# Investigating Ruminal Nitrogen Metabolism

*In Vitro* and *In Vivo* Studies Using $^{15}$N-labelled Forage Nitrogen Fractions

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Cover: Growing for science, red clover, timothy, silo and N fractionation (photo: M. Vaga)

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## Abstract

Nitrogen losses from dairy production have negative impacts on the environment and on production economics. In Northern Europe, dairy cattle are mainly fed forage-based diets supplemented with protein feeds such as rapeseed meal. This thesis sought to develop and evaluate new methods for forage protein evaluation and to improve the value of locally produced protein feeds.

Ruminal metabolism of the grass silage soluble nitrogen (SN) fraction and of $^{15}$N-labelled ammonia was modelled based on degradation kinetics determined *in vivo*. Kinetic models were developed to provide an optimal fit between predicted and observed values of $^{15}$N atom excess in different rumen N pools. Microbial N synthesis was around 20% greater with soluble non-ammonia N (SNAN) than with ammonia N, proving that SNAN stimulates microbial growth. The protozoal contribution to direct protein degradation in cows fed at high intake was rather small, with around 95% of protozoal N originating from bacterial N. An estimated 12.5% of SNAN escaped ruminal degradation.

The *in vitro* degradation rates of SN fractions were very similar to those observed in other *in vitro* studies, but much lower than those measured *in vivo*. Immediate predicted microbial uptake of SN in grass silage was similar to that observed *in vivo* (20% and 14%, respectively), but significantly greater for red clover SN fractions (> 50%). Proportionally more grass silage insoluble N was degraded (detected) to ammonia N than of red clover, and of dried forages than of silage. Dried forage had a higher concentration of utilisable crude protein (uCP) than silage.

Heat treatment of legume seeds (field bean, lupin and peas) increased uCP concentrations, but high temperatures (over 140°C in oven or 120°C in autoclave for more than 30 min) also significantly increased the indigestible N fraction in the feeds.

The *in vitro* method shows potential for estimating uCP concentrations, but requires further development to estimate degradation of proteins *in vivo*. Using $^{15}$N-labelled forages is a promising method for future protein metabolism studies.



*Keywords:* dairy cow, modelling, utilisable crude protein, silage, hay, microbial protein, soluble protein, *in vitro*, heat treatment, protein value



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# Contents

# List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I M. Vaga, M. Hetta and P. Huhtanen (2016). Effects of heat treatment on protein feeds evaluated *in vitro* by the method of estimating utilisable crude protein at the duodenum. *Journal of Animal Physiology and Animal Nutrition*, doi:10.1111/jpn.12646.

II S. Ahvenjärvi, M. Vaga, A. Vanhatalo, and P. Huhtanen (2017). Ruminal Metabolism of Grass Silage Soluble N fractions. *Journal of Dairy Science* (submitted)

III M. Vaga and P. Huhtanen. *In vitro* investigation of the ruminal digestion kinetics of different nitrogen fractions of $^{15}$N-labelled timothy. (manuscript).

IV M. Vaga and P. Huhtanen. *In vitro* investigation of the ruminal digestion kinetics of different nitrogen fractions of $^{15}$N-labelled red clover. (manuscript).

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The contribution of Merko Vaga to the papers included in this thesis was as follows:

I Planning the experimental work jointly with the co-authors, performing the experiments, processing the data and writing the manuscript

II Processing the data and writing the manuscript

III Planning the experimental work jointly with the co-authors, performing the experiments, processing the data and writing the manuscript

IV Planning the experimental work jointly with the co-authors, performing the experiments, processing the data and writing the manuscript

# Abbreviations

| | |
|---|---|
| $^{15}$NEP | $^{15}$N atom% excess pool |
| AA | Amino acid |
| ADIN | Acid detergent insoluble |
| ATP | Adenosine triphosphate |
| AUC | Area under the curve |
| CP | Crude protein |
| DM | Dry matter |
| EPD | Effective protein degradability |
| FAS | Formic acid treated silage |
| ISN | Insoluble N |
| ME | Metabolisable energy |
| NAN | Non-ammonia N |
| NDF | Neutral detergent fibre |
| NDIN | Neutral detergent insoluble N |
| OM | Organic matter |
| PPO | Polyphenol oxidase |
| RUP | Rumen undegradable protein |
| SN | Soluble N |
| SNAN | Soluble non-ammonia N |
| uCP | Utilisable crude protein at the duodenum |
| $uCP_{16}$ | uCP at duodenum estimated with assumed 16 h retention time |
| uSCP | Utilisable substrate CP at duodenum |
| $uSCP_{16}$ | uSCP at duodenum estimated with assumed 16 h retention time |
| UTS | Untreated silage |
| VFA | Volatile fatty acids |

# 1 Introduction

## 1.1 Ruminants and humans

Ever since the domestication of cattle and sheep around 7000 BC, ruminants have played an important role for human livelihoods (Van Soest, 1994). Ruminants are a significant source of food and provide various other necessary commodities such as clothing materials, fertilisers, transport and traction. Therefore in some cultures domestic ruminants are a symbol of economic status and wellbeing. The successful symbiotic relationship between humans and ruminant livestock developed largely because ruminants did not compete for food with humans. For a very long time in history, grass, straw and other fibrous plant materials that are not suitable for human food have been the main source of food for ruminants. Ruminants in return provide humans with high-quality proteins in the form of milk and meat. Furthermore, there is evidence that animal proteins, especially milk proteins, have higher biological value for humans than human-edible plant proteins (Hoffman and Falvo, 2011). Human-edible plant proteins are food proteins that are typically used directly for human consumption and have nutritional value for humans, such as grains and legume seeds. Today the importance of quality food production is as high as ever. In 2014, over 326 million beef animals and 813 million dairy animals (buffalo, cattle, sheep, goats) provided over 68 million tonnes of meat and over 801 million tonnes of milk for humans on a global scale (FAOSTAT, 2014). Beef and sheep meat make up 26% of the total amount of meat produced in the world from livestock (FAOSTAT, 2014). The intensification of livestock production has led to higher yields, but also to changes in agricultural practices. To achieve high productivity in ruminant livestock, high-quality, human-edible grain and protein feeds have been included in their diet (Huhtanen and Hristov, 2009). Compared with other domestic animals such as swine and poultry, the conversion ratio of total protein for lamb and beef production is many times higher, on average 30 and 24 kg/kg compared with

4.3 and 3.0 kg/kg total protein for pig and poultry meat (Figure 1), and total protein conversion for milk is about 5.6 kg/kg total protein (Wilkinson, 2011). However, the conversion factor of human-edible protein to animal proteins used in milk and beef production is only 0.71 and 0.9 kg/kg human-edible protein, compared with 2.6 and 2.1 kg/kg for pig and poultry meat, respectively (Wilkinson, 2011). It is possible to produce high-quality protein in growing or lactating ruminants even without inputs of human-edible proteins, *e.g.* by keeping the animals on pasture without any concentrate supplements. Therefore, the much higher conversion ratio of human-edible protein to animal proteins in other livestock diets compared with ruminants makes them less efficient animals to produce quality dietary proteins for humans. These ratios of course change depending on the intensity and global location of the production systems.

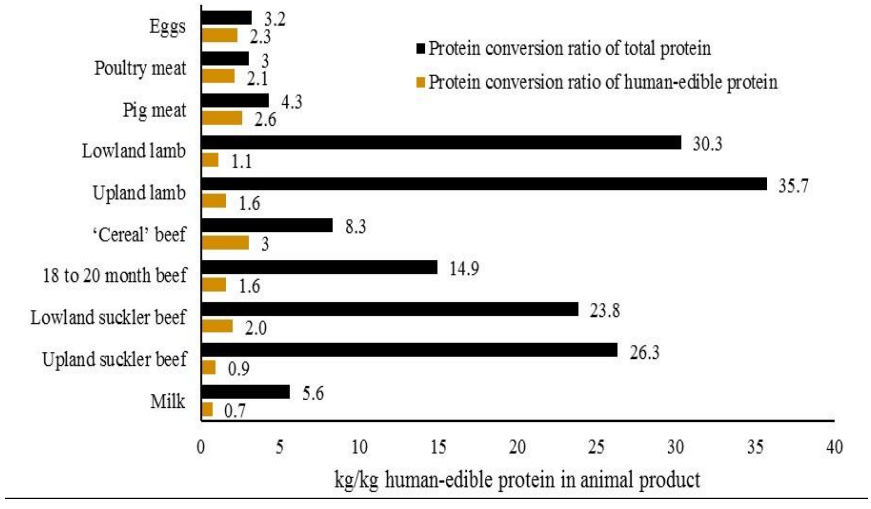


*Figure 1.* Total and human-edible protein conversion ratio (input per unit of output) (Source: Wilkinson, 2011)

As for every other living organism, protein is an essential feed component for ruminants. Proteins are needed for cell growth and production. With intensive farming practices and growing awareness of the need for environmental sustainability, official demand for efficient utilisation of feed proteins and reduced nitrogen (N) excretion from dairy farming is growing (EU Directive 2000/60/EC). The overproduction of milk and meat in Europe and the USA in recent years (Eurostat, 2016; USDA, 2017) shows that it is not necessary to reach maximum production, but rather to improve the efficiency. From a farmer's point of view, efficient use of N is important for profits, since

protein is the most expensive feed component and usually has a high impact on production.

## 1.2 The digestive tract and its function for nitrogen metabolism

The rumen is the largest and the most complex compartment of the ruminant digestion system, where the feed is degraded by microorganisms that live in symbiosis with the ruminant animal (Figure 2). Microbial digestion of structural carbohydrates is what gives ruminants the ability to utilise low-quality feeds more efficiently than monogastric animals. However, the

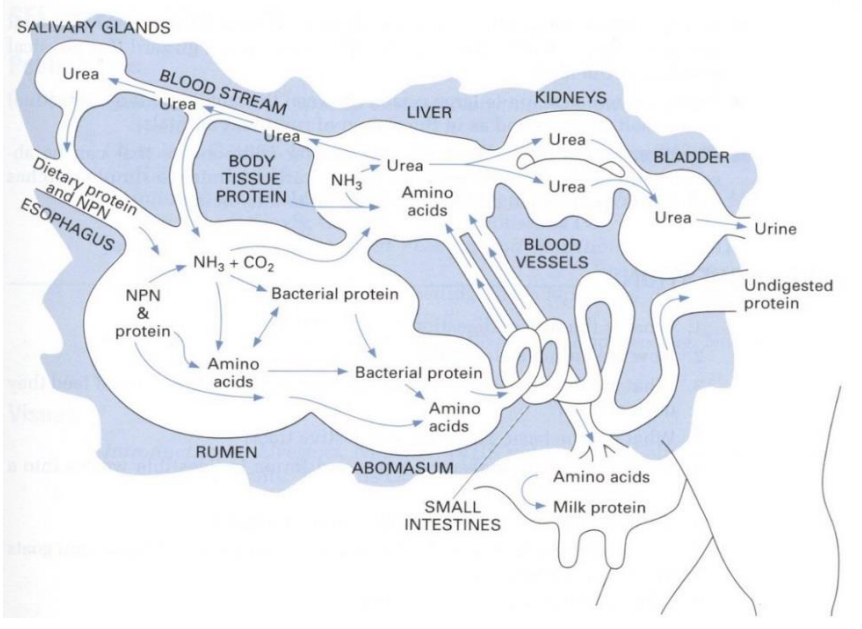


*Figure 2.* Protein pathways in the ruminant. (Source: J. Bryant and B. R., Moss Montana State University)

marginal efficiency of utilisation of supplementary protein is low in dairy cows fed typical Nordic high-quality diets. For example, it has been shown that only 10% of incremental crude protein (CP = N × 6.25) intake from soybean concentrate is converted to milk protein (Huhtanen *et al*., 2011). In growing cattle, marginal protein efficiency is even lower (Huuskonen *et al*., 2014). These low marginal responses question the sustainability of using high-quality protein supplements such as soybean meal for ruminants with low efficiency. The poor efficiency of protein utilisation may lead to overfeeding of expensive feed supplements, inefficient utilisation of protein feeds by cows and increased N excretion to the environment. However, in order to maintain reasonably high

production with dairy cows, an optimal level of high-quality protein and energy is needed.

According to NRC (2001), up to 23% CP in feed is theoretically needed to maximise milk production by dairy cattle. However, feeding such large protein quantities would lead to reduced milk N use efficiency (Figure 3; Huhtanen and Hristov, 2009). Meeting the N requirements of rumen microbes with minimal ammonia losses from the rumen and meeting the amino acid (AA) requirements of the host animal with a minimal amount of rumen-undegradable protein (RUP) is optimum from a biological point of view (Schwab *et al.*, 2005).

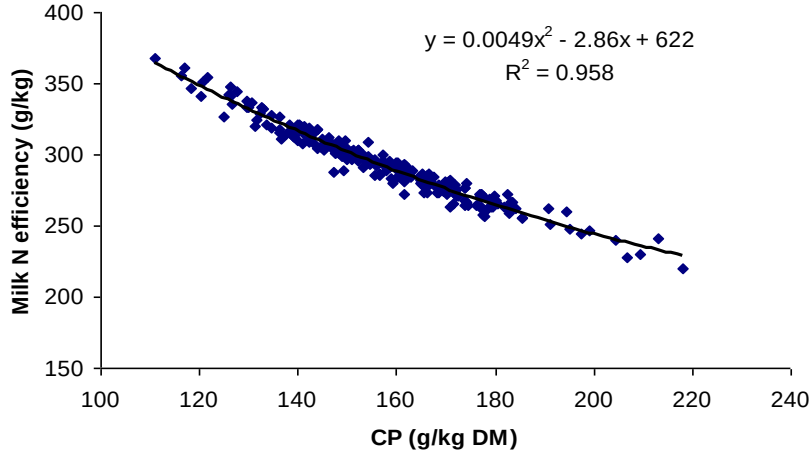


*Figure 3.* Relationship between CP concentration in the diet and milk N efficiency (Huhtanen and Hristov, 2009)

Ammonia production is a biological step in protein degradation, but sufficient energy is needed for microbial protein synthesis from ammonia (Russell *et al.*, 1983). Therefore there is no need to feed excessive amounts of protein feeds to dairy cows and expect maximum milk yields. Excessive protein supply and/or lack of energy supply to cows lead to overproduction of ammonia N and increased excretion of N in faeces and urine (NRC, 2001). Protecting proteins from microbial degradation in the rumen by processing methods (*e.g.* heat) may lead to low microbial efficiency (Van Straalen and Tamminga, 1990) and the increase in the supply of absorbed AA may be less than expected. Microbial protein synthesis is important, as it provides the majority of protein supplied to the small intestine, accounting for 50-80% of total absorbable protein in the small intestine (Storm and Ørskov, 1983). As ruminants and rumen microbes have evolved together, the AA composition of

microbial protein should be more favourable for the ruminant animal than protein from many other sources. However, the benefits of converting feed protein to microbial protein are lower when high-quality protein is fed.

