; a, b, c…P<0.05), otherwise statistically difference between urea treatments (T) are indicated by other superscripts (X, Y, Z…P<0.01; x, y, z…P<0.05).

Table 3. Effect of cereal type (C) and urea treatment (T) on the volatile fatty acid (VFA) and ammonia-nitrogen (NH_3-N) concentration in fermentation fluid at the halftime

	Treatment (T)[1,2]			Cereal (C)[2]		
	CTR	UT	UA	Barley	Corn	RMSE[3]
Total VFA (mM)	99.6	97.3	100.7	101.4	97.0	8.27
Individual VFA (mM/100mM)						
acetate	65.5	65.3	64.9	65.7^{a}	64.8^{b}	0.73
propionate	19.1	19.5	19.5	18.6^{B}	20.2^{A}	0.91
isobutyrate	0.92	1.03	1.02	0.96	1.02	0.11
butyrate	11.1^{A}	10.5^{B}	10.9^{A}	10.9	10.7	0.27
isovalerate	1.6	1.52	1.55	1.59	1.53	0.082
valerate	1.73	2.13	2.1	2.19	1.78	0.618
a:p[4]	3.45	3.37	3.34	3.55^{A}	3.23^{B}	0.175
NH_3-N (mg/dl)	18.7^{B}	25.9^{A}	24.1^{A}	26.5^{A}	19.3^{B}	2.87

[1] CTR: control; UT: urea-treated; UA: urea-added; [2] Means in the rows within T and C effects having different superscripts are statistically different (A, B…P<0.01; a, b…P<0.05); the fermentation run (block) was statistically significant for all variables except for acetate and valerate, while the interaction "C x T" was never significant; [3] Root mean square error; [4] Acetate to propionate ratio.

Figure 1. Ammonia N concentration and pH of the fermentation fluid at different incubation times for the feed treatments (CTR, UA and UT, a and b, respectively) and for the two cereals (c and d, respectively). (Ammonia MSE: 0.90; feed treatment, incubation time and cereal type P<0.01. PH MSE: 0.024; interactions feed treatment * incubation time and feed treatment*cereal type P<0.01).

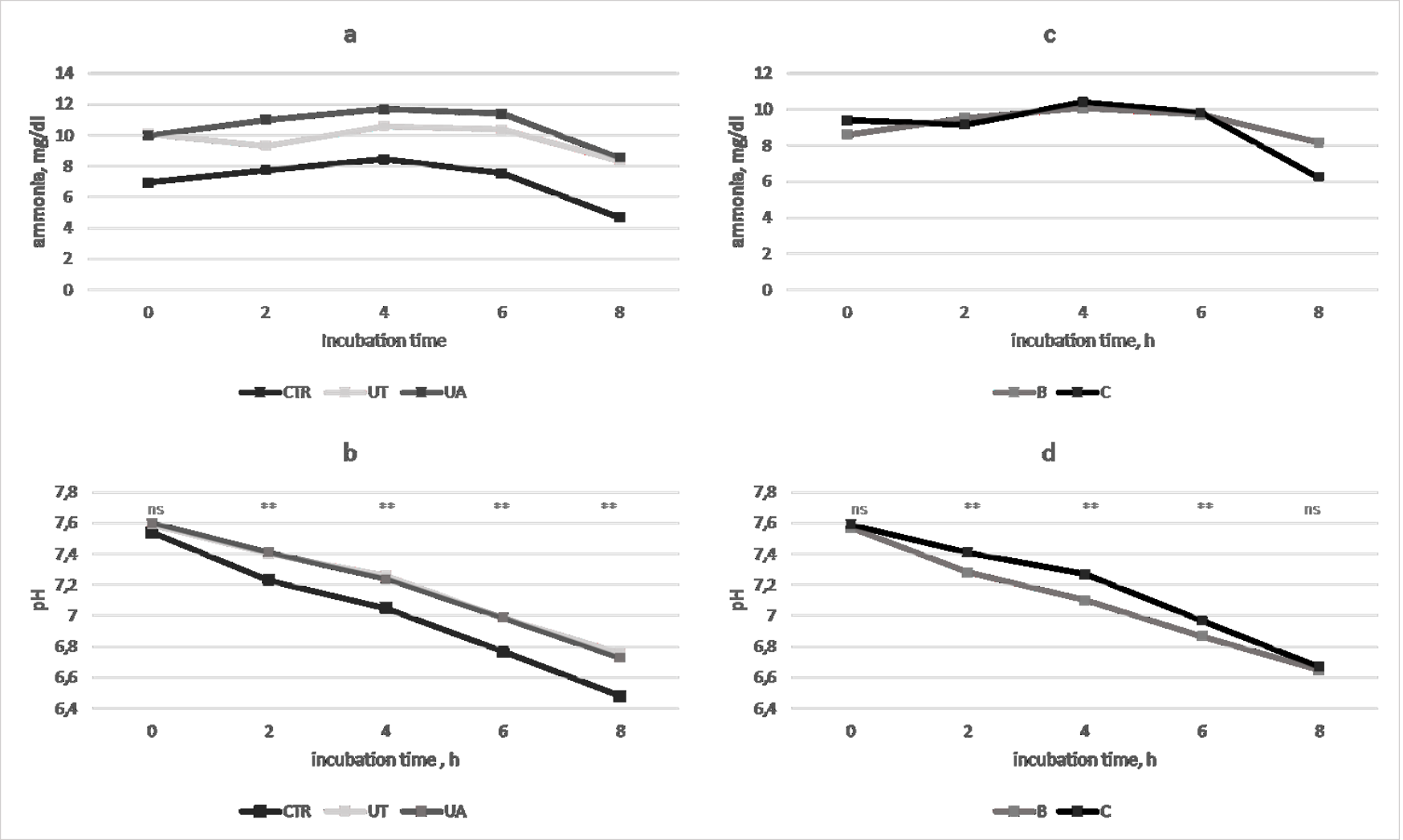
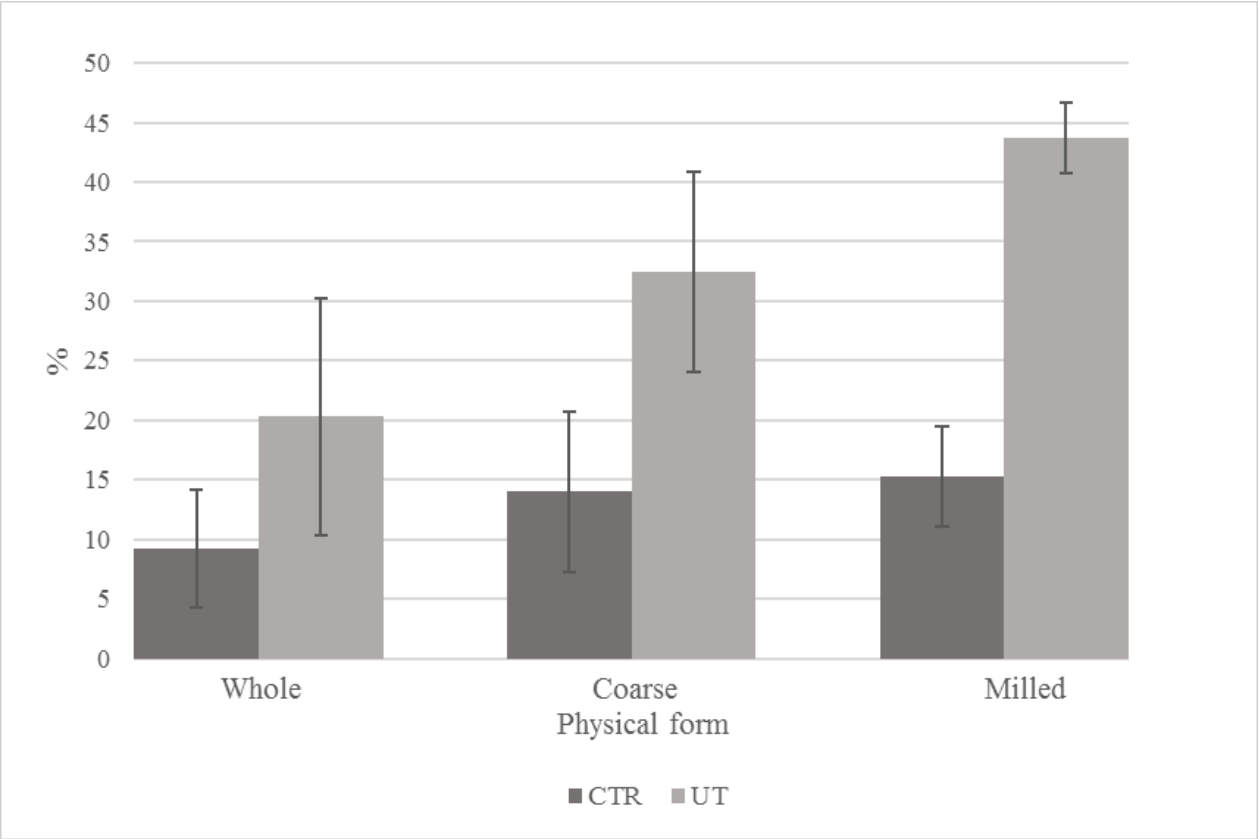