## 1.3 Nitrogen metabolism in ruminants

What makes ruminants different from most other mammals is the presence of the fore stomachs. The whole digestion system that is directly involved in the digestion process can be divided into the following compartments: rumen, reticulum, omasum, abomasum, small intestine, caecum and large intestine or hindgut. From a ruminant nutrition viewpoint, the most important compartments for protein digestion and N absorption are the rumen, small intestine and hindgut. Very different mechanisms are involved in the digestion process in various parts of the digestive tract and the presence of the rumen makes ruminant metabolism complicated to understand. For example, ruminal metabolism modifies dietary CP both quantitatively and qualitatively, making evaluation of the protein value of feeds and diets more complicated in ruminants than in simple-stomached animals.

Supporting microbial synthesis is paramount for ruminants, as microbes provide the necessary AA for the animal without requiring AA themselves. The microbial AA composition remains relatively constant despite the diet (Storm and Ørskov, 1983). Therefore dairy cows can survive and produce moderate levels of milk with diets that do not contain AA nitrogen. Virtanen (1966) conducted experiments where dairy cows were fed urea as a sole N source for extended period of times with cows still producing 4200 kg milk/year, well comparable to production level on practical farms at that time, without any ill effects. This demonstrates the importance of ruminal microbiology and the symbiosis with its host animal.

### 1.3.1 Rumen microbiology

The rumen and the reticulum are the first major compartments for digestion. However, the digestion of feed is not performed by the animal itself, but by a complex microbial community consisting of bacteria, protozoa, archaea and fungi (Van Soest, 1994). This complex community lives in symbiosis with the host animal, providing the host with energy in the form of volatile fatty acids (VFA) and AA through microbial degradation of plant material and *de novo* cell synthesis. Bacteria account for about half of the microbial mass in the rumen (Ørskov and Ryle, 1990), but they do most of the fermentation work and contribute the majority of microbial protein flowing to the duodenum (Sylvester *et al.*, 2005). The number of different bacteria species present in the

rumen is large and new species are found all the time (Bryant, 1957; Firkins and Yu, 2008). A simplified grouping of bacteria would be according to their general functionality for amylolytic, cellulolytic and proteolytic bacteria and then secondary fermenters who utilise the end-products of primary fermentation (Van Soest, 1994).

Protozoa are the second largest group of microorganisms and form about 20-50% of microbial biomass. Despite being such a large group, their contribution to total microbial protein flow from the rumen is relatively small, due to relatively long retention time in the rumen. The contribution of protozoa to microbial N flow was on average 23% when determined using different techniques, according to a review by Hristov and Jouany (2005). The contribution of protozoa to degradation is also low and is limited to breakdown of small insoluble particulate proteins (Hino and Russell, 1987) with some peptide and AA breakdown (Broderick *et al*., 1991). With small amounts of soluble protein, AA and peptides in the rumen, the contribution of protozoa to dietary protein degradation can be very small. It is widely assumed that ruminal protozoa do not assimilate ammonia directly, but instead rely on a combination of ingested bacteria and food protein for their N requirements (Williams and Coleman, 1992). The main role of protozoa in influencing digestion is the engulfment and digestion of bacterial and fungal cells (Walker *et al*., 2005). This increases microbial lysis and is considered a wasteful cycle of bacterial protein breakdown. Removal of the protozoa (defaunation) by chemical therapy (*e.g*. monensin) or freezing the rumen contents (requires rumen evacuation) usually results in decreased diet digestibility and therefore any benefits from increased microbial protein flow can be lost (Koening *et al*., 2000; Walker *et al*., 2005). Protozoa are a natural part of the rumen microbial community and none of the experimental antiprotozoal agents tested have made it into practical use (Williams and Coleman, 1992). Most of the studies investigating the effects of defaunation have been conducted in sheep fed at maintenance level.

The presence of anaerobic fungi in the rumen was discovered relatively recently (Orpin, 1975; Gordon and Phillips, 1998), as they were previously mistakenly considered to belong to the protozoa (Van Soest, 1994). The role of fungi in degradation mainly involves fibre break-down by very highly active cellulolytic enzymes (Lee *et al.*, 2000). Most studies conclude that fungi have some proteolytic activity, but that their contribution is low compared with that of other microbial communities (Walker *et al*., 2005). Their biological significance can be higher in specific instances, *e.g*. in breakdown of resistant fibre-bound proteins (Yanke *et al*., 1993).

About 0.3-3.3% of rumen microbial is archaea which are found free in the rumen fluid, attached to particulate material and rumen protozoa, associated as endosymbionts within rumen protozoa, and attached to the rumen epithelium (Janssen and Kirs, 2008). The ruminal archaea are strictly anaerobic methanogens using $H_2$ for growth and often formate for energy (Hungate *et al.*, 1970). Therefore their role in rumen metabolism is more important as secondary fermenters and do not contribute to direct degradation of feed (Henderson *et al.*, 2015). Since archaea contribute to $CH_4$ production in ruminants the interest in them is grown in connection to mitigation strategies (Hook *et al.*, 2010)

### 1.3.2 Proteolysis

Proteolysis is the hydrolysis of protein to AAs and non-protein nitrogen (Van Soest, 1996). This process of protein breakdown starts already when the forage is cut in the field, involving the activation of proteolytic plant enzymes in the forage and microbial degradation (Papadopoulos and McKersie, 1983). Proteolysis is hampered by drying the grass for hay, but in the process of ensiling the fresh forage goes through the first major step of microbial degradation. In the rumen, dietary protein breakdown is a complex process involving different microorganisms and step-wise degradation to peptides and AA with ammonia as the end-products (Broderick *et al*., 1991). A schematic representation of the process of protein degradation by rumen microbes is provided in Figure 4.

All ruminal microbes contribute to protein breakdown to some extent (Pfeffer and Hristov, 2005). However, bacteria are the main contributors to protein breakdown in the rumen. Up to 50% of the rumen bacteria may have proteolytic ability (Fulghum and Moore, 1963; Prins *et al*., 1983). The degradation of dietary proteins in the rumen begins with adsorption of soluble proteins to bacterial cell surfaces and bacterial attachment to insoluble proteins. Protozoa also exhibit proteolytic activity, but their role in rumen proteolysis is small (Nugent and Mangan, 1981). Microbes degrade dietary proteins to oligopeptides, small peptides, AA and ammonia. In experiments performed *in vitro* with casein it has been shown that there is an increase in peptides and a simultaneous decrease in proteins in the rumen, with gradual increases in AA concentration (Russell *et al*., 1983). Peptides are broken down in steps from oligopeptides to tri- and dipeptides and then further to AA, which are rapidly deaminated by microbes to ammonia. The concentration of free AA increases rapidly within 2 h after feeding, but then decreases rapidly within 2 hours thereafter (Brito *et al*., 2006). The increase in AA after feeding is due to a large proportion of the soluble protein in silage being converted to free AA (Hristov

and Sandev 1998). Soluble non-ammonia N (SNAN) entering the omasum mainly consists of peptides, suggesting that the rate of peptide breakdown is the main rate-limiting step in protein degradation (Choi *et al*., 2002; Reynal *et al*., 2007). The final step in breakdown of dietary proteins is the breakdown of AA to ammonia. Ammonia N is the major source of N for microbial protein synthesis.

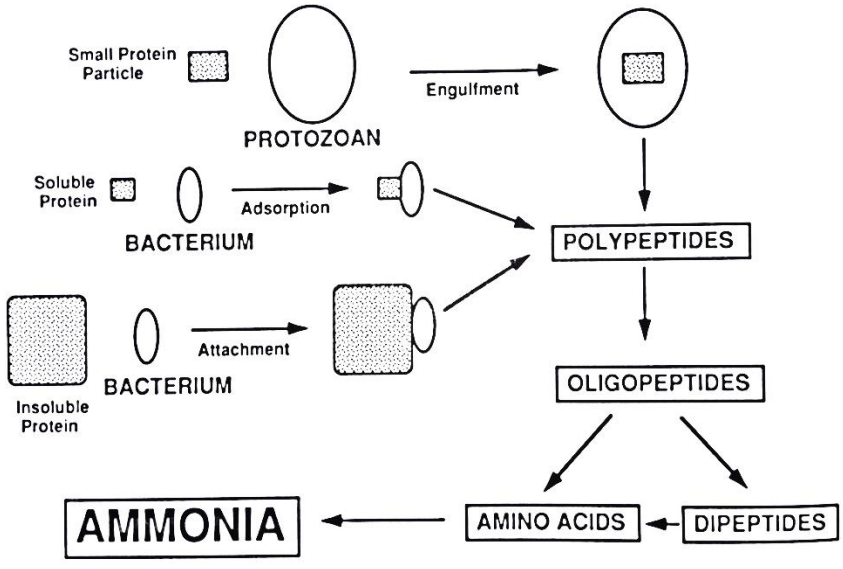


*Figure 4.* Pathway of degradation of intact protein to ammonia by microorganisms in the rumen (Source: Broderick *et al*., 1991).

### 1.3.3 Microbial synthesis

The end-products of protein degradation (peptides, AA and ammonia) meet the N requirements of rumen microbes (Schwab *et al*., 2005), while excess ammonia N is a product that the animal needs to excrete or recycle. With sufficient energy available, ammonia alone is an adequate source of N for protein synthesis (Leng and Nolan, 1984). It is important to have optimal concentrations of ruminal ammonia N to maintain maximised protein synthesis. Ruminal ammonia N concentrations of 50 to 85 mg/L have been suggested to be optimal to achieve maximum protein yield (Satter and Slyther, 1974; Kang-Meznarich and Broderick, 1980). The efficiency of microbial protein synthesis also depends on the balance between available dietary CP and energy (Russell *et al*., 1983). Newbold and Rust (1992) observed reduced

bacterial growth *in vitro* when either N or carbohydrates were restricted. Consequently, both carbohydrates (energy) and N need to be present in the rumen to maximise microbial growth. Synchronisation of available CP and energy is suggested (Sinclair *et al*., 1995), but this is not an easy task, with research giving confounding results without provided perfect solutions (Sauvant and van Milgen, 1995). According to a review by Chamberlain and Choung (1995), the benefits of dietary synchronisation of energy and protein supply are marginal. Since microbial protein synthesis requires energy, excessive protein concentration in the diet does not improve production, but just increases ruminal ammonia concentration. The excess ammonia N is absorbed through the rumen wall, converted to urea in the liver and eventually excreted as urea. However, urea recycling can also be a useful survival strategy for ruminants when their diet is low in N. Ruminants are able to transfer urea through the blood back to the rumen via the salivary glands, where the urea in the saliva supports microbial protein synthesis, thereby supplying the host animal with valuable AA (Kennedy and Milligan, 1980). For the animal, ammonia absorption is far less valuable than converting it to microbial protein. The post-ruminal digestibility of microbial protein is relatively consistent at around 78-85% and therefore highly valuable (Hvelplund, 1985). Most evaluation systems assume a constant value of 85% for microbial protein digestion. Ammonia absorption is negatively affected by decreased ruminal pH, which is a delay function of ammonia absorption after a meal to favour microbial uptake (Sauvant and Milgen, 1995). Ammonia may be enough for microbial N synthesis, but microbes are also the only means of converting non-ammonia N (NAN) into high-quality protein (Van Soest, 1994). Walker *et al*. (2005) suggest two possible benefits from AA supplementation: improved feed intake due to increased rate of microbial fermentation and increased growth yield of microorganisms. The presence of high concentrations of peptides and AA in the rumen has been shown to stimulate microbial protein synthesis. According to the Cornell net carbohydrate and protein system (Russell *et al*., 1992), the maximum relative improvement in the efficiency of microbial N synthesis is 0.187 for bacteria utilising non-fibrous carbohydrates. Broderick and Reynal (2009) replaced solvent-extracted soybean meal with lignosulphonate-treated soybean meal and urea in dairy cattle diets providing similar amounts of rumen-degradable and RUP. They found that microbial N flow decreased with an increasing proportion of treated soybean meal and urea, whereas ammonia N concentration increased, indicating that microbes were not limited by the ammonia N supply.

Increased feeding level increases passage rate and decreases microbial maintenance costs. The reduced ruminal retention time also increases the flow

of RUP. With high-quality dietary proteins, decreased degradation is generally considered beneficial (Van Soest, 1996). Predation on bacteria by ciliate protozoa leads to higher microbial protein turnover in the rumen. Increased passage rate leaves less time for predation and decreases ruminal recycling of bacterial protein (Walker *et al*., 2005). Diet quality and composition influence microbial synthesis in many ways. It is often claimed that ensiling forages has a negative influence on ruminal microbial protein synthesis. However, some of the few studies comparing hay and silage seem to suggest higher microbial N flow to the duodenum with silage (Holden *et al*., 1994; Martineau *et al*., 2007). Jaakkola and Huhtanen (1993) observed on average higher duodenal flow of microbial N with silage diets compared with hay diets. High-quality supplements of AA can increase microbial growth (NRC, 2001), but since proteins provide less energy (adenosine triphosphate, ATP) microbial synthesis from ammonia may be reduced. High-quality proteins also provide more digestible AA when they escape from ruminal degradation and are digested in the small intestine.

### 1.3.4 Rumen-undegradable crude protein

Rumen-undegradable CP contains potentially digestible fractions only digestible post-ruminally, but also some completely indigestible CP. Some of the proteins escaping ruminal degradation are digested and absorbed in the small intestine, but the amount depends strongly upon the source of RUP. Forage proteins that escape microbial degradation are often poorly digested, but processed grains and protein feeds can have higher value when digested in the intestine than in the rumen (Owens *et al*., 1979). Edmunds *et al*. (2013) compared various forages and estimated that about 10-20% of forage CP is RUP. However, they found that the proportion of acid-detergent insoluble N (ADIN) in RUP, which is considered to be also indigestible in the lower digestive tract (Van Soest, 1994), is very high in forages and this would significantly decrease the value of RUP. Many studies have evaluated the RUP value of grain- and protein-rich dietary supplements (*e.g*. Santos *et al*., 1998; Ipharraguerre and Clark, 2005). Rumen-undegradable CP consists mainly of insoluble N fractions (NRC, 2001), but more recent studies have also shown significant escape of soluble fractions from microbial degradation in the rumen (Ahvenjärvi *et al*., 1999; Choi *et al*., 2002; Reynal *et al*., 2007). Since both peptides and AA can be absorbed in the small intestine, the escaped soluble N is valuable (Webb and Bergman, 1991). Thus RUP provides additional valuable protein to the animal and increases milk protein production, but only when ruminal microbial protein requirements are met (Wright *et al*., 1998). In fact, RUP is often considered more valuable than rumen-degradable protein

(Ørskov, 1976), but this is probably overvalued (Van Soest, 1996). Most protein supplements high in RUP are of animal origin, such as fishmeal, or are heat-treated in a process to protect them against ruminal degradation. In a short communication, Wang *et al.* (2008) reported increased N urinary excretion with higher rumen degradable protein to RUP ratio, but no effect on milk yield. Schwab *et al.* (1976) used AA infusion to the abomasum in six trials, but found no significant effect on milk production except for sodium caseinate, which increased fat-corrected milk production and milk protein concentration. The reviews by Santos *et al.* (1998) and Ipharraguerre and Clark (2005) concluded that increased RUP concentration in the diet compared with solvent-extracted soybean meal only marginally increased the total NAN outflow. The overall benefits with increased essential AA flow to the abomasum were lessened due to decreased microbial N flow. Furthermore, only marginal increases in milk or milk protein yield were found with high RUP supplements, mostly with feeds of animal origin which are no longer permitted for use in ruminant feeds in the EU.