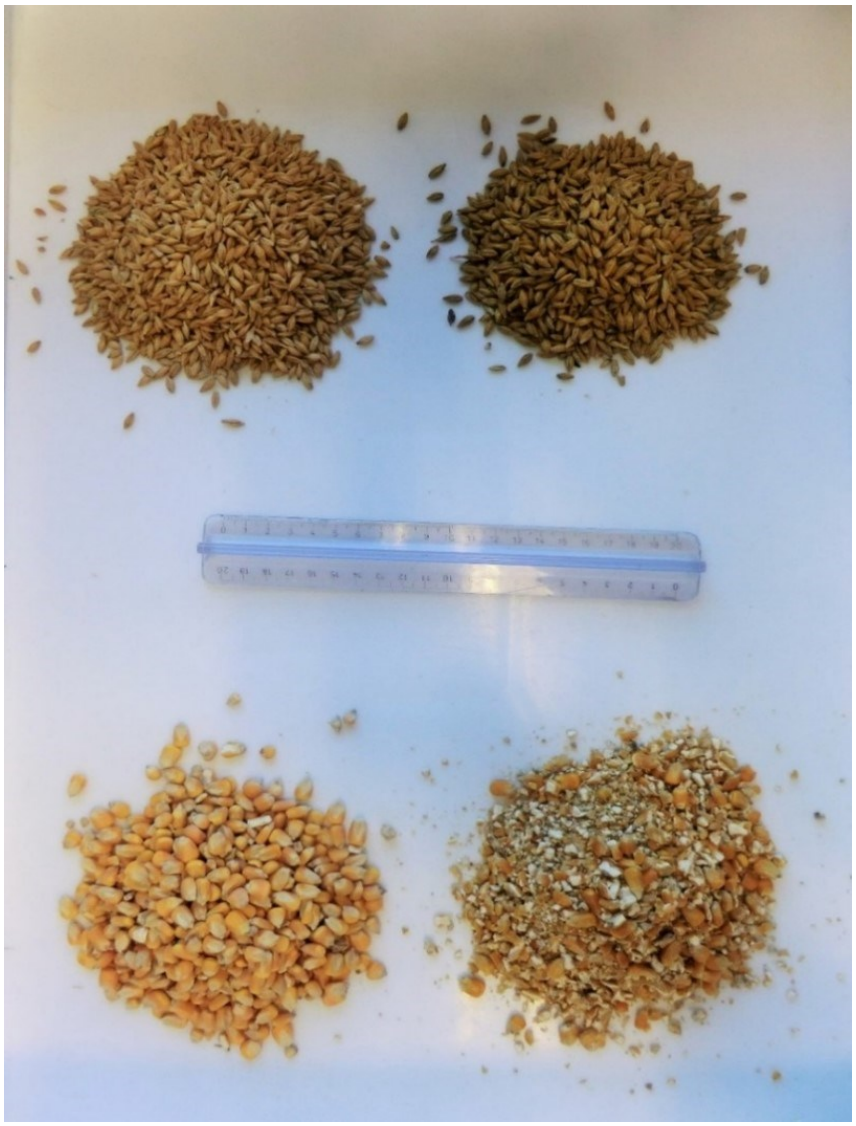
Figure 2. Nitrogen solubility (%) of cereal grains not treated or urea-treated (CTR and UT, respectively) and prepared in 3 physical forms: whole grain (whole), coarsely milled (coarse) and milled to 1 mm (milled). Interaction "substrate type x physical form" of statistical model used in Exp. 2 was statistically significant and different letters (A, B, C, D) over the bars indicate statistical different means ($P<0.01$, root mean square error equal to 7.26).



Picture 3. Batch culture incubation (Exp.1) and N solubility in water trial (Exp. 2).

Picture 4. Gas-test incubation.

Picture 5. A visual browning effect of the urea-treatment in barley and corn grains. From left to right: before and after urea-treatment. From top to bottom: barley and corn.



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Use of an *in vitro* gas production procedure to evaluate rumen slow-release urea products

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CHAPTER 4. Use of an *in vitro* gas production procedure to evaluate rumen slow-release urea products

ABSTRACT

The bulk of the gas produced in the *in vitro* rumen gas production technique originates from bicarbonate buffer, which reacts with volatile fatty acids (**VFA**) to release CO_2. Ammonia, which is produced from fermentation of nitrogenous substrates, neutralizes the VFA and prevents them from reacting with the buffer. We hypothesized that the *in vitro* gas test could be a suitable method to differentiate slow-release urea (**SRU**) products basing on the intensity of ammonia release and consequent reduction in gas produced. The study was composed by two *in vitro* gas experiments (*i.e.,* Expt.'s 1 and 2), which were conducted using graduated 100 ml syringes with gas measurements at 1, 2, 4, 6, 8, 10 and 24 h of incubation (in Expt. 2, also at 0.5 and 18 h). In Expt. 1, 500 mg of corn (*Zea mays subsp. mays*) meal (CM), alone or added with four progressively higher urea doses (*i.e.,* 25, 50, 75, 100 mg) were examined to evaluate the patterns of gas reduction and modifications of the fermentation process with urea addition. Increasing urea doses caused a consistent depression in gas produced at each measurement point ($P < 0.01$) and an increase ($P < 0.01$) in ammonia N concentration in the fermentation fluid after 24 h of incubation. When urea added exceeded 50 mg, a decrease ($P < 0.01$) of substrate apparent degradability (from 0.76-0.77 to 0.68-0.71) occurred. In Expt. 2 a group of 9 commercial SRU products, containing from 361 to 420 g of N/kg of dry matter, were added to 500 mg of CM in amounts equivalent to 50 mg (on an N basis) of feed grade urea (*i.e.,* **CM + SRU**), CM alone and CM added with urea (*i.e.,* **CM + U**), were incubated as in Expt. 1. At all measurement points, gas production was highest for CM and lowest for CM + U substrates ($P < 0.01$) while all CM + SRU mixtures were intermediate (e.g. from -30/40 to -70/80% of the CM gas yield, at 4 h). The *in vitro* gas procedure can be used to evaluate SRU products in terms of reduction of gas volumes due to the ammonia release. The procedure seems to be of high sensitivity because can well rank different commercial SRU products, mainly in the first hours of incubation. Further research might consider using the procedure by modern equipment and performing experiments to test accuracy and precision.

Keywords: rumen, ammonia, slow-release, urea, gas test

Abbreviations: CM, corn meal; CM + SRU, CM plus slow-release urea; CM + U, CM plus urea; DM, dry matter; EE, ether extract; N, nitrogen; SRU, slow-release urea; VFA, volatile fatty acids.

INTRODUCTION

Urea is a dietary additive fed to ruminants to supply soluble N for the rumen bacterial population. The very rapid conversion of urea into ammonia often exceeds the capacity of rumen bacteria to utilize all the ammonia with the surplus being absorbed through the rumen wall into the blood (Satter and Roffler, 1975). A way to avoid problems of toxicity due to absorbed ammonia, and to improve the efficiency of bacterial capture, is to slow the conversion rate of urea into ammonia by coating the granules with substrates able to protect them from rapid degradation (Cherdthong and Wanapat, 2010).

The efficacy of urea protection has been generally tested *in situ* by incubation of slow release urea (SRU) products in porous bags incubated in the rumen (Taylor-Edwards et al. 2009; Highstreet et al., 2010; Ceconi et al., 2015; Goncalves et al., 2015). As this technique requires ruminally cannulated cattle, and is labor expensive, ethical and economic issues suggest the need for an alternative method primarily based on laboratory techniques.

The *in vitro* gas production method is a well-accepted technique which allows rapid and repeatable measures, and is widely used in the nutritional evaluation of feeds for ruminants (Menke et al., 1979; Blummel and Becker, 1997; Cone et al., 1998; Cone and van Gelder, 1999; Spanghero et al., 2017b). Ammonia released from fermentation contributes little to the gas produced because it neutralizes the volatile fatty acids (VFA), which are no longer available to react with bicarbonate in the fermentation and so to release CO_2. Previous papers have clearly indicated that ammonia from nitrogenous compounds reduces gas production (Raab et al., 1983; Cone and Van Gelder, 1999), as also measured in other recent studies (Cherdthong et al., 2011, Cherdthong and Wanapat, 2014). These results led us to hypothesize that the *in vitro* gas test could be a suitable method to differentiate the urea release rate of SRU products based on their intensity of ammonia release.

Our objectives were to measure the gas production response in controlled conditions due to increasing ammonia levels during *in vitro* fermentation (*i.e.,* by adding increasing levels of feed

grade urea to a fermentable substrate) and to apply the method in ranking a group of commercial SRU products representative of those available commercially.

MATERIALS AND METHODS

Two rumen in vitro experiments were performed at the University of Udine, Department of Agricultural, Food, Environmental and Animal Sciences (Udine, Italy), from January to July 2017. A preliminary gas test (Expt. 1) aimed to support the experimental hypothesis that gas production is depressed by urea additions without adverse effects on fermentation. Second gas test (Expt. 2) was conducted to differentiate a set of commercial SRU products.

Fermentation substrates and rumen liquid handling procedure.

A total of 11 substrates were used, being a corn meal (CM) sample, urea (U), and 9 commercial SRU products available commercially (*i.e.,* NitroShure™, Optigen®, and 7 products from Sintal and Nutristar feed companies).

Rumen liquid for each *in vitro* gas run was obtained at a slaughterhouse from cull dairy cows (~4 cows per rumen inoculum) and was delivered to the laboratory within half an hour in airtight glass-bottles refluxed with CO_2 and immersed in thermoses filled with 39°C water. In the laboratory, the rumen fluid was strained through a 1 mm wire mesh filter and added to the nutrient solution under constant infusion of CO_2.

Gas production from substrate added with increasing levels of feed grade urea (Expt. 1).

Corn meal, 500 mg milled to 1 mm (Pulverisette; Fritsch, Idar-Oberstein, Germany), alone or with 4 progressively higher urea doses (*i.e.,* 25, 50, 75, 100 mg) was incubated in 100 ml calibrated glass syringes in triplicate with 40 ml of incubation medium according to Menke et al. (1979) as modified by Blummel and Becker (1997). The incubation medium for each syringe contained 10 ml of filtered rumen fluid and 30 ml of bicarbonate-mineral-distilled water mixture (2:1:3 on a volume basis). Syringes were placed vertically in a water bath at 39°C with three syringes without substrate used as blanks. Gas production was manually recorded after 0, 1, 2, 4, 6, 8, 10 and 24 h of incubation. Syringes were manually agitated at every reading and gas production was corrected for that of the blanks. At the end of the fermentation the syringe contents, including blanks, were centrifuged at 20,000×*g* for 10 min at 20°C (Blummel and

Lebzien, 2001), and the supernatant was sampled for ammonia N and VFA analyses (*i.e.,* 5 ml samples acidified with H_2SO_4 1.0 and 0.1*N,* respectively). The pellet was dried at 60°C and substrate apparent degradability was determined after correction for the blanks. Each run was repeated.

Gas production from substrate added with the SRU products (Expt. 2).

Corn meal, 500 mg either alone or with 50 mg of urea (*i.e.,* CM + U) or with one of the 9 commercial SRU products in equivalent amounts (on an N basis) to 50 mg of urea (*i.e.,* CM + SRU) were incubated in triplicate in two fermentation runs following the same procedure as in Expt. 1. Gas production was manually recorded after 0, 0.5, 1, 2, 4, 6, 8, 10, 18 and 24 h of incubation and corrected for the blanks.