### 1.3.5 Small intestine

Peptic digestion, similar to that in non-ruminants, occurs in the abomasum, with the difference that the digesta passage in ruminants is a continuous process. To enable a high rate of microbial protein passage, secretion of lysozyme accompanies the acid and pepsin that quickly lyse microbes. The lysozyme in cows is approx. six-fold more resistant to pepsin inactivation than that found in monogastric animals and therefore protects microbial AA from pepsin digestion (Dobson *et al.*, 1984). The main activity of the small intestine in ruminants is to absorb amino acids and peptides (Webb and Bergman, 1991). Different amino acids are absorbed at different locations in the intestine (Williams, 1969), indicating that the intestine is adapted to maximise AA absorption according to specific transporters of AA. In general, the transport of AA through the intestine mucosa is divided into simple diffusion, facilitated diffusion and active transport (Webb and Bergman, 1991). Peptide absorption has been proven to occur in the small intestine (Newey and Smith, 1960). Later studies have shown that a peptide and AA mixture has up to 80% higher absorption rate than AA alone, but peptides are absorbed against a concentration gradient, making this an energy-dependent process (Addison *et al.*, 1975). There are some indications of peptide absorption from the omasum (Webb *et al.*, 1992), but the significance of this function has not been studied.

### 1.3.6 Hindgut

Hindgut fermentation in ruminants is a similar process to that which occurs in monogastric mammals. Undigested substrates such as neutral detergent fibre (NDF), starch and proteins go through a secondary microbial degradation process similar to that in the rumen (Feldhamer *et al*., 2014). The similarity of the processes has been demonstrated by the fact that ruminant faecal matter is sometimes used as an *in vitro* inoculant and produces a similar degradation pattern to typical rumen fluid (Mould *et al*., 2005; Ramin *et al*., 2015). Protein sources available for hindgut fermentation are undigested feed and microbial N, endogenous N originating from the animal and ammonia N from recycled urea N. Fermentation end-products (and ammonia) can be absorbed from the hindgut, but synthesised microbial mass is lost in faeces. However, the importance of this process is when estimating total protein and nutrient digestion. Not taking this into account would lead to overestimation of the concentrations of metabolisable protein available to the animal.

## 1.4 Methods for measuring protein value

Microbial protein, RUP and, to a lesser extent, endogenous CP contribute to supplying metabolisable protein to the small intestine (NRC, 2001). All modern feed evaluation systems need accurate parameter estimates of the microbial N and RUP supply. The three general techniques used to measure feed protein quality and ruminal protein metabolism are the *in situ* or nylon bag method, *in vitro* rumen simulation and the *in vivo* whole animal method (Weiss, 1994).

### *1.4.1 In vivo* methods

Evaluating protein degradation *in vivo* should be the preferred option, as the conditions are most natural and therefore the results represent most accurately the conditions for the animal. The requirements for this method are accurate feed intake and output measurements, cannulated animals and reliable digesta and microbial markers. Abdominal and/or duodenal cannulas in addition to the more widely used rumen cannula are often used. In principle, all methods for estimating protein metabolism are similar. The total nutrient intake of the animal is recorded and the flow and composition of the nutrients are estimated by taking samples through cannulas surgically inserted into the digestive tract. Measurements are made as frequently as possible/necessary to estimate the rate of digestion and passage of digesta. However, omasal sampling (Huhtanen *et al*., 1997) through the rumen cannula has become the preferred method for

estimating microbial and RUP flow from the rumen, as it is less invasive and demonstrates good repeatability (Broderick *et al*., 2010). The greatest problem with all *in vivo* methods is the reliability of the digesta markers used (Lopez, 2005). The marker must not interfere with the digestion process, should not be absorbed (unless that's the purpose) and must follow accurately the dietary materials with which it is associated. Various studies have compared different digesta markers (*e.g*. Ahvenjärvi *et al*., 2003; Hansson, 2004; Kozloski *et al*., 2014) and microbial markers (Broderick and Merchen, 1992; Reynal *et al*., 2005). Most of these studies have concluded that the best results can be obtained using a combination of double markers (Facihney *et al*., 1975) or even triple markers (France and Siddons, 1986). The most frequently used marker combination today is probably CoEDTA as a liquid phase marker in combination with ytterbium (Yb), chromium (Cr) and/or indigestible NDF as particle markers and $^{15}N$ salts and purines as microbial markers (Broderick and Merchen, 1992; Ahvenjärvi *et al*., 2003). An advantage of $^{15}N$ compared with other markers is that it is a stable isotope with high analytical precision and that it permits determination of other N kinetics variables, *e.g.* the contribution of ammonia N to microbial N synthesis. The isotope $^{15}N$ is naturally occurring in very stable concentrations everywhere in the environment and in all flora and fauna that contain any N. This provides the possibility to use $^{15}N$-enriched (excess $^{15}N$ over natural abundance) fertilisers or chemicals to label proteins in feeds or ammonia N, which can then be utilised in detailed N metabolism studies on animals and plants (Hristov *et al*., 2001; Huss-Danell and Chaia, 2005; Hristov *et al*., 2005). However, the partitioning of $^{15}N$ within the animal creates differences in natural delta values between substrates such as ruminal ammonia, blood urea, microbial N and urine and others, so these differences need to be considered (Cheng *et al*., 2012). In plant and environmental sciences, the atmospheric natural abundance of $^{15}N$ and delta values of different plants, soils and animal faeces have been widely used to measure N transfer (Unkovich *et al*., 2008; Sjörgersten *et al*., 2010).

Overall, the *in vivo* methods for evaluating protein degradation provide valuable data and, at least for time being, remain the 'gold standard' against which to validate other methods. However, the amount of work and the need for surgically modified animals make *in vivo* methods too costly and impossible to use in routine work.

### *1.4.2 In situ* methods

The most widely used technique for estimating ruminal feed protein degradability is the *in situ* method. A method involving incubation of small amounts of feed in silk fibre bags within the rumen of sheep was first used by

Quin *et al*. (1938). However, the mathematical tools to estimate effective protein degradability (EPD) using *in situ* techniques developed by Ørskov and McDonald (1979) were the key factor behind *in situ* methods being such an extensively used method today (Hvelplund and Weisbjerg, 2000). The equation to estimate EPD from kinetic parameters is as:

$$EPD = A + B \times c / (c + k),$$

where A is the proportion of CP degraded at time = 0, B is the fraction of protein that is degradable in infinite time, c is the degradation rate of fraction B and k is the passage rate of fraction B. NRC (2001) use also fraction C in the equation to describe completely undegradable fraction. The general concept of the *in situ* method is simple compared with other types of methods. Small amounts of feeds are weighed into nylon bags and placed into the rumen through a rumen cannula. The assumption is that the conditions in the bags are similar to those in the surrounding rumen. The disappearance of the contents of the bags is measured at different time points, usually up to 24-48 h (longer for forages) to determine the rate and extent of degradation. This is relatively easy to use and provides the possibility to evaluate many feeds routinely, which are the main reasons contributing to the popularity of this method. Another advantage is that the standard *in situ* method can be extended by using mobile bags to estimate total tract digestibility of RUP (Hvelplund *et al*., 1985; Vanhatalo *et al*., 1996). Most feed evaluation systems rely on the protein degradation parameters estimated *in situ*. However, almost every textbook description of the *in situ* method mentions several associated limitations (Broderick *et al*., 1991; Hvelplund and Weisbjerg, 2000). For example, the physical barrier of the nylon bag can restrict microbial access to the feed inside the bag compared with the surrounding digesta (Meyer and Mackie, 1986). Huhtanen *et al*. (1998) found the activity of cell wall-degrading enzymes within the bags to be only 35% of that in the surrounding rumen digesta. Moreover, microbial contamination of undegraded protein particles in the bag causes underestimation of protein degradation (Varvikko and Lindberg, 1985; Nocek and Grant, 1987). This is not a major problem for protein-rich feeds, but causes significant errors in prediction when using forages or other feeds with low CP and high NDF concentrations. Much research has been done to determine the best possible bag material characteristics for different analyses (Nocek, 1988; Valente *et al*., 2015). Another important limitation of the *in situ* method is the inability to estimate degradation of soluble N fractions of feeds. The soluble N and small feed particles escape the bags without being degraded and are therefore assumed to be degraded at an infinite rate (Ørskov and MacDonald, 1979; NRC, 2001). However, studies using an omasal sampling technique (Choi *et al*., 2002; Reynal *et al*., 2007) or $^{15}$N-labelled feed N

fractions (Peltekova and Broderick, 1996; Choi *et al*., 2002;) have demonstrated that from 6.7% to around 20%, and possibly up to 30%, of SNAN from intake N escapes ruminal degradation (Hristov and Broderick, 1996). Part of the SNAN flowing to the duodenum can be of microbial origin (Choi *et al*., 2002; Reynal *et al*., 2007), but considering the rapid absorption of soluble proteins onto microbial surfaces, this may allow some of the soluble N to escape ruminal degradation before being incorporated into microbial cell protein. Broderick *et al*. (1988) compared degradation of several protein feeds and found that *in situ* measurements significantly underestimated the degradation rates of proteins compared with *in vitro* measurements. Taking into account that soluble proteins are not degraded at an infinite rate in protein evaluation systems is an improvement, but considering the wide range of degradation rates of various soluble protein sources, using constant degradation is still not ideal (Broderick *et al*., 1988; Huhtanen, 2017). These limitations and possible animal and diet differences contribute to low repeatability of the *in situ* method. Madsen and Hvelplund (1994) reported very poor reproducibility results from ring tests involving 23 laboratories in 17 different countries.

### 1.4.3 *In vitro* methods

The *in vitro* technique is another relatively easy method for determining protein degradation kinetics. The basic principle of the *in vitro* technique is to incubate the substrate of interest in a biological medium to imitate ruminal conditions. In general, the variety of techniques used can be simply divided into methods estimating parameters by analysing the end-substrate composition or methods measuring and analysing degradation end-products such as gases and ammonia production. The pioneering work of Tilley and Terry (1963) was based on incubating small amounts of feed in ruminal fluid collected through rumen cannulas, or sometimes through the oesophagus using a stomach tube (Ramos-Morales *et al*., 2014). Analysis of the residues at the end of digestion allows the extent of microbial degradation to be determined. A second step involving acid-pepsin to simulate post-ruminal digestion and estimate *in vivo* organic matter (OM) digestibility may be included (Tilley and Terry 1963; Van der Meer, 1986). A similar pepsin-cellulase technique has been often used to eliminate the need for rumen fluid, collection of which requires surgically modified animals. As the name suggests, the pepsin-cellulase technique uses enzymatic degradation to estimate *in vivo* OM digestibility (Jones and Hayward, 1975). Considering the mixed rumen microbial population involved in protein degradation, it is unlikely that enzymatic methods can give more than a good empirical correlation with *in vivo* rumen degradation (Broderick *et al*., 1991).

Despite some alternatives, the more realistic conditions that can be achieved with rumen fluid-based *in vitro* methods mean that such methods are generally the primary choice for scientific purposes. *In vitro* systems like that described by Cone *et al.* (1996) use gas production caused by microbial fermentation to estimate degradation rates at different stages and total degradation. Several modifications have been proposed over time to estimate *in vitro* protein degradation parameters (Raab *et al.*, 1983; Broderick, 1987; Krishnamoorthy *et al*., 1990; Lebzien and Voigt, 1999). A closed batch system *in vitro* removes the problem of escape of SNAN and does not limit microbial access to the feed, problems that occur *in situ*. However, these same advantages also set other limitations. *In vitro* methods lack the dynamic nature of the real rumen and the time required to reach a given digestibility or degradability can be shorter in a batch system because there is no escape of particles, and therefore predicted time is used to correspond to *in vivo* conditions (Varvikko and Vanhatalo, 1991). However, the lack of energy or substrate that arises without the balanced diet found *in vitro* may restrict the microbial efficiency. Raab *et al*. (1983) described a technique in which graded amounts of carbohydrates are added to rumen fluid and the resulting gas and ammonia production after 24 h incubation is used to calculate microbial protein. The method is based on extrapolation of the linear regression between ammonia N and gas production measured at different time points. The intercept then represents the ammonia N produced with zero carbohydrate addition and a degradation curve can be obtained. Theodorou *et al.* (1991, in Theodorou *et al.*, 1994) presented a method where substrate is incubated in gas-tight bottles and accumulated gas pressure is measured by a pressure transducer connected to a digital voltmeter. Repeating this procedure at regular intervals during fermentation enables gas accumulation profiles to be constructed (Theodorou *et al*., 1994). This method has been further modified by Pell and Schofield (1993) to include a computer monitoring system, to allow fully automated continuous gas recordings. Moreover, Karlsson *et al*. (2009) have modified the system to allow multiple ammonia samplings from the same incubation vessel.

Ammonia is the main end-product of microbial degradation, so it can be used for estimation of microbial activity. However, quantifying the extent and rate of degradation by measuring ammonia N produced *in vitro* poses several difficulties. For example, microbes utilise degradation products for microbial N synthesis, which results in underestimation of degradation (Broderick, 1994). Another *in vitro* method, the IIV technique, inhibits microbial protein synthesis during degradation using hydrazine sulphate and chloramphenicol (Broderick, 1987). The ammonia N and total AA production is then measured to calculate rumen degradable protein. A severe limitation of this system is the

accumulation of ammonia and inhibition of microbial N synthesis, which sets a time limit of 4 h to maximum 6 h. This is too short a time for estimation of the degradation of slowly degraded proteins. Another aspect to consider is that microbes do not degrade all proteins to ammonia, but a large proportion of proteins, especially soluble N, is directly taken up by microbes (Schwab *et al*., 2005). Hristov and Broderick (1994) further modified the system by including $^{15}NH_3$ to correct for microbial uptake. This method uses $^{15}N$ as a marker for ammonia N and allows degradation rates to be estimated by quantifying microbial N synthesis from ammonia N by determining changes in $^{15}N$ pool sizes of ammonia and/or non-ammonia (Ahvenjärvi *et al*., 2009).

Determining microbial N synthesis and feed protein degradation separately increases the prediction error by adding errors from different estimation methods. One solution to avoid the tedious work of separating microbial and undegraded feed N and problems associated with *in situ* measurements is to predict the total utilisable protein at the duodenum as a single value (Lebzien and Voigt, 1999; Edmunds *et al*., 2012). A procedure described by Steingaß and Südekum (2013) uses ammonia N concentration at the end of *in vitro* incubation to quantify utilisable CP (uCP), which represents the NAN flow (microbial + feed) to the duodenum. Edmunds *et al*. (2012) found that this method required different incubation duration when forages or concentrate feeds are evaluated. Estimating uCP for complete diets may be more preferable, as it better reflects the *in vivo* animal conditions and balances protein and energy availability for microbial protein synthesis. There are suggestions that *in vitro* fermentation is optimal with about 24% water-soluble carbohydrates in the diet (Burke *et al*., 2011). Using pre-incubation of rumen fluid, Karlsson *et al*. (2009) improved the repeatability and a good relationship between *in vitro* uCP and omasal NAN flow or milk protein yield has been observed (Gidlund *et al*., 2017, unpublished).

## 1.5 Influencing forage protein value

Protein quality is a major issue when feeding domestic animals. While ruminants are able to utilise different proteins sources, mainly forages, the efficiency of CP utilisation (milk N/N intake) is generally low, ranging from 16.4 to 40.2% (Huhtanen and Hristov, 2009). Furthermore, increasing dietary CP levels above the optimal range does not significantly increase milk yield (Wright *et al*., 1998) but instead increases urinary N output and milk urea N (Olmos Colmenero and Broderick, 2006), thereby decreasing the protein efficiency in the production system. Several studies have shown that increasing

dietary CP concentration from on average 160 to 190 g/kg CP does not further improve milk yield (Broderick, 2003; Groff and Wu, 2005; Olmos Colmenero and Broderick, 2006). Good quality forages, especially legumes, usually have a sufficient CP concentration to meet the above-mentioned requirements for dairy cattle. Unfortunately, the CP efficiency of forages is low, due to high degradability, fermentation losses in silages or particle losses during processing if stored as hay (McDonald, 1981; Van Soest, 1994).

### 1.5.1 Ensiling

Ensiling forages is the most cost-efficient method of forage preservation. However, the ensiling process usually impairs protein quality and some energy (ATP) is lost through VFA and lactic acid production in the silo (Chamberlain, 1987). The basic idea of ensiling is to preserve forages (or some grains like barley) in acidic conditions caused by microbial fermentation of sugars under anaerobic conditions. Lactic acid production is the preferred end-product of silage fermentation. However, other less favourable processes such as carbohydrate and protein degradation to VFA, ammonia and $CO_2$ also occur during ensiling (Van Soest, 1994). These losses represent energy losses for both rumen microbes and host animals, but in relative terms the losses of available energy are much greater for rumen microbes. Different additives can be used either to restrict bacterial degradation or to stimulate lactic acid production during ensiling in order to improve the forage quality. Most commonly, formic and/or propionic acid-based additives to inhibit fermentation have been used for the past few decades. Formic acid-based additives decrease the pH of silage faster, restricting microbial activity, while the main benefit of propionic acid is to inhibit yeast and moulds and thus improve aerobic stability of silage. Jaakkola *et al.* (2006) compared the effect of different application rates (0 to 6 L/t fresh herbage) of formic acid on grass silage and observed a decrease in total acids from 126 to 27 g/kg and in soluble N from 746 to 610 g/kg N. While the formic acid treatment in that study effectively decreased protein degradation in the silo, ruminal CP degradability and flow of feed N at the duodenum were not significantly influenced by formic acid level. However, the flow of microbial N to the duodenum increased significantly with formic acid treatment, from 49.0 to 65.4 g/d (Jaakkola *et al.*, 2006). Similarly, Nagel and Broderick (1992) reported 65% greater dietary NAN flow from the rumen of lactating Holstein cows when fed formic acid-treated alfalfa silage compared with untreated silage, while milk production increased from 29.2 to 32.6 kg/d and milk protein content increased from 2.74 to 2.90%. However, one of the reasons for these higher production responses is most likely the higher DM intake, and consequently higher N

intake, usually observed with formic acid-treated restrictively fermented silages (Derbishire *et al*., 1976; Huhtanen *et al*., 2003).

Wilting of forage material is typically included in silage making. Wilting increases the DM concentration in forage and lowers the extent of fermentation during ensiling (McDonald, 1981). The extent of wilting has different effects on DM intake and ruminal degradability. For example, Verbič *et al*. (1999) observed numerically lower DM intake with highly wilted silage than with un-wilted when fed *ad libitum* to sheep. However, sheep have different preference of forage type than cows and therefore wilting has generally results in higher silage DM intake with cows (Huhtanen *et al.,* 2007). Verbič *et al*. (1999) also observed decreased ruminal degradability, but increased microbial N flow, with silages with increased extent of wilting. Lower *in situ* ruminal protein degradability of wilted grass silage compared with unwilted silage has been reported (Tamminga *et al*., 1991). Hristov and Sandev (1998) reported lower *in situ* degradability of CP for wilted lucerne alfalfa silage compared with unwilted, but no differences in total DM disappearance. The effect of silage DM concentration on N flow was studied by Edmunds *et al*. (2014), who found significantly increased estimated uCP flow to the duodenum with increased silage DM. Dawson *et al.* (1999) compared formic acid- or bacterial inoculant-treated wilted and unwilted grass silages from four different cuts and found lower digestibility values for wilted silage in three out of four cuts. The positive production responses associated with wilting are most likely due to the higher DM intake observed on feeding wilted silage (Roffler *et al*., 1967; Dawson *et al*., 1999).

### 1.5.2 Hay

Preserving forage as hay is the traditional method. However, in recent decades hay making has been mostly abandoned in countries in Northern Europe, mainly due to the need for dry weather or huge drying facilities during processing, which makes hay making risky compared with silage making. However, it is often reported that the efficiency of microbial protein synthesis is higher in animals fed hay-based diets than in animals fed silage (Chamberlain, 1987; Thomas and Rae, 2013). The ruminal protein degradation in dairy cattle fed hay-based diets was about 5% lower than that in cattle fed silage-based diets (Jaakkola and Huhtanen, 1993). These authors also observed decreased ruminal ammonia N concentration in duodenally cannulated cattle when fed hay compared with silage in the diet and concluded that the main reason is probably that hay has a lower CP concentration than silage. However, they found no differences in NAN flow due to higher microbial protein flow. A greater difference (about 15%) in ruminal CP degradation of grass silage

compared with hay was observed in a study by Verbič *et al.* (1999) and this led to much higher microbial protein content with hay than with silage, although the direct-cut silage used was reported to be badly fermented in that study. Significant differences in protein fractions in hay and silages affect microbial degradation. Hristov and Sandev (1998) compared the chemical composition of lucerne silages and hay and found that the non-protein nitrogen concentration was approximately 33% lower in hay (206 g/kg N) compared with silage (619 g/kg N). The free AA and ammonia N concentration in that study was 59 and 8 g/kg N, respectively, in hay, compared with 444 and 109 g/kg N, respectively, in silage. Silage fermentation is said to reduce available true protein for rumen microbes, but the consistent reports of lower degradability of hay do not support this claim (Hristov and Sandev, 1998). Most importantly, the experimental evidence discussed by Huhtanen and Broderick (2016) provides no support for greater protein value of grass hay compared with well-fermented grass silage. Feeding experiments conducted with alfalfa have found higher milk protein production in cows fed hay diets than silage diets, but with supplemented diets no differences between hay and silage diets have been observed (Broderick 1995; Vagnoni and Broderick, 1997). However, no silage additives were used in the alfalfa diets in those studies. Overall, the production responses observed have been mainly related to DM intake. The difficulties in ensuring good quality in hay (weather conditions) and high losses in processing (Van Soest, 1994), especially with legumes, have led to decreased usage of hay in recent decades.

### 1.5.3 Legumes *versus* grass

Red clover (*Trifolium pratense*) is a good source of feed for dairy production. Being a legume, it can fix atmospheric $N_2$ and therefore requires less N fertiliser, making it especially beneficial for organic farming systems (Wilkins and Jones, 2000). As mentioned above, DM intake is generally accepted as the main factor behind production responses (Huhtanen *et al.*, 2007). Feeding red clover silage has also been shown to increase milk production compared with grass silage (Steinshamn, 2010).

The unique advantage of red clover compared with other forages is that it contains polyphenol oxidase (PPO), an enzyme that deactivates plant proteases (Jones *et al.*, 1995a; 1995b; Lee, 2014). This reduced activity of proteases prevents protein breakdown during ensiling and can increase true protein content in silages (Broderick *et al.*, 2001; Vanhatalo *et al.*, 2009). This can explain the higher microbial synthesis observed in cows fed red clover silage diets compared with grass silage (Dewhurst *et al.*, 2003a; Vanhatalo *et al.*, 2009). Red clover itself has a higher CP concentration and, together with

reduced proteolysis during ensiling, the protein supply to the animal can be expected to be greater from silage containing red clover than from pure grass silage (Bertilsson and Murphy, 2003; Vanhatalo *et al*., 2009). This leads to higher N intake despite similar or even lower silage DM intake with red clover silage diets (Heikkilä *et al*., 1992; Vanhatalo *et al*., 2009). Vanhatalo *et al*. (2009) reported higher NAN flow from the rumen to the omasal canal and higher N digestibility in cows fed red clover silage compared with grass silage. However, despite this higher NAN flow milk production was not increased and milk protein composition was in fact decreased with red clover silage. Very similar results have been reported by Dewhurst *et al*. (2003a). In a review paper, Steinshamn (2010) reported a small increase in milk production, on average from 25.7 to 27.1 kg/day, when cows were fed red clover silage in the diet compared with grass silage. However, lower milk fat content (39.9 and 41.8 g/kg milk, respectively) and a tendency for lower milk protein (30.9 and 31.4 g/kg milk, respectively) was also observed with red clover silage compared with grass silage. This small increase in milk yield comes at the expense of lower N efficiency through increased N excretion in faeces and urine (Bertilsson and Murphy, 2003; Vanhatalo *et al*., 2009). The lower post-ruminal utilisation of red clover protein is a problem that is still not well explained. While red clover silage as a sole feed has no effect on milk yield over grass silage, feeding a mixed diet of grass and clover has been found to give similar or higher milk production compared with feeding clover silage alone, without any major reduction in N digestibility and N efficiency (Heikkilä *et al*., 1992; Vanhatalo *et al*., 2009; Kuoppala *et al*., 2009). Various studies have been conducted on grass and clover mixtures and general conclusions seem to suggest that a 50:50 or higher red clover:grass ratio provides the best combination of milk yield and N efficiency (Dewhurst *et al*., 2003b; Moorby *et al*., 2009).

### 1.5.4 Protein supplements

Additional concentrate feeds are required to supply energy and RUP to meet the protein requirements of high-producing dairy cows during lactation. Soybean meal has been widely used as a protein supplement in recent decades due to its high concentration of good quality protein. However, the alternative use of soybean as human food and high production demands, combined with impractically long transportation distances, has led to a search for other sources of locally produced protein. Several types of protein feed can be produced in colder regions of Northern Europe, *e.g*. rapeseed (*Brassica napus*) and various legume feeds (seeds) like field bean (*Vicia faba*), lupin (*Lupinus angustifolius*) and pea (*Pisum sativum*) (May *et al*., 1993; Tufarelli *et al*., 2012). Rapeseed

meal (canola meal) has been proven to be good substitute for soybean meal (Shingfield *et al*., 2003; Gidlund *et al*., 2015). Furthermore, legume feeds have the benefit of fixing atmospheric N and are therefore especially suitable for organic production systems. Unfortunately, legume grain-based feeds are reported to be less favourable in dairy cow diets due to high protein solubility in the rumen (Mustafa *et al*., 2003), which leads to lower protein quality for milk production. In a recent study (Puhakka *et al*., 2016), milk production was found to be lower for cows fed field bean as a protein supplement compared with an isonitrogenous rapeseed meal supplement. Soybean and rapeseed meals are heat-treated to improve the protein value and heat treatment could also be used on legume seeds. Previous research has shown that heat treatment decreases protein degradability and improves protein utilisation by dairy cows (Broderick and Craig, 1980; Goelema *et al*., 1999). The extent of heating can have different effects on feeds. Overall, feed digestion and/or AA availability can be improved with low levels of heat exposure (Stern *et al*., 1985; Undi *et al*., 1996), but the benefits of heat treatment for protein value may be lost with excess heat, which can cause a proportion of the protein to become unavailable to the animal due to the Maillard reaction (Van Soest, 1994).

Despite the advances in forage preservation techniques and the numerous studies conducted to evaluate different forages, there are still major gaps in knowledge on how to achieve the best feed efficiency or protein quality.

# 2 Objectives

The overall objective of this thesis was to develop and improve *in vitro* methods of evaluating forage protein degradation kinetics. The existing methods are either too costly for routine use or related to high prediction errors. *In vitro* methods would be useful for routine usage and can have high capacity. Improving *in vitro* prediction estimates would be valuable for routine screening of feedstuff to evaluate protein efficiency. Specific objectives were:

- To use heat treatment to improve protein quality of domestic produced legume seeds.
- To evaluate protein quality in feeds using utilisable CP approach and evaluate the method itself.
- To compare the effect of preservation methods on protein ruminal degradation kinetics of grass and red clover.
- To develop models to estimate ruminal digestion kinetics of different forage protein fractions *in vivo* and *in vitro.*

# 3 Materials and methods

## 3.1 Paper I

Three Swedish-produced legume protein feeds (field beans, lupines and peas) were heat-treated for 30, 60 and 90 min using two methods and three temperature levels (dry oven at 120, 140 and 160 °C, autoclave at 105, 120 and 135 °C). The effect of heat treatment on protein was evaluated by analysing changes in chemical composition and by estimating uCP at the duodenum according to the method described by Steingaß and Südekum (2013). Each legume feed was mixed with grass silage and barley to create an isonitrogenous diets with a CP concentration of 180 g/kg DM, by replacing barley with legume seeds. Feed portions of 1000 mg were incubated for 48 h in 60 mL buffered rumen fluid collected from two fistulated Swedish Red cows fed grass silage and commercial concentrate (60:40 on DM basis). A modified gas *in vitro* system (Karlsson *et al.*, 2009) that allows replicate sampling from the same vessel was used to determine the ammonia N concentration in the liquid phase at 8, 24 and 48 h, together with automatic gas production recordings (Cone *et al.*, 1996). The ammonia production from the different diets (degradation product) and blanks (N contribution from rumen fluid for microbial synthesis) was used to calculate uCP at the duodenum at 16 h ($uCP_{16}$), which represents estimated undegraded feed protein and microbial N flowing out of the rumen.