Chemical analysis

The SRU products, urea and CM samples were analysed for dry matter (DM), ash and N contents, while the SRU products were also analysed for ether extract (EE). The N was determined by a Kjeldahl method (976.05; AOAC, 2000), DM was at 105°C for 3 h (930.15; AOAC, 2000), ash content by incineration at 550°C for 2 h (942.05; AOAC, 2000) and EE by solvent extraction (954.02; AOAC, 2000).

Samples for ammonia N determination were centrifuged and analysed spectrophotometrically using a modified Berthelot reaction method (Krom, 1980) with the continuous flow SAN++ analyser (Skalar Analytical B.V., Breda, The Netherlands). For VFA analysis of the fermentation fluids of Expt.1, aliquots of the supernatants obtained after centrifugation were filtered through polypore 0.45 μm filters (Alltech Italia, Milan, Italy). The filtrate was injected into a high-performance liquid chromatograph (Perkin-Elmer, Norwalk, CN, USA), set to 220 nm according to Martillotti and Puppo (1985).

Statistical analysis.

The data of Expts. 1 and 2 were statistically analysed as factorial designs according to the model:

$Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$

Where: y is the measure of the experimental unit (*e.g.,* analytical or gas measurement on each syringe), μ is the overall mean, α_i is the random effect of the fermentation run (i = 1,2, block), β_j is the fixed effect of the treatment (j = 1,5 and j = 1,11, respectively in Expt. 1 and 2) and ε_{ijk} is residual error.

Student-Newman-Keuls multiple range test was used to compare averages within the substrate type and significant differences were declared if $P < 0.05$ and if $P < 0.01$. Orthogonal polynomial contrasts were used to test statistical significance of the linear and quadratic components of treatments in Expt.1.

RESULTS

Chemical composition of CM, feed grade urea and SRU products are in **Table 1**. The N content of the SRU products ranged from 780 to 900 g/kg DM. Ash and EE contents varied among SRU, with 4 SRU products containing only fat in addition to urea (*i.e.,* 110 or 150 g/kg DM), while the others were composed of ash and EE in approximately equal amounts (*i.e.,* 40 to 60 g/kg DM).

Experiment 1: gas production from substrate added with increasing levels of feed grade urea

Progressively higher urea additions to the CM resulted in depressed gas production at each measurement time ($P < 0.01$), and an increase ($P < 0.01$) in the concentration of ammonia N in the fermentation fluid at the end of fermentation (24 h) (*i.e.,* linear and polynomial regressions between gas production and the doses of feed grade urea had $R^2 > 0.79$ and are in **Figure 1**). When urea added exceeded 50 mg, a decrease ($P < 0.01$) of apparent degradability of substrate occurred (*i.e.,* 0.76-0.77 to 0.68-0.71). Total VFA concentrations reached their highest and lowest values with urea additions of 25 and 75 mg respectively, and only butyrate concentrations differed among treatments (*i.e.,* 11.3-11.5 to 13.3-13.4 mM, $P < 0.01$) when added urea exceeded 50 mg, while valerate had its lowest values at 50 and 75 mg of urea added and highest values for the highest urea-added dose.

Experiment 2: gas production from substrate added with the SRU products

The gas production of CM, CM + U and CM + SRU products are in **Table 3**. At all measurement points the gas production (**Table 3**) was highest for CM and lowest for the CM + U substrates ($P < 0.01$) with the CM + SRU products intermediate (e.g. from -30/40 to -70/80% of the CM gas yield, at 4 h as shown in **Figure 2**).

DISCUSSION

In feeding and metabolism studies where SRU products were fed in substitution for feed grade urea, the rumen protection of the SRU products was not measured (Huntington et al., 2006; Holder et al., 2013; Calomeni et al., 2015) or was measured by a gravimetric method using ruminal *in situ* nylon bags (Taylor-Edwards et al., 2009; Highstreet et al., 2010; Ceconi et al., 2015; Goncalves et al., 2015). There is concern regarding use such *in situ* methods for urea and SRU products because the washing of the bags after incubation can partly solubilize the residual urea, leading to an overestimation of the ruminal disappearance. For this reason, analyses of urea on unwashed bag residues has been suggested (Holder, 2012). Moreover, simultaneous *in situ* incubation of several large bags (*e.g.*, various products for many incubation times) could supply an excessive amount of urea to the rumen with risks of toxic effects.

There are some studies where gas production has been used to evaluate feed substrates with added urea (Cherdthong et al., 2011; Cherdthong and Wanapat, 2014; Phesatcha and Wanapat, 2016). However as there are none that utilised gas production in standardised conditions to measure the rumen degradation of SRU products, we examined the hypothesis that the gas test can be used to evaluate and rank the SRU products in terms of estimated ammonia release in the rumen.

It is well known that gas produced in the Menke and Staingass *in vitro* system (1988) originates in part from fermentation of the feed substrate (~40% of total gas), while the remainder is produced from the buffers. This is because for every mole of VFA produced, the bicarbonate buffer used releases about one mole of CO_2 (Makkar, 2005). According to Cone and van Gelder (1999), gas produced from fermentation of casein is only a third of that from fermentation of carbohydrates, on an equal weight basis, and for every 10 g of crude protein per kg of substrate, gas production is reduced by 2.48 ml/g of organic matter incubated. This

depression of gas production when nitrogenous compounds are fermented is mainly due to production of ammonia, which neutralizes the acids and reduces gas production from the buffer.

The role of ammonia in gas production was considered by Raab et al. (1983), who proposed gas production as a method to estimate protein degradability of common feeds (*e.g.,* protein and starchy meals or forages) and was based on correction of gas measurements by the amount of ammonia produced. However, if the SRU products are added to the same source and amount of fermentable organic matter the comparison based on gas production is not biased by differences in the carbohydrate contents and fermentability and solubility is inversely related to the amount of gas produced.

The SRU products considered in the current study were commercial additives, not selected by the technology of protection or encapsulation.

In Expt. 1 there was a consistent depression of gas produced when 4 progressively incremental amounts of 25 mg of feed grade urea were added to a basal amount of corn meal. While for short incubation times (*i.e.,* < 4 h) the gas reduction followed a curvilinear shape, in subsequent times of incubation the trend was linear. Moreover, the amount of ammonia N measured at the end of fermentation in the fermentation fluid increased consistently with the doses of urea and followed a linear trend of accumulation. This was expected, as the concentration of bacterial ureases in rumen fluids is assumed to be abundant and not limiting the conversion of urea to ammonia (Cook, 1976). The ammonia N accumulation during the fermentation with the urea-added substrates reached high concentrations (range 24 to 61 mM), which are 2 to 4 times the values suggested to be required for optimal rumen bacterial growth (*i.e.,* 13.8 mM, Mehrez et al., 1977) or double the *in vivo* post-feeding (2 h) peak of ammonia N which ranges between 20 and 30 mM (Aschenbach et al., 2015). However urea addition did not change *in vitro* apparent digestibility up to 50 mg of feed-graded urea, while further increments (*e.g.,* 75 and 100 mg) resulted in a significant reduction. We suggest that the highest ammonia concentration inhibited bacterial growth, although there were concomitant high VFA accumulations, with similar VFA profiles, apart from an increase in butyrate at the highest additions which confirms that high rumen ammonia concentrations promote a fermentation oriented towards butyrate production (Spanghero et al., 2017a).