## 3.2 Paper II

The main objective of **Paper II** was to develop dynamic models describing rumen metabolism of ammonia N and SNAN. Soluble NAN isolated from $^{15}N$-labelled timothy grass silage and ammonia N labelled with $^{15}N$ were

administered into the rumen contents of four ruminally cannulated Finnish breed cows. Ruminal N pools were determined by manual evacuation of rumen contents. The passage rate of soluble N fractions out of the rumen was determined using LiCoEDTA as a marker (Udén *et al*., 1980). Grab samples of rumen digesta were collected frequently between 0-72 h after administering the $^{15}N$ dose. The $^{15}N$ atom% excess was determined in ruminal N fractions of ammonia N, $B^1$-fraction, microbial N and protozoal N. The models were developed from the kinetic data to estimate N fluxes using the WinSAAM software (Wastney *et al*., 1998). A three-compartment kinetic model was developed for $^{15}N$-labelled ammonia N treatment to provide an optimal fit between predicted and observed values of $^{15}N$ atomic excess pools, including ammonia N, bacterial N and protozoal N. For the SNAN treatment, an additional compartment of SNAN was included. The fluxes between pools were predicted using estimated parameter values in a dynamic mechanistic model developed by Powersim software. Calculations of area under the curve (AUC) ratios of $^{15}N$ were used to estimate proportions of N fractions originating from another or preceding N pool (Nolan and Leng, 1974).

## 3.3 Paper III

Timothy grass (*Phleum pratense*) was grown on two replicate 2 $m^2$ experimental plots. The grass on one of the plots was labelled with $^{15}N$ by fertilising it with $^{15}N$-enriched $NH_4NO_3$, while the other plot received non-enriched fertiliser. Harvested grass was chopped and wilted before being preserved as dried grass or as formic acid-treated silage (FAS) or untreated silage (UTS) ensiled in 1-L glass jars. The silages were freeze-dried and separated into three N fractions: soluble N (SN), insoluble N (ISN) and neutral detergent-insoluble N (NDIN). Soluble N was the water-soluble fraction and the NDIN fraction was prepared by boiling water-insoluble residues in neutral detergent solution. The three N fractions were mixed together with additional isolated NDF, soybean meal and/or carbohydrate components to obtain diet mixtures with similar CP and NDF concentration to intact forages. The intact forages were incubated as sole feeds. The intact forages and three N fractions from $^{15}N$-labelled and unlabelled forages were incubated in pre-incubated rumen fluid *in vitro* (Karlsson *et al*., 2009). The ammonia N in rumen fluid for unlabelled forage fractions was labelled with $^{15}N$ (as $(^{15}NH_4)_2SO_4$). Two normal blanks (buffered rumen fluid without feeds) and two blanks with $^{15}N$-labelled ammonia N were incubated in every run. Frequent samples of incubation medium were collected to determine ammonia N and soluble N

concentration and the excess $^{15}N$ atom%. Ammonia concentration was used to estimate uCP according to Steingaß and Südekum (2013). The atom% excess value was used to estimate kinetic parameters of *in vitro* degradation of different forage N fractions, net microbial uptake of ammonia N and utilisable proportion of feed N.

## 3.4 Paper IV

The aim of the **Paper IV** was to estimate the *in vitro* degradation kinetics of the N fractions in red clover (*Trifolium pratense)*, using methods identical to those described above for **Paper III**. The only exceptions were that for labelling N in red clover, a fertiliser with five-fold higher $^{15}N$ enrichment was used to achieve similar $^{15}N$ enrichment (about 2 atom% excess) in red clover N fractions as in timothy grass, and that the *in vitro* buffer solution for incubation contained 2 g/L ammonium carbonate, compared with 1 g/L in **Paper III**. Dynamic models were developed with WinSAAM using measured $^{15}N$ enrichment in ammonia N and SN pools.

# 4 Results

## 4.1 Paper I

The heat treatments decreased soluble protein and increased neutral detergent-insoluble CP concentration linearly ($P < 0.01$) with increasing treatment time and/or temperature in all protein feeds. The inclusion of untreated lupin or pea to the basal diet increased diet $uCP_{16}$ concentration only numerically, from 160 to 166 and 172 g/kg DM, respectively, while untreated field bean had no effect. Increased treatment time and temperature increased $uCP_{16}$ concentration linearly ($P < 0.01$) in the field bean and pea diets, but had no linear effect on the lupin diets. The $uCP_{16}$ concentration in lupin was increased already with the low and medium treatment levels and increasing the treatment time or temperature further had no significant effect. The autoclave treatment used lower temperatures than the dry oven treatment, but was more effective in increasing $uCP_{16}$ in all protein feeds. The combination of longest treatment time and highest temperature in the autoclave (90 min at 135 °C) significantly increased neutral detergent insoluble CP concentration to 183, 245 and 366 g/kg CP in field bean, lupin and pea, respectively. Subtracting acid-detergent insoluble N from $uCP_{16}$ reduced the digestible $uCP_{16}$ concentration in extensively treated protein feeds.

## 4.2 Paper II

The soluble N fraction in grass silage comprised 93% of NAN and 7% of ammonia N. The mean proportions of bacterial N, protozoal N and feed N in the rumen solid phase were 59, 20 and 21 %, respectively. Models describing the N metabolism of ammonia N and SNAN gave a good fit to the $^{15}N$ data. The rapid $^{15}N$ enrichment of microbial N with the SNAN treatment was not

possible to model, so initial values were estimated by the model. More than 90% of the ammonia $^{15}N$ dose disappeared from the ammonia N compartment within 2 h. Based on the rapid $^{15}N$ enrichment of the protozoal pool, it was not possible to describe the protozoal pool only by first-order transfer of $^{15}N$ from the bacterial N pool. However, the protozoal N pool was described accurately when a parameter describing physical attachment of bacteria to the protozoal pool was added, in addition to flux representing predation. The model predicted that a significant proportion (7-8%) of bacteria were physically attached to protozoa. Based on AUC ratio, the recovery of $^{15}N$ as microbial N was about 20% greater with the SNAN treatment than with ammonia N. Area under the $^{15}N$ enrichment curves indicated that most (95%) of the protozoal N was derived from bacterial N, but only about 15% of microbial N flowing to the duodenum was of protozoal origin. Approximately 16% of bacteria were recycled back to ammonia N or to the SNAN pool. An estimated 12.5% of SNAN escaped ruminal degradation. Based on compartmental modelling, the total recovery of $^{15}N$ as NAN was 36% greater when $^{15}N$ was dosed as SNAN rather than ammonia N (505 compared with 370 mg/g). The results also indicated that the value of silage SNAN was greater than that of ammonia N, partly because it was a better N source for rumen microbes, especially when estimated from AUC ratio, and partly because of escape.

## 4.3 Paper III

All silages were of good quality, but untreated silages had higher pH and ammonia N concentrations than formic acid-treated silages. The $^{15}N$-labelled silages had higher CP and also soluble N concentrations compared with dried grasses. The $^{15}N$ atom% in the soluble N fraction was slightly higher and in the NDIN fraction about 3.5% lower than in intact forages. Ammonia N concentration in the soluble N fraction was about 10% and 1.4% for silage and dried grass, respectively. A high proportion (25-38%) of $^{15}N$-labelled ammonia disappeared from all feeds and blanks within the first 15 min of incubation, but some of the $^{15}N$ reappeared in the ammonia $^{15}N$ atom% excess pool ($^{15}NEP$) in the following 15 min. According to model predictions, 20% of the soluble N dose from $^{15}N$-labelled dried grass was immediately taken up by microbes, but only 0.9% of the soluble N from the FAS and UTS silages. Fractional disappearance rate of soluble N from $^{15}N$-labelled FAS, UTS and dried grass during the first 6 hours was 0.145, 0.125 and 0.115 /h, respectively. After the initial disappearance of $^{15}N$ from the ammonia N pool, there were no changes in ammonia $^{15}NEP$ size with silage soluble N fractions, but ammonia $^{15}NEP$

size decreased with dried grass soluble N. The estimated fractional degradation rate of the ISN fraction to ammonia was 0.049, 0.057 and 0.091 /h for $^{15}$N-labelled FAS, UTS and dried grass, respectively, and by the end of the incubation 14, 30 and 24% of $^{15}$N from the respective fraction was found in ammonia $^{15}$NEP. Only about 12% of $^{15}$N from the $^{15}$N-labelled NDIN fraction was recovered in ammonia $^{15}$NEP in the end of incubation. The $uCP_{16}$ and utilisable substrate CP ($uSCP_{16}$) values were higher for dried grass compared with silages. An estimated 18-37% of $uCP_{16}$ was from the contribution of microbial N originating from the rumen fluid and was higher for dried grass than for silage.

## 4.4 Paper IV

All red clover silages were of good quality and representative $^{15}$N-labelled and unlabelled silages had very similar composition and fermentation quality. The $^{15}$N atom% was slightly lower in soluble N fractions and higher in NDIN fractions than in the intact forages. About 2.4-fold less $^{15}$N from $^{15}$N-labelled dried red clover appeared in ammonia $^{15}$NEP in the first 30 min, compared with silages, but for the rest of the incubation time the appearance rate of $^{15}$N in ammonia $^{15}$NEP was similar for all forages. The calculated disappearance of the $^{15}$N dose (195 µg) from the ammonia N pool was 38-65% in the first 15 min, after which the $^{15}$NEP size increased again in the following 15 min. The $^{15}$N uptake from ammonia $^{15}$NEP was lower with dried red clover compared with silage. The $^{15}$NEP size in blank with $^{15}$N-labelled ammonia N decreased in the first nine hours, but slightly increased again during the last 19 hours. The soluble N consisted of 14-27% ammonia N, with the percentage being lower for dried forage than for silage. Over 70%, 65% and 61% of the $^{15}$N from the soluble N fractions of $^{15}$N-labelled FAS, UTS and dried red clover, respectively, disappeared from soluble $^{15}$NEP in the first 15 min. The respective fractional disappearance rate of soluble N fractions, after the initial rapid uptake of soluble N, was 0.273, 0.126 and 0.206 /h, respectively. These fluctuations in $^{15}$NEP size in the first hours of incubation were not possible to model satisfactorily. In the first nine hours of incubation, only 1.6-3% of $^{15}$N from ISN fractions appeared in ammonia $^{15}$NEP, but by the end of incubation (28 h) about 12% was in ammonia $^{15}$NEP. There were no significant differences between silage ISN fractions and/or dried red clover ISN. The estimated microbial synthesis rate from ammonia N was 0.279 and 0.329 /h with formic acid and untreated silage ISN fractions, respectively. A maximum of 3% of $^{15}$N from NDIN fractions was recovered in ammonia $^{15}$NEP after 28 h of incubation. The $^{15}$N-labelled ammonia $^{15}$NEP size decreased only until 6 h

and remained almost unchanged thereafter. Dried red clover had the highest $uCP_{16}$ concentration and formic acid-treated silages had higher $uCP_{16}$ than untreated silages. The estimated proportion of $uCP_{16}$ derived from rumen fluid was 11% for dried red clover and 21% for $^{15}N$-labelled silages.

# 5 Discussion

## 5.1 Evaluation of protein quality *in vitro*

Alternative systems such as *in vitro* and *in situ* methods are routinely used to estimate ruminal degradation kinetics *in vivo*. The estimates produced with these systems need to reliably represent the conditions prevailing *in vivo* in the rumen. *In vitro* systems try to replicate rumen conditions on a small scale and this allows several feeds to be evaluated simultaneously and many parameters to be measured in controlled conditions. However, the conditions are not identical and there are various factors that affect the accuracy of the predictions, and thus *in vitro* measurements need to be 'translated' to give *in vivo* estimates.

### *5.1.1 In vitro* versus *in vivo*

The rumen is a continuously changing dynamic system harbouring a complex anaerobic microbial community. The batch system differs from the situation *in vivo* in that the handling of rumen fluid affects microbial activity and being a closed system, the substrate may become limiting. Furthermore, the degradation products cannot escape (except gas) since no absorption (rumen wall) or passage takes place, but also no recycling of urea occurs.

The rumen protozoa are difficult to cultivate *in vitro* (Bonhomme, 1990), but they play an important role in ruminal protein metabolism. The protozoa can contribute to protein breakdown via peptide and AA uptake, but their main effect comes from bacterial predation and intraruminal recycling of N. Previous studies have reported a 35-50% increase in microbial flow from the rumen with defaunation of rumen contents (Walker *et al.*, 2005). However, those studies were mostly conducted at maintenance level feeding in sheep. In **Paper II**, it was estimated that about 95% of rumen protozoa was derived from bacterial predation. This estimate is higher than typically reported in the

literature. One reason could be that in studies using continuous $^{15}N$ infusion, $^{15}N$ enrichment had not reached steady state due to much slower turnover time of protozoa than bacteria (Hristov *et al.*, 2001; **Paper II**). With reduced activity of protozoa *in vitro* bacterial recycling would be reduced, which would increase degradation rates compared with the case *in vivo*. However, it should be emphasised that reduced ruminal digestibility of OM and NDF is often observed with defaunation (Eugine *et al.*, 2004). The lack of protozoal contribution to peptide degradation and no absorbance of ammonia N through the rumen wall would also change the dynamics of degradation of highly available soluble N *in vitro.* Accumulation and disappearance of peptides in *in vitro* systems with mixed rumen microbes given casein as a protein source (Russell *et al.*, 1983) were slower than those observed *in vivo* (Chen *et al.*, 1987).

Estimating protein degradation rates *in vitro* using only ammonia production underestimates the degradation rates, because microbes use ammonia N for microbial synthesis. Peptides and AA can also be used directly for microbial synthesis without first being degraded to the external ammonia N pool (Wallace *et al.*, 1999; Carro and Miller, 1999). In **Paper II**, it was estimated that about 15% of soluble NAN was directly incorporated into microbial N and the proportion of microbial N synthesised from ammonia N was two-fold higher with ammonia than with SNAN treatment. According to Russell *et al.* (1983), when enough carbohydrates are available then most of the microbial protein is synthesised directly from AA without going through the ammonia N pool. Quantifying *in vitro* microbial synthesis from ammonia N is further complicated by the change in available energy during the incubation. Using the data from **Papers III** and **IV**, an attempt was made to estimate microbial N synthesis from the disappearance of $^{15}N$-labelled ammonia N and appearance of $^{15}N$ in the ammonia N pool from corresponding $^{15}N$-labelled N fractions, but the results overestimated microbial N synthesis. This discrepancy was attributed to different ammonia N uptake rates at different stages of incubation, due to changes in available fermentable organic matter.

High variability of background ammonia concentrations *in vitro* has been reported in other studies (Broderick *et al.*, 2004; Karlsson *et al.*, 2009) and has also been observed in our laboratory (unpublished). Previous authors have used pre-incubation of rumen fluid to reduce the background ammonia N variability. Pre-incubating rumen fluid was used in **Papers III** and **IV** with promising results, as no unexplained ammonia concentration peaks were detected and blanks used within every run had nearly identical ammonia production curves. However, the pre-incubation step leaves microbes deprived of ammonia N, which can result in immediate uptake of ammonia, thereby influencing the

estimates of rate of microbial synthesis. On average, 44% of the initial dose of $^{15}N$ given in incubation vessels as $(^{15}NH_4)_2SO_4$ disappeared from the ammonia $^{15}N$ pool during the first 15 min (**Papers III** and **IV**). In **Paper II**, the disappearance of the ammonia $^{15}N$ dose given in the rumen was about 14% in the first 13 min. However in **Paper II**, the recovery of the ammonia $^{15}N$ dose given in the rumen was only about 75%. Blake *et al.* (1983) observed high microbial N enrichment already after 2 min when steers were given $^{15}N$-labelled $^{15}NH_4Cl$ in the rumen. The enrichment of AA was much slower, indicating that uptake of ammonia N is not equal to microbial N synthesis (in the short term). Moreover, the rate and extent of microbial $^{15}N$ enrichment were lower when ammonia $^{15}N$ was given together with urea rather than with decorticated groundnut meal. The differences in initial ammonia N uptake by microbes between *in vitro* and the rumen could then be explained by the pre-incubation and also the availability of other N substrates in the rumen.

The optimal ammonia N concentration to support maximal microbial growth is reported to be at least 50 mg/L *in vitro* (Satter and Slyter, 1974) or 85 mg/L under *in vivo* conditions (Kang-Meznarich and Broderick, 1980). However, using too high an ammonia N concentration in the buffer solution to support microbial activity (McDougall, 1948) leads to problems of variability, as discussed above. Moreover, according to the data used in a meta-analysis of omasal sampling studies (Broderick *et al.*, 2010), the ruminal ammonia N concentration has no significant ($P = 0.22$) effect on the efficiency of microbial protein synthesis when used as a second variable in addition to DM intake. The buffer solution used in every *in vitro* study in this thesis had an ammonia N concentration of at least 40-80 mg/L, well within the suggested range (**Papers I, III** and **IV**). The *in vitro* ammonia production data reported in **Papers III** and **IV** ranked the forages and forage protein fractions as would be expected based on general knowledge of the chemical composition of silage and hay, but degradation rates of soluble N fractions in of grass and red clover were slower than observed *in vivo* (**Paper II**). However, the degradation rates of the SN fraction in red clover silage were similar to those reported for the soluble fraction of lucerne by Peltekova and Broderick (1996). This could reflect the limitation of batch systems regarding changes in the microbial community, reduced protozoa activity and substrate imbalance. In the *in vitro* experiments carried out in our laboratory, 4 g/L $(NH_4)_2CO_3$ is typically used for preparing the buffer solution (Menke and Steingaß, 1988). Moreover, additional peptone is sometimes added to stimulate microbial growth in the early stage of incubation when forages with low CP concentration are used (Hetta *et al.*, 2008). To avoid the above-mentioned problems with variable ammonia N, a reduced amount of ammonium carbonate was used in **Papers III** and **IV**, but

this could have reduced microbial activity and be a significant cause of the lower degradation of proteins to ammonia observed in those studies. This speculation is supported by the lag phase (about 30-60 min) observed in gas production data. However, the lag phase could also be because the pre-incubated rumen fluid had almost no fermentable substrate left (based on zero grass production from blanks) and therefore it takes time before sufficient feed substrate is digested to show up in gas recordings. Overall, the gas production kinetics could be considered normal, or perhaps slightly lower than normal (Ramin and Huhtanen, 2012). There is currently no good solution to hand and more practical testing is needed to modify the method in this regard.

### 5.1.2 *In vitro* versus *in situ*

Degradation estimates made *in situ* are the basis of many feed evaluation systems. In theory, the *in situ* approach is the simplest available method for routine screening of feedstuffs. The placement of feeds in the rumen within nylon bags should expose feeds to the natural environment of the rumen. However, the *in situ* method has several disadvantages, prompting a search for more reliable methods. Escaping feed particles, reduced microbial activity in the bags and microbial contamination of degradation residues are the main methodological problems associated with the *in situ* method (Hvelplund and Weisbjerg, 2000). These problems explain why the reproducibility of the *in situ* method was found to be very poor in a ring test involving 23 laboratories in 17 countries (Madsen and Hvelplund, 1994).

The first major disadvantage of the *in situ* method is the escape of soluble materials and feed particles from the bag without being fully degraded (Hvelplund and Weisbjerg, 2000). This is especially important for estimating degradation of soluble proteins. According to the equation developed by Ørskov and McDonald (1979) to estimate EPD, the soluble protein fraction is considered to be degraded at an infinite rate. In older feed evaluation systems such as CNCPS (Sniffen *et al.,* 1992) and NRC (2001), the degradation rates used for soluble CP were so high that it could be concluded that all soluble protein is degraded in the rumen. *In vivo* studies using ruminal evacuation or omasal sampling techniques have demonstrated that significant proportions of soluble NAN escape ruminal degradation (Hristov and Broderick, 1996; Choi *et al.,* 2002). In **Paper II**, it was estimated that 12.5% of soluble NAN escaped ruminal degradation. Considering the high proportion of soluble fractions in silages and many protein feeds, accurate estimation of the degradability of soluble proteins is important. The average *in vitro* degradation rates found here for the soluble N fractions of grass forage (0.12 /h; **Paper II**) and red clover

(0.24 /h; **Paper III**) were slower than those reported by many others. For example, Peltekova and Broderick (1996), using an *in vitro* technique, estimated degradation rates of 0.48 /h for casein and 0.27 and 0.29 /h for soluble proteins of lucerne hay and silage. Hedqvist and Uden (2006) reported *in vitro* degradation rates of 0.18-0.62 /h for soluble proteins in 11 feedstuffs (four forages). However, all those reported *in vitro* degradation rates are lower than those observed *in vivo* (Nugent and Mangan, 1981; **Paper II**) and would lead to the assumption that more than 50% of the soluble fractions in forage could escape ruminal degradation. The degradation rates of soluble NAN in grass silage in **Paper II** were several-fold greater (1.22 /h) than those observed *in vitro* by Peltekova and Broderick (1996) and Hedqvist and Uden (2006). Hedqvist and Uden (2006) also observed that only 33% and 26% of the soluble proteins in rapeseed cake and pea, respectively, were recovered 30 min after dosing to the rumen, while after 1 h the concentrations had reverted back to the baseline.

The escape of soluble fractions from the bag is not the only problem with the *in situ* method. Vanhatalo *et al.* (1996) reported that about 17-35% of acid detergent insoluble N (ADIN) in grass residues incubated for 96 h in the rumen disappeared from mobile bags within the intestine. In **Paper I**, the extensive heat treatments significantly increased the ADIN concentration of legume seeds. Since ADIN is considered to be indigestible, the partial escape of ADIN from *in situ* bags would overestimate the degradability and digestibility of proteins.

Various *in vitro* systems have been successfully used to predict methane production and feed degradation with reasonable accuracy from gas production kinetics (Getachew *et al.*, 1994; Huhtanen *et al.*, 2008; Ramin and Huhtanen, 2012). Estimating protein degradation *in vitro* poses more difficulties, because the degradation end-products (ammonia N and AA) are used for microbial synthesis, resulting in underestimation of the rate and extent of degradation (Broderick, 1994). To overcome this problem, Broderick (1987) used hydrazine sulphate and chloramphenicol to inhibit microbial synthesis during degradation. In a comparison of the inhibitory effect *in vitro* and *in situ*, Broderick *et al*. (1988*)* found that the degradation rates of seven protein feedstuffs were on average 36% slower *in situ*. The reason for slower rates *in situ* is that soluble N is not taken into account, but *in vitro* the faster degradation of soluble N increases overall degradation values. Another reason could be that the physical barrier of the nylon bag reduces microbial access to feed particles (Meyer and Mackie, 1986). Ramin *et al.* (2013) reported lower methane production when feeds were incubated inside filter bags *in vitro*, indicating reduced microbial degradation.

Our main objective in **Papers III** and **IV** was to use ammonia N as the main indicator to estimate degradation parameters. This proved to be more difficult than expected. The ability to quantify ammonia N and soluble N in the incubation medium made it partially possible to estimate the degradation kinetics of the soluble N fraction. Estimating microbial N synthesis and degradation of intact feeds and insoluble N fractions was less successful. The idea was to use replicate $^{15}$N-labelled and unlabelled feeds and measure the $^{15}$N appearance from feed to ammonia and correct for microbial synthesis by measuring $^{15}$N disappearance from the labelled ammonia N pool. However, since microbes use some feed N directly without degrading it to the external ammonia pool and because of microbial recycling, it was impossible to estimate the actual protein degradation rate and extent with this method alone. Using $^{15}$N-labelled plant N fractions and modifying the inhibitory *in vitro* method (Broderick, 1987) could be one possibility to improve this technique, at least for soluble fractions. The accumulation of ammonia and AA limits the incubation time of the inhibitory *in vitro* system to about 6 hours, which may be too short a time for more slowly degradable proteins. Considering the results presented in this thesis, the *in situ* technique would be a more accurate method for evaluating ruminal degradation of insoluble N fractions. However, the microbial contamination of degradation residues *in situ* can still cause underestimation of degradation, especially with the insoluble N fraction with low CP concentrations (Hvelplund and Weisbjerg, 2000). Protein degradation of feeds with low CP and high NDF concentration can lead to up to 30% underestimation due to microbial contamination (Michalet-Doreau and Ould-Bah, 1992).

One criticism of the present work could be that the N fractions were incubated within mixed diets including other carbohydrate and protein substrates (**Papers III** and **IV**). With $^{15}$N-labelled forages using mixed diets should not cause any problems, because the excess $^{15}$N can only originate from the labelled feeds. However, incubating N fractions *in vitro* as mixed diets makes it difficult to assess the microbial N synthesis from ammonia N, because the mixed diet contents and available carbohydrates would affect the microbial uptake of $^{15}$N-labelled ammonia N (Russell *et al.*, 1983). On the other hand, incubating N fractions as single substrates would lead to inaccurate estimates because microbial synthesis would be influenced by limited energy or protein, especially with single fractions.

### 5.1.3 Utilisable CP approach

With many errors associated with determining microbial N and RUP separately, it may be useful to instead estimate total NAN flow to the duodenum as a whole (Lebzien and Voigt, 1999). Existing *in vitro* methods make it possible to obtain frequent liquid samples during incubation and determine ammonia N production (Karlsson *et al.,* 2009). Ammonia measurements alone do not allow microbial synthesis or degradation to be determined separately. Determining uCP from ammonia N production *in vitro*, as described by Steingaß and Südekum (2013), is a promising method, at least for ranking the feeds. Edmund *et al.* (2012) pointed out that forages and protein feeds require different *in vitro* incubation times to be estimated accurately, because of the variability in ammonia N concentrations. As discussed before, some of the variability can be removed by normalising rumen fluid with pre-incubation. The other thing to consider would be that feed composition influences microbial activity. Possible interactions between diet components can influence the total diet digestion and N utilisation in a diet (Huhtanen *et al.,* 1991; Moss *et al.,* 1992). In **Paper I**, heat-treated protein feeds were incubated together with silage and barley mixed as complete diets. Incubating feedstuffs as a sole feed *in vitro* is common practice, but does not represent rumen conditions accurately, especially with feeds high in CP, as the imbalance of energy or protein supply for microbial synthesis can reduce microbial growth (Russell *et al.,* 1983). Considering that dairy cows are normally fed mixed diets of forages and concentrates, it would be logical to evaluate the effect of individual feeds on uCP by incubating them as mixed diets. Forages can be incubated as single feeds. Heat treatment of feedstuffs increases the insoluble N fraction ($B_2$) and decreases ruminal degradability (NRC, 2001). Decreased ruminal degradation by drying forages or heat-treating feeds with a high proportion of SN, such as legume seeds, should increase NAN flow from the rumen (Van Soest, 1994; NRC, 2001). The estimated $uCP_{16}$ concentration of legume seeds was significantly increased by the heat treatments tested in **Paper I**. However, only marginal increases in uCP were observed for untreated legume seeds, as also found in the *in vivo* study by Vanhatalo *et al.* (2004). In their study, pea supplementation did not increase omasal NAN flow compared with a grass silage-barley diet. In **Papers III** and **IV**, the estimated $uCP_{16}$ was significantly higher for timothy and red clover hay than for the respective silages. The value of uCP estimated *in vitro* still needs to be evaluated *in vivo*, but preliminary results obtained by Gidlund *et al.* (2017, unpublished) show a good relationship between $uCP_{16}$ and omasal NAN flow measured *in vivo*.

## 5.2 Using $^{15}N$ in rumen studies

### 5.2.1 Microbial marker

Microbes use different N sources for microbial cell synthesis. Stable isotope $^{15}N$ is routinely used in ruminant nutrition studies to label the microbial population in the rumen (Broderick and Merchen, 1992). *In vivo* experiments often use $^{15}N$ to reach stable enrichment of microbial protein to measure microbial N flow from the rumen (Mathison and Milligan, 1971; NRC, 1985). Typically, a certain period before the sampling time, a continuous flow of $^{15}N$ solution is infused into the rumen through a rumen fistula to establish stable $^{15}N$ enrichment. However, it should be considered that protozoal N enrichment takes a longer time to reach stable conditions, which could lead to underestimation of microbial N flow. In **Paper II**, the $^{15}N$ enrichment of protozoal N was much slower than that of bacterial N, presumably because protozoal N is derived mainly from bacterial predation (Walker *et al.*, 2005). Protozoa flow is rarely determined separately, but if protozoa enrichment is lower than bacteria enrichment, this would underestimate the total microbial N flow (Broderick *et al.*, 2010). For instance, if the protozoal contribution to microbial N flow is 20% and protozoa enrichment is 50% of that of bacteria, then microbial N flow would be underestimated by about 10%. An advantage of $^{15}N$ as a microbial marker compared with others is that it is relatively easy to separate microbial N from feed N, because $^{15}N$ in NAN represents only microbial N (Reynal *et al.*, 2005). A bolus injection of $^{15}N$-labelled substrate can be used to measure microbial synthesis from that particular substrate, by determining $^{15}N$ changes in different N pools (Hristov *et al.*, 2001). In **Paper II**, a single dose of $^{15}N$-labelled N fractions was injected into the rumen and the enrichment curves of microbial N and other N pools were used to estimate fluxes between pools. Since $^{15}N$ isotope is stable and naturally occurring, it does not influence microbial synthesis. Although small differences in $^{15}N$ enrichment in different N fractions in the rumen and whole animal have been reported (Steel and Daniel, 1978; Cheng *et al.*, 2012), but these differences are small and can be corrected using background measurement. A mass spectrometer equipped with an isotope analyser used for $^{15}N$ measurement has very high sensitivity (Tunlid *et al.*, 1985), making it possible to use very low amounts of excess $^{15}N$ in experiments to label microbial or any other protein fraction. In **Paper II**, it was noted that the ammonia $^{15}N$ pool increased and decreased slightly at regular intervals related to the feeding time of the cows. These changes were small but still detectable. The conclusion drawn was that these changes were caused by slightly higher $^{15}N$ enrichment of grass silage over the stable atmospheric $^{15}N$ concentration (Huss-Danell *et al.*, 2007; Cheng

*et al.*, 2011). In future, we would recommend determining $\delta^{15}N$ values for all feedstuffs, or at least the full diet fed to cows, in experiments where $^{15}N$ is used as a marker. The microbial contamination of *in situ* residues can be estimated using $^{15}N$ to label microbes and estimate their contribution to remaining feed in the nylon bags (Varvikko and Lindberg, 1985)

### 5.2.2 Ammonia and urea $^{15}N$ kinetics

Microbial synthesis from ammonia N can be estimated by labelling ammonia N with $^{15}N$. This method is robust and relatively easy to conduct. Peltekova and Broderick (1994) estimated microbial synthesis and degradation of N fractions of lucerne hay and silage *in vitro* by labelling the ammonia N pool with $^{15}N$ and determining the enrichment changes during the incubation for ammonia $^{15}N$ pool, isolated bacterial $^{15}N$ pool and total solids $NA^{15}N$ pool. A similar method to estimate ruminal degradation of rapeseed meal *in vitro* was developed by Ahvenjärvi *et al.* (2009). Their method is promising, but the need to have many replicate samples to determine $^{15}N$ enrichment in total residues makes it very laborious. In **Paper II** the $^{15}N$-labelled ammonia used to quantify microbial synthesis *in vivo* gave meaningful results and high repeatability. In **Papers III** and **IV** it was partly possible to estimate microbial synthesis from ammonia N with $^{15}N$-labelled ammonia N. With $^{15}N$-labelled ammonia N the recycling of microbial N was estimated to occur, which should be taken into account in the future when estimating protein degradation based only on ammonia N measurements, even if the difference can be small (Nolan and Leng, 1972). Urea recycling can play a major role in rumen *in vivo* estimates (Nolan and Leng, 1974). If $^{15}N$ is used as a marker *in vivo*, the recycling of urea back to the rumen could interfere with rumen degradation estimates. Using double-labelled ($^{15}N^{15}N$) urea and considering that the fractionation of urea by microbes (Cheng *et al.*, 2012) would lead to production of $^{14}N^{15}N$, the change in this ratio ($[^{14}N^{15}N]$:$[^{15}N^{15}N]$) can be used to determine the extent of urea recycling to the rumen (Lapierre and Lobley, 2001; Marini and Van Amburgh, 2003).