Based on results from the Expt.1 we tested the sensitivity of the new procedure in ranking commercial SRU products, which were kept anonymous because the study of their specific slow release aptitude was not an aim of present paper. The SRU products were added to CM in an amount equivalent to 50 mg of urea (approx.100 g/kg). Such a concentration of urea is higher than that used previously *in vivo* (maximum of 10 to 20 g/kg DM in: Taylor-Edwards et al., 2009; Highstreet et al., 2010; Holder et al., 2013; Ceconi et al., 2015), but it does not impair rumen fermentation *in vitro* and allows to work with not milled substrates, such as encapsulated urea. Thus, the gas production for CM and CM + U resulted in values very close to those previously obtained for the same substrates. For all incubation times, the SRU products were intermediate between CM and CM + U and were well ranked, mainly in the first hours of incubation, as shown in **Figure 2**. In fact, at 4 h of incubation they ranged from as low as 30 to 40% (high releasing) to as high as 70 to 80% (low releasing) of the gas measured from the CM substrate alone. In the subsequent 4 h of incubation, the low releasing SRU had a gas production of 75 to 80%, while the high releasing products were 45 to 50 % of the CM gas production. It is possible that differences in fat content of products (range from 50 to about 150 g/kg) slightly affected *in vitro* gas production and therefore SRU comparisons should be done at same fat coating level.

During the progression of fermentation differences among SRU products resulted attenuated, due to a probable exhaustion of the buffering effect of urea.

CONCLUSIONS

The *in vitro* gas procedure can be used to evaluate SRU products in terms of reduction of gas volumes due to the ammonia release. The procedure seems to be of high sensitivity because can well rank different commercial SRU products, mainly in the first hours of incubation. Further research might consider using the procedure by modern equipment and performing experiments to test accuracy and precision.

Overall the procedure proposed allows to select the best SRU products and to increase and better utilise urea in productive ruminant diets.

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Table 1. Chemical composition (g/kg dry matter) of the substrates.

	Substrates[1]										
	CM	Urea	SRU								
			A	B	C	D	E	F	G	H	I
DM, g/kg	870	990	919	990	975	923	985	982	983	918	912
Ash	12	0	71	0	0	68	0	0	0	70	71
Nitrogen	12	450	361	376	376	390	391	348	420	403	416
Ether extract	40	0	49	111	117	80	149	147	86	61	47

[1]CM: corn meal; SRU: slow-release urea products, coded from A to I.

Table 2. Gas production during 24 h fermentation and end-point measures of apparent substrate degradability (ASD), ammonia N and VFA concentrations in the fermentation fluid of the syringes containing corn meal (500 mg) added with increasing amounts of feed grade urea (*i.e.,* 25, 50, 75, 100 mg).

	Urea added[1]					Components[2]		
	0	25	50	75	100	L	Q	RMSE[3]
Measurement time points (h), ml/g DM								
1 h	19.9	10.2	8.4	5.8	5.6	**	**	1.88
2 h	32.2	16.6	10.7	5.8	5.3	**	**	2.41
4 h	60.7	36.6	21.0	7.9	4.1	**	**	4.06
6 h	102.8	68.7	43.0	16.0	8.1	**	**	8.01
8 h	156.4	116.6	80.3	37.1	20.2	**	**	7.10
10 h	220.1	187.9	146.9	83.4	63.X	**	.12	7.92
24 h	342.8	298.3	266.9	200.4	175.2	**	.15	12.58
ASD	0.763	0.770	0.761	0.706	0.680	**	.22	0.0322
Ammonia N, mM	12.2	24.3	35.6	45.5	61.2	**	.30	3.72
VFA, mM	128	160	147	113	132	.30	.48	26.0
Individual VFA, mM/100mM								
acetate	67.9	68.2	69.4	68.8	67.4	.78	.15	1.91
propionate	13.9	13.3	12.7	11.9	12.1	.06	.64	1.68
butyrate	11.3	11.5	11.4	13.3	13.4	**	.60	0.81
isobutyrate	0.99	1.10	0.99	0.80	0.80	**	.59	0.172
valerate	3.52	3.57	3.29	3.13	4.11	.58	**	0.472
isovalerate	2.35	2.33	2.13	2.00	2.21	.16	.35	0.364

[1] mg of urea added to 500 mg of corn meal in each syringe.
[2] L: linear; Q: quadratic.
[3] Root mean square error.

Table 3. Gas production during 24 h fermentation in syringes containing corn meal (500 mg) alone, or added with 50 mg of feed grade urea (CM+U) or added with one of 9 commercial SRU products in amounts equivalents to 50 mg of feed grade urea.

	Substrates[1]											
	CM	CM+U	SRU									RMSE[2]
h			A	B	C	D	E	F	G	H	I	
0.5	6.8^{a}	1.3^{c}	6.6^{a}	6.4^{a}	6.1^{a}	5.9^{a}	4.5^{b}	4.4^{b}	4.1^{b}	4.0^{b}	3.0^{bf}	0.94
1	13.6^{a}	2.1^{g}	11.6^{ab}	11.7^{ab}	10.5^{bc}	9.9^{bcd}	7.6^{ed}	8.8^{ecd}	7.6^{ed}	6.8^{ef}	4.9^{f}	1.69
2	27.6^{a}	4.9^{f}	22.4^{b}	22.3b	19.4^{bc}	19.1^{bc}	13.4^{d}	18.8^{bc}	15.9^{cd}	12.7^{d}	8.9^{e}	2.90
4	57.9^{a}	18.0^{d}	43.4^{b}	39.3^{bc}	35.4^{bc}	34.1^{bc}	29.9^{bcd}	39.7^{bc}	36.3^{bc}	24.3^{cd}	23.6^{cd}	9.53
6	110.8^{a}	40.2^{e}	83.1^{b}	78.1^{b}	63.2^{c}	71.2^{bc}	59.5^{cd}	83.4^{b}	70.7^{bc}	56.7^{cd}	48.1^{ed}	9.44
8	164.4^{a}	78.1^{g}	130.0^{b}	123.1^{bcd}	107.2^{ed}	112.7^{cde}	101.5^{fe}	128.8^{bc}	113.2^{ced}	98.6^{fe}	87.0^{fg}	10.61
10	218.4^{a}	126.3^{f}	179.1^{b}	169.9^{bc}	153.1^{cd}	160.2^{cd}	148.8^{d}	178.4^{b}	156.4^{cd}	145.3^{ed}	132.8^{fe}	11.06
18	323.3^{a}	234.7^{d}	277.8^{b}	271.6^{b}	261.8^{bc}	265.4^{bc}	251.7^{cd}	275.0^{b}	243.7^{d}	244.1^{d}	237.0^{d}	11.01
24	351.6^{a}	260.0^{d}	299.7^{b}	292.4^{b}	287.8^{bc}	286.4^{bc}	272^{cd}	293.3^{b}	261.0^{d}	263.4^{d}	257.5^{d}	11.64

$^{a–g}$,Significant differences among treatments are denoted by lowercase letters ($P \leq 0.01$).
[1]CM: corn meal; CM+U: corn meal added with feed grade urea; SRU: slow-release urea products, coded from A to I.
[2] Root mean square error.

Figure 1. Linear (blu, solid line) and polynomial (red, dotted line) regressions between the gas production (ml/g DM, vertical axis) and addition of feed grade urea (mg, horizontal axis) to corn meal (500 mg) for each of 7 incubation times (*i.e.,* 1, 2, 4, 6, 8, 10 and 24 h, respectively). In parenthesis the R^2 of the regression and the statistical significance P (<0.01 or <0.05 or not significant, ns) of the equation coefficients, respectively.

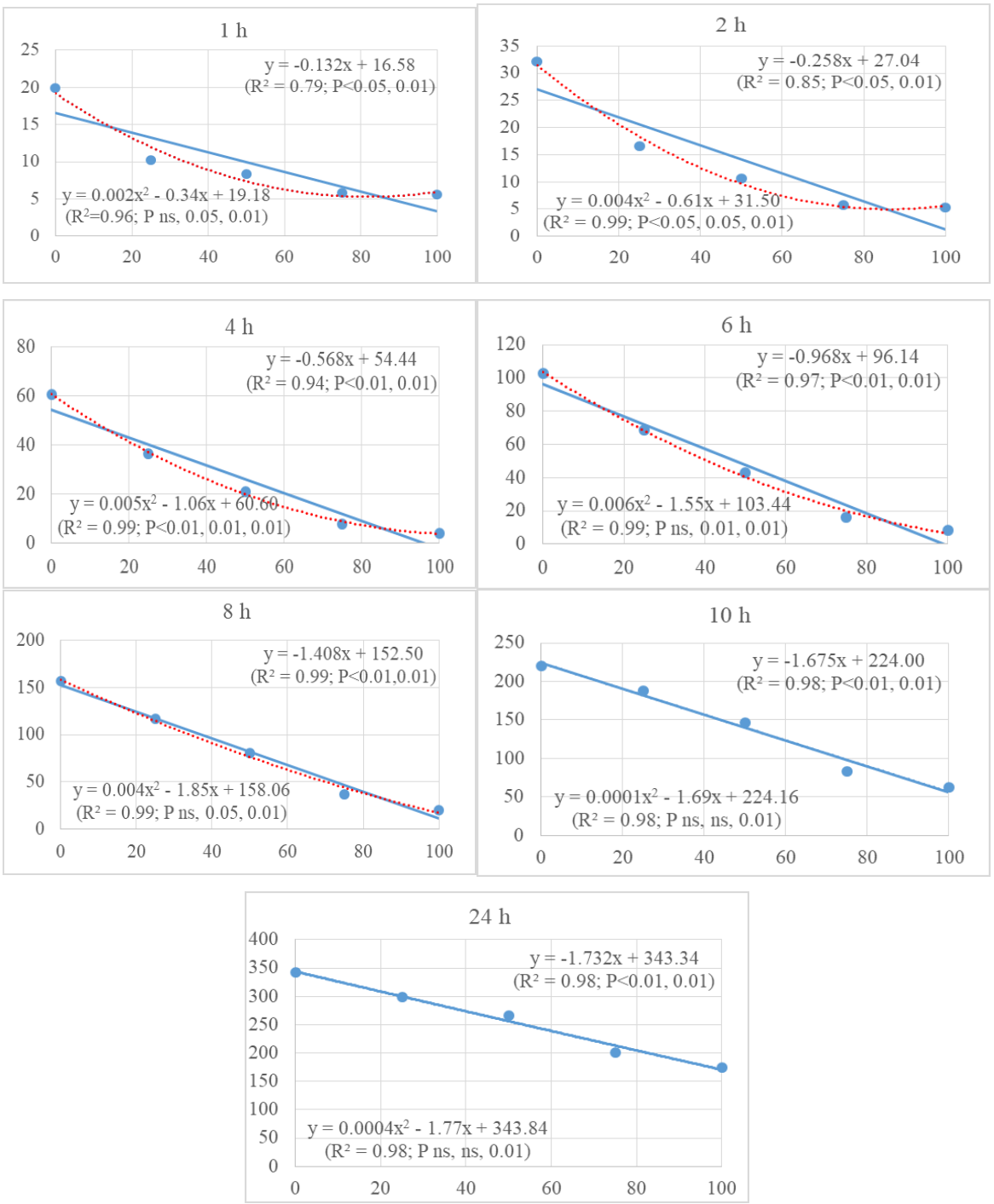
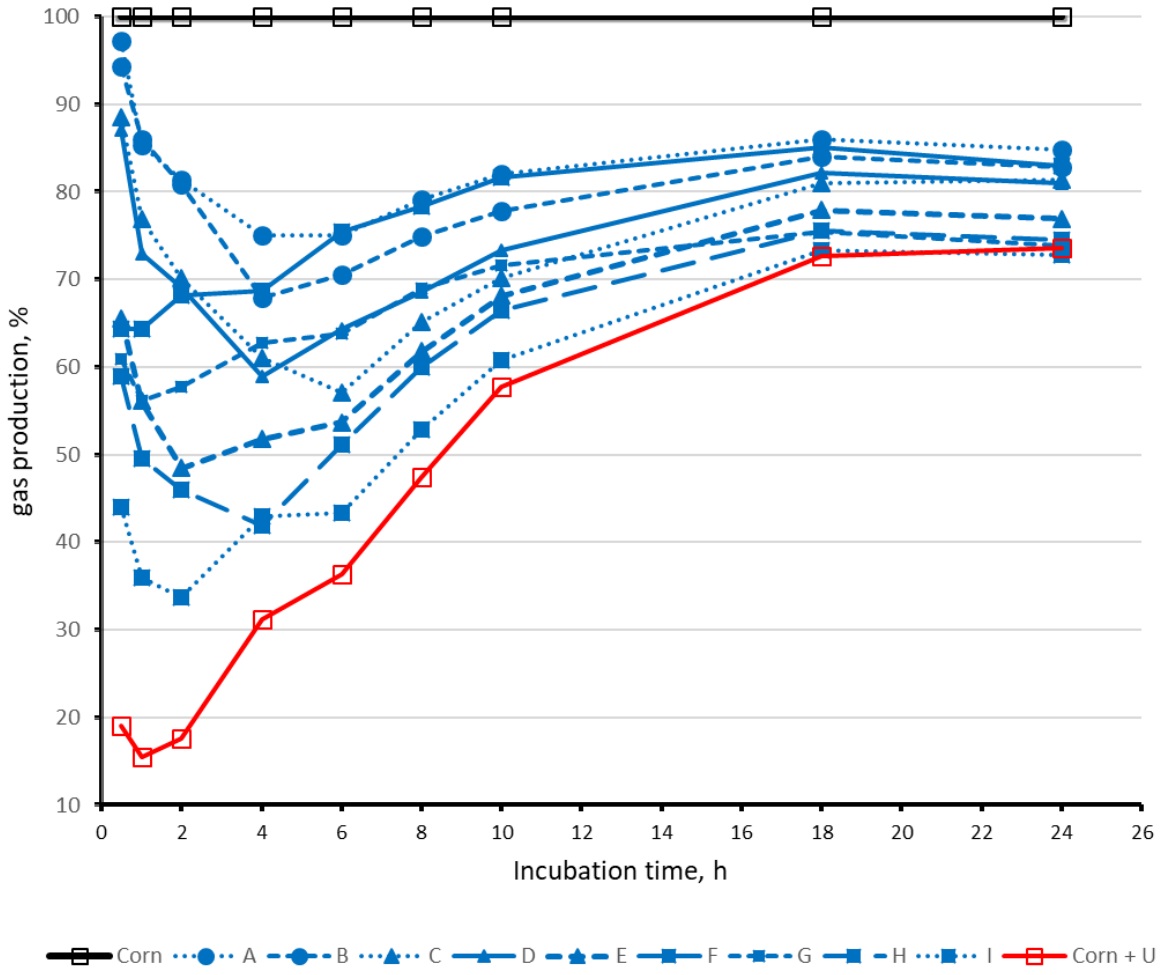