### 5.2.3 Using $^{15}N$ labelling of plant N fractions

In **Papers II-IV**, intrinsically $^{15}N$-labelled forage N fractions were used to study rumen metabolism of plant N fractions. This method had proven useful in a previous study by Hristov *et al.* (2001), who used $^{15}N$-labelled lucerne silage and hay in *in vivo* studies. The advantage of labelling feedstuffs with $^{15}N$ is that it represents feed N only, therefore allowing fluxes between different N

pools to be accurately estimated. Using area under the $^{15}N$ enrichment curve following single injection of $^{15}N$-labelled substrate (Nolan and Leng, 1974) enables fractionation of plant N to other pools, such as bacterial N, protozoal N and ammonia N, to be estimated. **Paper II** showed a consistent enrichment pattern with both $^{15}N$-labelled ammonia N and SNAN and the models developed accurately fitted the enrichment data of individual cows. The bacterial N recycling to ammonia N in **Paper II** was rather similar (Nolan and Leng, 1974) or slightly lower than reported in other studies (Koenig *et al.,* 2000; Oldick *et al.,* 2000). The lower recycling was most likely due to the higher feeding level in **Paper II**, which increased ruminal passage rate and reduced microbial retention time and thus increased microbial cell yield per unit energy fermented (Russell *et al.,* 1992). An important observation in **Paper II** was that silage-based diets can have positive $\delta^{15}N$ values (Cheng *et al.,* 2011; Huss-Danell *et al.,* 2007) and even though the external inputs of $^{15}N$ are small, their contribution can have quite an important effect on estimates of recycling. Comparison of the results obtained with $^{15}N$-labelled ammonia N and SNAN in **Paper II** showed that SNAN was a more optimal N source for rumen microbes than ammonia N. Although ammonia N can provide all microbial requirements, peptides and AA have beneficial effects on rumen fermentation (Walker *et al.,* 2005). In the CPNCP model, microbial growth efficiency of non-cellulolytic bacteria is reported to improve by up to 18.7% with preformed AA (Russell *et al.*, 1992).

As far as we know, there are no reports of using $^{15}N$-labelled forages or plant N fractions in previous *in vitro* studies. All $^{15}N$ recovered in the ammonia $^{15}N$ pool represents feed $^{15}N$ degradation to ammonia N. However, as mentioned above, this method can currently only determine soluble N degradation because the soluble N can be quantified in the incubation medium. Insoluble feed N fractions would require $^{15}N$ determination of the whole digesta and separation of microbial and feed N fractions. However, the appearance of $^{15}N$ measured in the ammonia $^{15}N$ pool when forage NDIN fractions were incubated in **Papers III** and **IV** could indicate direct microbial utilisation, rather than degradation of NDIN to extracellular ammonia N. Similarly, in *in vivo* studies with $^{15}N$-labelled silage ISN and NDIN fractions, no $^{15}N$ appeared in the fluid phase (**Paper II** unpublished data)

The disadvantages of using $^{15}N$-labelled feedstuffs for research are that it takes a long time to grow the plants and the high cost of $^{15}N$ chemicals makes it expensive to produce labelled plant material for *in vivo* studies. However, considering the high sensitivity and precision of $^{15}N$ analysis, only low levels of enrichment are needed for *in vitro* experiments.

## 5.3 Improving forage protein value

Since forages normally form the main proportion of cattle diets, many strategies have been used to improve forage protein value. It is important to optimise growing and harvesting methods to achieve the highest possible quality and yield. Forage quality alters during the stages of plant development and during the growing season. The effect of preceding development of the plants is well known to have an effect on animal production (Steen, 1992). Early cut forages have higher digestibility (McDonald, 1981), but higher solubility can lead to higher losses during ensiling and to reduced N efficiency compared with late cut forages (NRC, 2001). The negative changes in forages during later growth are an increase in structural carbohydrates and a decrease in CP and soluble components (McDonald, 1981). The forage protein concentration and fractionation can be affected by the rate of N fertilisation (Fox and Brown, 1969; Peyraud and Astigarraga, 1998). The increased CP concentration and production yield with fertilisation can be accompanied by increased non-protein N concentration (Van Soest, 1994) and decreased protein efficiency (Shingfield *et al.,* 2001). In a review, Peyraud and Astigarraga (1998) also reported slightly higher OM digestibility with high compared with low N fertilisation. However, the higher CP in grasses due to higher N fertilisation promotes no significant increase in NAN flow (g/kg DM intake) to the duodenum (Vanhatalo and Toivonen, 1993).

### 5.3.1 Formic acid and formaldehyde treatment

Fermentation inhibitors are widely used to improve silage quality (McDonald, 1981). Formic acid-based silage additives are possibly the most commonly used in practice. These additives usually contain a mixture of formic acid as a fermentation inhibitor and propionic acid to improve aerobic stability (Driehuis *et al.,* 1995). The function of fermentation inhibitors is to accelerate the decrease in pH of ensiled forage matter. Since fermentation products such as VFAs provide little or no energy to microbes, extensively or poorly fermented silages reduce microbial protein synthesis (Harrison *et al.,* 2003). In a meta-analysis, Huhtanen *et al.* (2003) found a negative relationship between the extent of fermentation and milk yield and milk fat and protein concentrations. In **Papers III** and **IV,** formic acid application reduced silage pH and ammonia N concentration. The *in vitro* incubations showed higher degradation of soluble N to ammonia with untreated compared with formic acid-treated silages. With grass silages, the uptake of ammonia N for microbial synthesis was increased with N fractions of formic acid-treated silages compared with untreated silages, but this effect was not observed in red clover silages (**Paper IV**). The formic

acid treatment also had a smaller effect on the ammonia concentrations of red clover silages compared with grass silages. This could be because of lower soluble protein concentration in the red clover (NRC, 2001) and the naturally reduced fermentation of red clover due to the polyphenol oxidase (PPO) content (Jones *et al.,* 1995a; 1995b). Despite small differences observed in *in vitro* degradation of the insoluble N fractions in red clover, the estimated uCP concentration was significantly higher for formic acid-treated silages compared with untreated silages. These differences are most likely attributable to differences in soluble N composition. Higher losses of soluble N to ammonia instead of microbial synthesis would reduce the estimated NAN flow to the duodenum. This agrees with the higher production responses observed with restricted fermentation silages (Huhtanen *et al.,* 2003). With grass silages (**Paper III**) the uCP concentration was numerically higher in untreated silages, but the uSCP was not significantly different. The higher observed uCP could be because of proportionally higher microbial synthesis from ammonia N with insoluble N fractions of untreated compared with formic acid-treated silages. Considering that the fermentation quality of the experimental silages was relatively good, then the small differences between untreated and formic acid-treated silages are not unexpected. It has been concluded that increased production responses are mostly due to higher DM intake with formic acid-treated silages compared with untreated (Huhtanen *et al.,* 2003).

In the past, formaldehyde has been used as a silage additive to reduce proteolysis in the silo and increase the supply of RUP. In studies conducted in the 1970s with sheep, marked increases in duodenal NAN were reported, but this was not reflected in improved performance in production studies, probably because the digestibility of CP and also OM were decreased (Huhtanen and Broderick, 2016).

### 5.3.2 Hay versus silage

Compared with fresh grass, during the silage fermentation process a high proportion of true proteins in forage are degraded to more soluble fractions of ammonia N, free AA and peptides (McDonald, 1981; Van Soest, 1994). Some proteolysis also occurs during wilting and drying and therefore hay typically has a higher non-protein nitrogen concentration than fresh forages (NRC, 2001). Since grass was dried in forced air oven at 50°C in this thesis, proteolysis probably occurred to a lower extent than it would be in field-dried hay (**Paper III**). In **Paper III** the degradation of soluble protein in dried grass was proportionally similar that of soluble protein in formic acid-treated silages in the first 6 hours. However, significantly more ammonia N was used for

microbial synthesis with dried grass. With red clover (**Paper IV**), the degradation of soluble protein and microbial synthesis from ammonia was similar to that in grasses. Since fermentation products provide little or no ATP to rumen microbes (Chamberlain, 1987), it can be concluded that hay promotes higher microbial protein synthesis compared with silages, as long as degradability does not become limiting (Huhtanen and Broderick, 2016). The significantly higher uCP concentrations found for dried grass and red clover compared with silages in **Papers III** and **IV** are consistent with previous findings that ruminal *in situ* determinations consistently show lower degradability of hay compared with silages made from the same sward. For example, around 5-21 %-units lower ruminal degradation has been reported for hay compared with silage (Jaakkola and Huhtanen, 1993; Verbič *et al.*, 1999; Grabber and Goblentz, 2009). The greater proportion of microbial synthesis from ammonia with dried grass and red clover than with silages also agrees with previous reports of lower ruminal degradation and increased microbial efficiency with hay compared with silages. However, production results do not support these findings, as higher milk production is normally observed with silage-based diets compared with hay (Figure 5). In almost all of the experiments used for Figure 5, the forages were supplemented by concentrate feeds. Considering that energy might be the limiting factor for silages, these higher production responses can be due to an effect of supplementation. As Professor emeritus G. A. Broderick puts it: "It's the energy that drives the bus!"

As discussed above, the appearance of $^{15}N$ from labelled NDIN fractions was at least partly due to microbial lysis (**Papers III** and **IV)**. A numerically greater proportion of $^{15}N$ from NDIN was recovered from the ammonia N pool in dried grass and red clover compared with the corresponding silages, indicating either higher degradation of dried grass NDIN fraction and/or higher microbial N synthesis from direct incorporation of dried grass NDIN, which would appear in ammonia after microbial lysis (**Papers III** and **IV**). It is possible that part of the NDF-bound N is hydrolysed during silage fermentation, reducing the available NDIN content for rumen microbes (Jones *et al.*, 1992; Rinne *et al.*, 1997). Significantly lower $B_3$ fraction (fibre-bound N) has been reported for grass silages compared with the corresponding grass before ensiling (Rinne *et al.*, 1997)

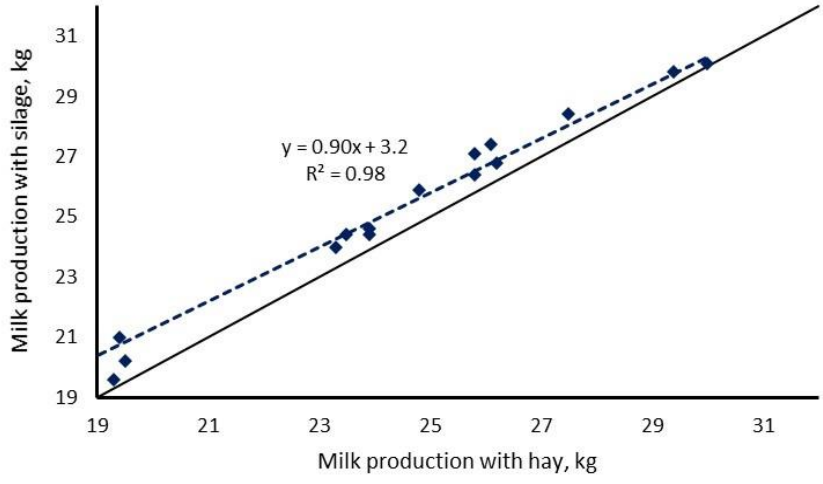


*Figure 5.* Comparison of milk production with hay and silage based diets. The markers above the middle line (y = x) represent higher production with silage and markers below represent higher production with hay. (Source: Bertilsson, 1983; Broderick *et al.*, 1985; Nelson and Satter, 1992; Heikkilä *et al.*, 1991(ref. Huhtanen, 1993); Coulon *et al.*, 1997; Murphy *et al.*, 1999; Shingfield *et al.*, 2002, 2005; Martineau *et al.*, 2007; Villeneuve *et al.*, 2013)

### 5.3.3 Red clover versus grass

The main benefits of red clover over grasses are the higher CP concentration and the reduced need for N fertiliser. The PPO in red clover should also reduce the fermentation losses during ensiling (Jones *et al.*, 1995b). However, poor silage fermentation quality is often reported with red clover (Taylor and Quesenberry, 2013) which is usually attributed to low DM content and high buffering capacity of red clover and legumes (McDonald, 1981). Therefore, improved silage quality can be obtained with formic acid treatment. However, the effect on the protein fraction is likely to be lower than with grasses. In **Paper IV**, significant differences in *in vitro* degradation profiles were only found for red clover-soluble proteins, while with grasses significant changes were observed also in other fractions (**Paper III**).