Figure 2. Plot of gas produced by corn meal (CM) with slow-release urea products (coded from A to I) and by corn plus feed grade urea (CM+U) as proportion of that produced by the CM sample alone (=100).



CHAPTER 5: COMMENTS AND IMPLICATIONS

1. First trial

The *in vivo* trial with bulls (first contribution) was a feeding trial conducted in an experimental farm, with conditions very close to those adopted in fattening units. The rumen fluid collected from the bulls at the end of the trial was used in an experiment with a continue fermentation system to examine the effect of dietary treatments on fermentation parameters and microbial population. All diets were formulated to have the same FOM content, and the dietary treatments were obtained with very limited variations in amounts of ingredients. In fact, a mixture of about 50 g of urea plus 350 g of corn meal substituted 400 g of SBM in the daily rations (9.3 kg DM/d) differing for urea content (**+U** and **–U**, respectively). Two levels of CP content (12.8 and 11.0% DM, medium and low, respectively) were obtained by including around 450 g of SBM or corn meal. Moreover, the level of CP, even at the medium level, was calibrated to determine an insufficient amount of rumen degradable N with respect to the available fermentable energy. The substitution of SBM with urea increased diet RDP content, thus, providing more rumen available N for MPS. Due to greater amount of high quality AA from MPS, +U diets were expected to enhance animal performance. Despite this, no differences were detected between +U and –U diets. This result could be due to the greater rumen urea-recycling mechanism in +U diets, where N absorbed was rather in a form of ammonia than AA (Lapierre and Lobley, 2001). The excess of rumen ammonia from +U diets was rapidly absorbed and then recycled back into the rumen, providing a constant urea influx to the rumen N pool thus allowing favourable conditions for rumen microbes to capture ammonia. However, the recycling mechanism is very expensive, due to the considerable losses of N in urine and feces, accounting for around 40% of N recycled (Lapierre and Loble, 2001). It is likely, that in our experiment any positive effect of urea supplementation (e.g. increase on microbial growth and in feed digestibility) on animal performance was levelled by inherent N losses during urea-recycling mechanism.

Supplementation with SRU products provides a slower but constant ammonia supply, may synchronize better rumen available N and energy and decreases the urea-recycling together with the annexed N losses in the environment. Apart from the origin of N absorbed (Lapierre and

Lobley, 2001), the intensity of urea-recycling is also determined by the level of N intake (Batista et al., 2017). Therefore, at the higher level of CP, which theoretically has greater urea-recycling than lower CP diets, the use of SRU may improve the assimilation of the rumen available N by microbes and decrease N losses. Thus, supplementation with SRU products instead of urea may favor the microbial growth and, consequently, animal performance.

We tried to compensate the lack of *in vivo* results with the continuous culture rumen simulation experiment. Apart from the expected substantial variations in ammonia concentrations, in general, the dietary treatments *in vitro* had only minor impacts on the fermentation end-products with the only exception for the butyrate concentrations. A fundamental concern regarding the *in vitro* systems is their capacity to simulate what happens in the rumen. The comparison between the *in vivo* and the *in vitro* conditions by a specific small experimental contribute is reported in the Appendix of this thesis (Nikulina et al., 2017). The results indicate lower values of total VFA, of acetate molar proportions and, consequently, acetate to propionate ratio in fermentation liquid (**FL**) comparing to rumen inoculum (**RI**). These findings agree with those of Hristov et al. (2012) from the extensive review made over variabilities between continuous culture experiments and *in vivo* data. The decline in total count, diversity and richness of rumen bacteria in FL in our study was in line with Soto et al. (2012) who assumed the filtration and exposure to oxygen during the preparation of the RI for inoculation may eliminate the most abundant solid-associated and anaerobic bacteria species. Same authors also concluded an influence of *in vitro*-related factors, such as, pH variations, dilution and passage rates and others on the bacteria counts and the selection of certain bacteria strain. Despite the above commented differences between *in vivo* and *in vitro* data, the environment of the given continuous-culture fermenter allowed to maintain the structure of the bacterial community at the Phyla level found in RI.

Finally, the use of urea in substitution of vegetable proteins for ruminant livestock has some valuable economic impact on feeding costs. According to the current commodity prices, the economic advantage, when 1 kg of SBM is substituted with the mix of corn meal and urea (0.88: 0.12, accordingly), is around 0.15 EUR, and this value varies substantially depending on the actual prices. In feeding systems for bulls applied in Italy the average inclusion of SBM averages

0.8 kg/d per animal (Cozzi, 2007), therefore, the values given in **Table 5.1** represent a general estimation of the economic benefit of urea supplementation due to the price variations. Thus, while influence of the urea prices is negligible due to the comparatively small quantities applied, the changes in the SBM costs have a great economic impact. Overall, high price for SBM and low corn meal quotations are the more favorable economic condition for urea supplementation to the cattle diet. According to Clal.it, current prices in Italy are the lowest in a last 5 years (0.33 EUR for SBM, 0.18 EUR for corn meal and 0.31 EUR for the urea). However, an average saving of 0.19 EUR/d per animal is about 10% of the daily feed expenses (Montanari et al., 2012).

2. Second trial

The discussion of advantages connected with the preservation of high-moisture grains is not a part of the present thesis, where only nutritional aspects are considered. The main result of the study was the demonstration that both UT cereals exhibited an appreciable SRU effect, being the least in the milled, intermediate in the cracked, and the greatest in the whole kernels. Our trial demonstrated that N solubility almost doubled when the physical form of grain is changed from the whole to the milled. These results may have important practical applications, considering the fact that fattening bulls' rations generally utilize substantial amounts of cereals given in different forms: whole, coarsely or finely ground. In the Italian feeding practices based on corn silage and on specialized breeds, the use of grains is around 1 kg per 100 kg of animals' live weight in beef specialized breeds (Cozzi, 2007). However, in the bull fattening systems of other countries (e.g. Spain or North America) the inclusion is often much higher (NRC, 2016). The usage of whole kernels is a diffuse practice in feeding fattening bulls, especially in the first part of the growing period (below a live weight of 400-500 kg), as they are able to efficiently masticate the kernels during rumination. Thus, ammonia kept inside the urea-treated whole kernels will likely to be gradually introduced to the rumen environment during the mastication and rumination processes. Moreover, the utilization of urea-treated kernels would be important to reduce the risks of mixing errors in the ration. In fact, the simple addition of urea to feed ingredients given in the limited amounts per head (e.g. 80 – 100 g/d), requires elevated standards of "precision feeding" and homogenous urea distribution in a substrate to avoid the risk of urea toxicity. The addition of urea to grains in a dose of 14 kg/t allows calculating a daily dose of urea ranging from 60 to 80

g/d which bulls receive with the diets containing 4-5 kg of treated cereals. This amount is per se diluted in the whole volume of grain, which is mixed with corn silage and therefore the intake is fractionated according to the meal frequency.

However, the nature and the mechanisms of ammonia binding inside the kernel during urea-treatment is yet unknown. Ammonia permeates into different grain layers (browning effect) enhancing the CP content of the original cereal, and the release of the ammonia from the deeper layers needs more time comparing to the outer coats, but as was demonstrated in the present work, finer grinding destroys the grain matrix leveling this difference in ammonia release. The pattern of gas and ammonia production differed from barley to corn. It is possible that apart from physical adhesion, some chemical linkage between ammonia and kernel constituents might take a place. Depending on physical properties of the grain, the characteristics of these bounds may also differ and be partially or entirely altered by the milling. Thus, in barley higher than corn in fiber content, ammonia might bind to the fibrous part. A similar gas production between treated and the control barley was probably due to the cellulolytic bacteria which readily utilized ammonia bound to fiber, thus subtracting it from the reaction with the buffer affecting gas production. Although the treatment did not improve TSD, it might have promoted the growth of these bacteria, as they use ammonia as a major N source (up to 80% of cell N; Atasoglu et al., 2001).

During the urea-treatment, free ammonia may bind to the constituents of the kernel. Thus in the urea-treated corn, ammonia reacts with the saturated lipids and the sugars creating ammonia-compounds of low solubilization in the rumen (Srivastava and Mowat, 1980). It is likely that ammonia binding to the grain constituents in barley and corn has different nature which affected the gas production patterns in our study. However, due to this, the synchrony between rumen available energy and N in both kinds of cereal was likely improved favoring the ammonia utilization and as a consequence, MPS. Nevertheless, the differences between cereals caused by the urea treatment were not detected in our experiments (*in vitro* ammonia kinetic and water-dissolving trial), as the interaction between cereal and the treatment was never significant, the gas-test appeared to be a promising method able to detect the differences in ammonia utilization from various urea feeds by rumen microbes comparing to the conventional urea.

3. Third trial

The results of the previous experiment showed new perspectives of the traditional gas-test technique utilization and forced us to explore further the capacity of the method to respond to the fluctuations in ammonia concentration in the fermentation fluid.

Conventional techniques used to evaluate the capacity of SRU products for ammonia release are either based on the measure of the substrate N disappearance, such as *in situ* nylon bags technique (Ørskov et al., 1980) and Daisy incubation (Method 3, Ankom Technology, 1998) or ammonia appearance (Cooke at al., 2009). The major disadvantage of the traditional *in situ* methodologies in their use for the SRU products evaluation is the toxicity of urea, which limits the number of samples to be inserted in the rumen at the same time. Another drawback is the lack of standardization for the *in situ* procedure with the emphasis on the bag's rinsing, leading to a large variability in inter-and intra-laboratory results (Vanzant et al., 1998). Therefore, apart from the inherent gravimetrical error, the techniques based on the residual substrate measurements, may overestimate the N disappearance due to the washing of the bag's residue, thus becoming less useful for the soluble substrates evaluation. Hence, the use of residual urea measure not requiring the washing from bacterial contamination is more appropriate for this purpose (Holder, 2012). Nonetheless, the urea analysis also appears to be time and labor-consuming. The measure of *in vitro* ammonia appearance in the fermentation liquid over the time (Cooke at al., 2009) has its limitations too, as all the samplings are taken from the same fermentation flask, which may lead to the underestimation or distortion of the true ammonia concentrations. Increasing the amount of the bottles according to the sampling times may overcome this problem, but it would require additional equipment and labor. Simultaneous ammonia uptake by rumen microbes greatly driven by the carbohydrate source (Hoover and Stokes, 1991) may also give misleading results of the true ammonia release in batch culture.

In a context of the above-described shortcomings of the current methodologies evaluating the SRU products, a new utilization of the gas test technique was proposed. This innovative approach was suggested after examination of the literature, where the comparison of urea-based products, with the various degree of ammonia release, showed a difference in the gas yields. As a

result of the study, the capacity of *in vitro* gas procedure to detect the difference in ammonia release in various SRU product was confirmed.

CHAPTER 6. CONCLUSIONS

The use of urea in low CP diets for bulls allowed to maintain the satisfactory growth in Italian Simmental finishing bulls. However, the controlled reduction in the rate of urea hydrolysis by the means of whole urea-treated cereal grains and SRU products may improve N utilization by rumen microbes and, consequently, animals' performance. With the cereals as the principal feed ingredient of beef-diets, feeding whole urea-treated cereal grains, as an alternative to mere urea addition, may favor the efficient rumen N utilization. Moreover, a variety of SRU products available on the market require being tested. The *in vitro* gas-test appears to be a promising technique to detect ammonia release patterns in SRU products with respect to rumen metabolism.

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Table 5.1. Variations of the diet cost (EUR), by the substitution of 1 kg of soybean meal (SBM) with a corn mixture: urea (88:12, 46% CP on DM basis) according to minimum (Min), medium (Med) and maximum (Max) commodity prices for SBM, corn meal (Corn) and urea in the last 5 years (2012-2017)[1].

	Corn	Urea	Corn + Urea	SBM		
				Min	**Med**	**Max**
		Min		0,32	0,40	0,57
Min	0,15	0,31	0,17	0,15	0,23	0,39
Med	0,19	0,31	0,21	0,11	0,19	0,36
Max	0,28	0,31	0,28	0,04	0,11	0,28
		Med				
Min	0,15	0,426	0,18	0,13	0,21	0,38
Med	0,19	0,426	0,22	0,10	0,18	0,34
Max	0,28	0,426	0,29	0,02	0,10	0,27
		Max				
Min	0,15	0,52	0,19	0,12	0,20	0,37
Med	0,19	0,52	0,23	0,09	0,17	0,33
Max	0,28	0,52	0,31	0,01	0,09	0,26

[1]According data of CLAL.it (https://teseo.clal.it/clal20/en/index.php)