The higher CP concentration of red clover and greater NAN flow observed in dairy cows fed red clover silages compared with grass silages does not result in a proportional increase in milk production in those studies (Dewhurst *et al.*, 2003a; Vanhatalo *et al.*, 2009). However, the degradation rates measured by the *in situ* method can be misleading. With red clover based diets, much more feed N was flowing in the small (<38 µm) feed particles than is the case with

grass (Huhtanen *et al.*, 2014) indicating that the *in situ* approach does not predict lower ruminal degradability of red clover. This indicates in turn that the utilisation of absorbed AA is lower with red clover than with grass-based diets (Huhtanen and Broderick, 2016). In general, higher milk production is observed when cows are fed red clover silages compared with grass silages (Figure 6A). However, these differences in milk production are related to higher DM intake (Figure 6B), indicating that the beneficial effects of red clover mainly derive from increased ME supply. In a review, Dewhurst (2013) pointed out that despite often lower digestibility of legume silages, the higher DM intake of red clover diets results in higher milk production compared with grass silages. Comparison of the $uCP_{16}$ concentrations obtained in **Papers III** and **IV** revealed no significant differences between formic acid-treated and untreated silages or between dried red clover and timothy. However, based on the $uSCP_{16}$ concentrations, a greater proportion of CP in red clover silages compared with grass silages was degraded to ammonia N and also more ammonia N was used for microbial synthesis. These results agree with the higher NAN flow observed occasionally when feeding red clover compared with grass silages (Dewhurst *et al.*, 2003a; Vanhatalo *et al.*, 2009). The higher degradation rates of soluble proteins in red clover to ammonia and greater direct microbial synthesis compared with grass silages also support higher NAN flows reported with red clover.

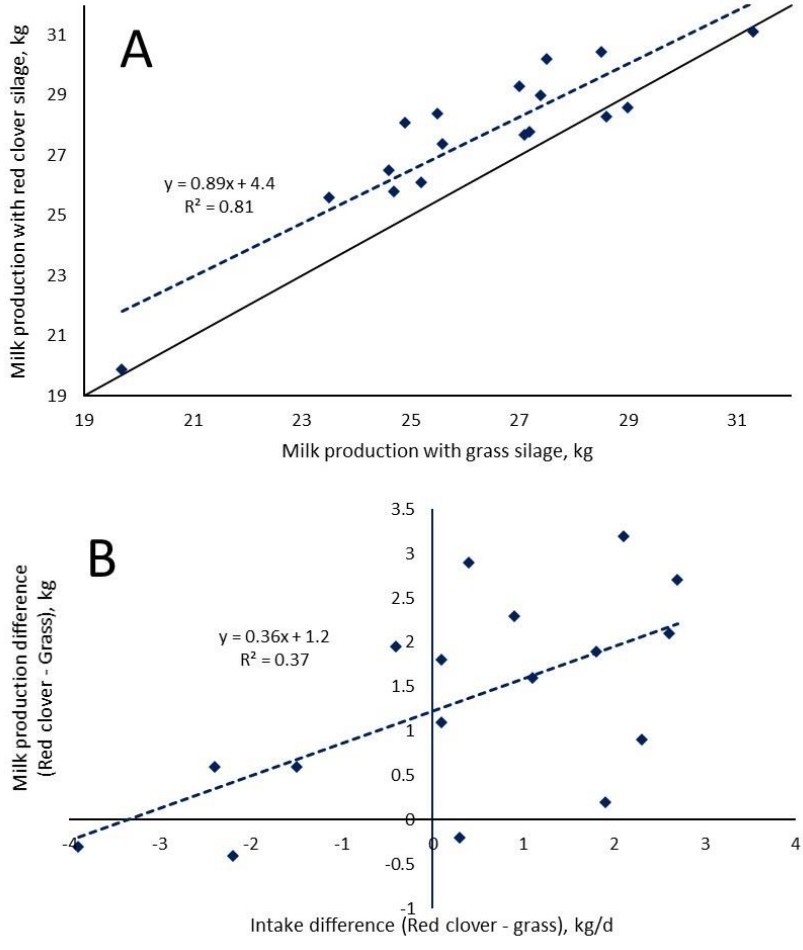


*Figure 6.* Comparison of milk production with grass silage and red clover based diets (**A**). The markers above the middle line (y = x) represent higher production with silage and markers below represent higher production with hay. Relationship between differences in milk production and DM intake with grass and red clover silages (**B**). (Source: Thomas *et al.*, 1985; Heikkilä *et al.*, 1992; Tuori *et al.*, 2002; Bertilsson and Murphy, 2003; Dewhurst *et al.*, 2003a; 2003b; Moorby *et al.*, 2009; Vanhatalo *et al.*, 2008, 2009; Halmemies-Beauchet-Filleau *et al.*, 2014)

## 5.4 Altering legume seed protein value by heating

Application of heat is used in many forms in animal feed processing, such as roasting, expeller processing, extrusion, drying and many more (Wallace and Chesson, 2008). Heat treatment can have an effect on feed hygiene (killing of pathogens) or change the nutritional value through the Maillard reaction, inactivation of toxins and protein inhibition (Van Soest, 1994; FAO, 2004). The Maillard reaction can be preferred to a certain extent, because it reduces ruminal degradation of feed proteins (NRC, 2001). In **Paper I**, the heat treatment of legume seeds significantly decreased the SN concentration in legume seeds and increased the insoluble protein fraction. This would decrease protein degradation in the rumen and could improve feed protein value (NRC, 2001), assuming that processed grains and protein feeds have higher intestinal digestibility (Owens *et al*., 1979). The inclusion of field beans or peas in a basal diet of grass silage and barley had only a small effect on uCP concentrations (**Paper I**). This indicates high ruminal degradability of untreated legume seeds and explains the low production responses of legume seeds (Robinson and McNiven, 1993; Singh *et al.*, 1995; Ramin *et al.*, 2015). The estimated uCP concentrations in diets of legume seeds in **Paper I** increased with increased extent of heat treatment, suggesting improved protein value. These results are in agreement with earlier findings (Aguilera *et al.*, 1992; Nowak *et al.,* 2005; Spörndly, 2013). Mild heat treatment was even observed to increase OM digestibility in **Paper I**, while moderate heat treatment can increase the amount of AA absorbed (Stern *et al.*, 1985). However, the positive effects of heat application are not unlimited. For example, some heat-sensitive AAs (mainly lysine and methionine) are easily damaged by relatively low temperatures (Moshtaghi Nia and Ingalls, 1995; Dakowski *et al.*, 1996) and decreased ruminal protein degradability can possibly limit the efficiency of microbial protein synthesis, which would reduce the total benefits of higher NAN flow (Santos *et al.*, 1998; Ipharraguerre and Clark, 2005). Considering the poor AA composition of legume seeds, *e.g.* they are low in methionine (NRC, 2001), heat treatment could reduce the value even more. On the other hand, peas for instance have a high energy value due to high starch content and therefore could be fed in combination with rapeseed meal (high in methionine) to achieve a favourable AA balance.

Moreover, extensive heating could cause proteins become completely indigestible (Webster, 1987; Van Soest, 1994). This was observed in **Paper I**,

where acid detergent-insoluble CP concentrations in legume seeds significantly increased when the seeds were exposed to high temperatures. Therefore the improved uCP concentrations in diets due to heat treatment became biologically less valuable. It should be emphasised that determining NDIN concentration is important when heat treatment is used in feed processing.

Heat treatment is a simple and effective way to improve feed protein value, but care has to be taken when choosing the procedures for specific feeds. The protein composition is different in feeds and therefore the responses to heating are also different (Aguilera *et al.,* 1992). Moreover, it was observed in this thesis (**Paper I)** that the optimal results (increase in uCP without increasing indigestible proteins too much) were obtained at different temperatures with different feeds.

# 6 Conclusions

The studies reported in this thesis demonstrate the potential for using $^{15}$N-labelled forage in ruminal N metabolism studies, especially *in vivo* studies. Producing $^{15}$N-labelled plants may be expensive, but the high detection limits and accuracy mean that only small amounts of labelled material are needed for the experiments. Current *in vitro* systems might not yet be sufficiently well equipped to exploit the full potential of $^{15}$N-labelled feed fractions, but the method could be already suitable (with some modifications and evaluations) for use for soluble N fractions. Use of $^{15}$N-labelled N fractions in *in vivo* studies in this thesis proved to be an accurate method for examining ruminal metabolism. It was also capable of detecting even small changes in the system and identifying external influences (*e.g.* external influence of feeding during experiment). It was concluded both *in situ* and *in vitro* that the soluble N fraction in forage stimulates microbial N synthesis and is a more valuable source of N for microbes than ammonia N. Soluble NAN increased microbial N synthesis and a significant proportion escaped the rumen undegraded. This escaped SNAN should be considered a valuable AA source, since it should be all digested post-ruminally. Intraruminal N recycling due to protozoal predation on bacteria is probably less important in dairy cows with high levels of feed intake. The potential benefits of reduced bacterial turnover are balanced out by reduced OM and NDF digestibility. The increased CP in forage or feed seems to have rather limited value unless related to improved digestibility. The production value of forages is mainly related to intake potential and digestibility, which are strongly correlated. Therefore ensuring silage digestibility and fermentation quality should be the main priority in dairy production and care should be taken when processing animal feeds so as not to reduce the digestibility. Drying, formaldehyde treatment or inclusion of red clover provide little evidence that reduced protein degradability has the same protein value as high quality protein supplements, and even with increased

NAN flow from the rumen the production responses are not what could be expected from increased protein supply to the small intestine. Moreover, the current protein evaluation methods do not predict production responses well. The only consistent trait of the *in situ* method is its poor repeatability, but *in vitro* methods fail to provide results that could be accurately related to *in vivo* conditions. The predicted degradation rates of forage N fractions *in vitro* were significantly and inconsistently lower than those estimated *in vivo*. Expensive or not, at present *in vivo* methods remain the only accurate way for estimating ruminal N metabolism. However, the *in vitro* methods hold potential to be improved in the future.

# 7 Future perspectives

The impact of human activity on the environment will continue to increase and the efficiency of food production will need to be improved. Evaluation of existing methods and development of new methodology for evaluation of nutritional efficiency in dairy production are still necessary. The method used in this thesis for estimating uCP *in vitro* indicated good potential, but needs to be validated *in vivo.* Production experiments will always be required for validation of other techniques, and therefore one recommendation would be to use dose response studies *in vivo* with simultaneous *in vitro* uCP measurements of the same diets. Some preliminary experiments using this method have shown promising results, with a good relationship between *in vitro* uCP and *in vivo* NAN flow and milk production.

Labelling with the isotope $^{15}N$ has proven to be a good tool in metabolism studies and much of its potential still remains untapped. Individual AA digestion and the effect on milk production have been studied for a long time, but some AAs in the feed are degraded and resynthesised by ruminal microbes, making it difficult to identify the real pathways of AAs in the animal and contribution in milk. Using $^{15}N$-labelled AA to study digesta flows *in vivo* and AA contribution in the milk could help understand the digestion of feed proteins.

Another potential use of $^{15}N$ in nutritional studies is in $^{15}N$-labelled N fractions. Producing sufficient amounts of forages with low levels of $^{15}N$ enrichment is not too expensive for *in vitro* experiments. Another option is to use small amounts of highly enriched forages for *in vivo* studies using pulse doses. The sample preparations are laborious but the high precision of $^{15}N$ measurements and available modelling techniques could reduce the number of samples needed. Since $^{15}N$ is substrate-specific and labelled proteins can only be degraded and used by microbes, it may not be necessary to measure $^{15}N$

enrichment in every digesta fraction separately. The $^{15}N$ isotope has been widely used as microbial marker, either to measure the flows or contamination of feed particles, but $^{15}N$-labelled ADIN, which is indigestible, could be used as a passage kinetic marker. This could solve most of the existing problems with passage kinetic markers, since it has the same digestibility and is naturally present in feed particles, meaning it would not influence the results.

# 8 Popular scientific abstract

The growing human population requires quality food and ruminants can provide that in the form of milk and meat, but increasing ruminant production also has an impact on the environment. Sufficient amounts of proteins are needed in the dairy cow diet to produce milk, but extensive amounts of proteins are not well utilised for production and are excreted to the environment. Efficient use of feed proteins is also important for the economics of production, since supplementary protein feeds are expensive and can often be used directly for human food. For example, soybean is a high quality protein source and is often used to increase milk production, but soybean is also used directly as human food or more efficiently for pigs and poultry. Moreover, since soybean cannot be grown in Northern Europe it needs to be imported from far away, which puts more strain on specific sites where it is grown. It is more important to improve the quality and efficiency of forage-based diets supplemented with locally produced concentrates or by-products. Legumes, such as field beans, lupins and peas, are N fixers and therefore require less N fertiliser, and the seeds are high in protein and have potential to be used as supplementary feeds for dairy cows. However, the protein quality in legume seeds is low for ruminants, but it could be improved by heat treatment. Experiments with heat treatment of legume seeds in this thesis indicated improved protein value, based on reduced degradability by rumen microbes and increased protein flow to the duodenum. However, different processing temperatures and methods should be used for different feeds, because extensive heating (on average over 120 °C in autoclave or 140 °C in oven and longer than 30 min) increased the proportion of indigestible protein in the feeds through the Maillard reaction. This would decrease the protein value for cows. However, AA profile of legume seeds is not ideal for supplementation of grass

silage-based diets, and therefore the effects of heat treatment need to be validated in production experiments.

This thesis sought to improve laboratory methods for estimating ruminal protein degradation kinetics. The feed evaluation models used to optimise dairy cow diets require accurate digestion parameters and understanding of ruminal degradation of feeds and different feed fractions. A naturally occurring nitrogen isotope $^{15}N$ was used to label forage protein fractions during growing. Since $^{15}N$ is natural, it does not influence the digestion process and is not harmful in any way. These forages were dried or preserved as silages treated with formic acid additive or without. The *in vitro* method, in which feeds were incubated in rumen fluid, was used to simulate rumen degradation and the *in vivo* method (real life) was used to study the degradation process, by following the changes in $^{15}N$ concentration in feed proteins and microbial protein. Formic acid treatment effectively improved the fermentation quality of grass and red clover silages and reduced ruminal degradation. Drying forages decreased ruminal degradation and increased estimated protein flow from the rumen, but previous studies have indicated that decreasing degradability does not improve milk production and that higher production responses are mainly related to increased feed dry matter intake.

Laboratory methods have been developed to simulate rumen degradation, in order to reduce labour and costs and increase repeatability (standardisation). Some methods (*in situ*, where feeds are incubated in nylon bags within the rumen) are routinely used to determine the degradation characteristics of feeds, but cannot be used with all protein feeds. For example, soluble proteins and even small feed particles escape the bags without being degraded. The *in vitro* method described in this thesis was used quite successfully for determining degradation of the soluble nitrogen fraction in forages, but is not yet suitable for estimating degradation of insoluble protein fractions. An *in vivo* experiment using $^{15}N$-labelled nitrogen fractions was laborious, but provided valuable input data to models for estimating rumen degradation and broadened the current understanding of ruminal protein metabolism. It was concluded that current laboratory evaluation methods need to be improved before they can be used to accurately estimate *in vivo* conditions, and therefore the current *in vivo* methods should still be utilised in ruminant metabolic studies.

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