The effect of red clover (*Trifolium pretense L)* accessions and ploidy on *in vitro* utilisable crude protein in red clover silage: a two-year comparative study

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Abstract

Variations in protein concentration and the ruminal nitrogen utilisation in diploid and tetraploid red clover (*Trifolium pretense L.*) silage across two years (2022 and 2023) were studied by using *in vitro* utilisable crude protein (uCP) method. A total of 45 in 2022 and 44 accessions in 2023 were assessed including four control varieties. Plant materials were harvested with a haldrup plot harvester, which was then allowed to wilt until they reached a dry matter (DM) concentration of 25% to 30%. After the determination of DM content, wilted forage was chopped and treated with a formic acid-based silage additive at a rate of 6 mL/kg fresh matter. Herbage was then vacuum-packed and placed to ensile for 100 days, after which the samples of the ensiled silage were taken for analysis and the rest was freeze-dried. The ammonia-N concentrations were determined during an *in vitro* incubation at 4, 9, 20 and 30h, used to determine the uCP at 16 h rumen retention time (uCP_{16}). In both years all silage samples exhibited good fermentation characteristics. Low pH values (lower than 4.06 in 2023) and high concentration of lactic acid (35.15 g/kg DM in 2022 and 81.22 g/kg DM in 2023) compared to other volatile fatty acids indicative of well-preserved silage. Diploid red clover (RC) was higher in crude protein (CP) and lower in neutral detergent fibre (NDF) content than tetraploid RCs in both years. A strong positive correlation ($R^2 = 0.78$) was indicated between uCP_{16} and CP content in the silage across the two years. A negative linear correlation between NDF and both uCP_{16} and CP indicates that higher content of fibre levels may compromise the ruminal protein utilisation. The uCP_{16} values of silages were significantly affected only by the year factor and not by ploidy. Three RC accessions had 5 to 9 g/kg DM lower uCP_{16} concentration than predicted by the model based on silage CP concentration, whereas 6 accessions had observed uCP_{16} values between 4 to 7 g/kg DM higher than predicted values. Those results indicate that selecting accessions with higher CP utilisation potential might be possible and that the CP concentration alone is not enough for selection for better CP efficiency in the rumen. Furthermore, numerical variations were observed in protein utilisation between tetraploids and diploids of RC accessions and significant differences between the years. Further research is warranted to evaluate the efficiency of protein utilisation in the rumen with red clover ploidies, and it is essential to further study, other parameters associated with protein utilisation in red clover.

Keywords: In vitro, Ploidy, Red clover, Ruminants, Silage, Utilisable crude protein

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Abbreviations

Ammonia-N	Ammonia nitrogen
CP	Crude protein
DM	Dry matter
LAB	Lactic acid bacteria
NDF	Neutral detergent fibre
PPO	Polyphenol oxidase
RC	Red clover
RT	Retention time
RUP	Rumen undegradable feed crude protein
SD	Standard deviation
SEM	Standard error of the mean
uCP	Utilisable crude protein
uCP16	uCP at 16 h of incubation
WSC	Water-soluble carbohydrate

Plant species

Latin name	English name
Brassica napus	Rapeseed
Dactylis glomerata	Cocksfoot
Glycine max	Soybeans
Linum usitatissimum	Linseed
Lolium arundinaceum	Tall fescue
Lolium multiflorum	Italian ryegrass
Lupinus perennis	Lupin
Lolium perenne	Perennial ryegrass
Medicago sativa	Lucerne
Phleum pratense	Timothy
Pisum sativum	Peas
Trifolium pretense L	Red clover
Vicia faba	Field beans

Introduction

Demand for milk and meat will be significantly boosted by the continuous rise of the global population. The Food and Agriculture Organization (FAO) pointed out in its 2013 report that the world's population, estimated at 7.2 billion in 2013, is expected to reach 9.6 billion by the year 2050. This global population growth underscores the increasing demand for dairy and meat products to satisfy the dietary requirements of a larger global population. This growing demand requires significant attention to optimizing animal feed. The feed cost is the main factor influencing the sustainability of the economy in the dairy industry (Lindberg *et al*., 2021).

Dairy cows in Nordic countries typically consume diets rich in grass, particularly fermented grass silage, which accounts for more than 50% of the diet's dry matter (DM) (Emanuelson, 2006). Creating balanced and appropriate animal diets is crucial for ensuring optimal intake of nutrients, including protein, sugars, fats, vitamins, and minerals. Among those components, efficient utilisation of protein sources with management of forage quality plays a crucial role in maximizing feed use efficiency because protein is a vital dietary nutrient for appetite, reproduction, milk production and growth. Enhancing production efficiency is the key factor in reducing environmental impact and increasing profitability in Swedish dairy production (Hessle *et al*., 2017). In addition to high-quality forage, rapeseed (*Brassica napus*), field beans (*Vicia faba*), and peas (*Pisum sativum*) are mostly cultivated for animal feeds in Sweden. Sweden also grows and processes small quantities of lupin (*Lupinus perennis*), linseed (*Linum usitatissimum*) and domestic soybeans (*Glycine max*) (Gidlund, 2017). In Sweden, soy is the main imported protein ingredient in animal feeds due to insufficient domestic production of substitutes such as rapeseed. Because of the volatility of feed prices on the global market, enhancement of the domestic production capabilities, and small improvements in forage protein utilisation can increase the security of feed supply management.

Since the early 1980s, the traditional use of preserved hay for indoor feeding of dairy cattle during the winter housing period in Sweden has gradually shifted towards ensiling. Year-round grazing is not feasible in Sweden, due to the harsh climatic conditions. The duration of the indoor period varies by geographical location, but it typically lasts 5 to 6 months on average (Jardstedt, 2019). The switch to preservation methods for silage has resulted in earlier harvesting phases and increased cutting rates, resulting in higher levels of energy and protein in the roughages (Karlsson *et al*., 2023). The improvement in roughage quality, combined with the fluctuation in the prices of cereal grains and protein feeds, resulted in an

increased proportion of roughage in lactating dairy cow diets, from around 35–40% in the 1990s to 50–55% in 2010, despite a simultaneous increase in milk yield per cow (Van Den Pol-Van Dasselaar *et al*., 2019).

Legumes, like lucerne (*Medicago sativa*) and red clover (*Trifolium pretense L*.), have lower water-soluble carbohydrate (WSC) levels and higher buffering capacities than grasses(McDonald *et al*., 2010). Water-soluble carbohydrates are the primary energy source for lactic acid bacteria (LAB) during silage fermentation. Low levels of WSC can lead to lower production of lactic and other organic acids during the fermentation process which may result in a higher final pH in legume silage than in grass silage (McDonald *et al*., 2010). However, it is entirely possible to produce high-quality silage from legumes alone or in combination with grasses, as long as proper management practices are followed. Legumes have significant advantages over grasses due to their high digestibility and sufficient crude protein (CP) concentration, which allows reducing use and costs of nitrogen fertilizer when compared with grasses (Finn *et al*., 2013). Compared with lucerne, red clover (RC) contains higher levels of sugar (Owens *et al*., 1999a), providing more substrate for LAB fermentation which reduces the pH of RC silage more than of lucerne (Owens *et al*., 1999b). In dairy cow production experiments (Broderick *et al*., 2001), it has been shown that RC silage is better suited than lucerne silage in terms of nitrogen use efficiency, and leads to lower milk urea due to its lower non-protein nitrogen fraction and higher digestibility of organic matter and neutral detergent fibre (NDF) (Sousa, *et al*., 2020).

Incorporation of RC into cattle diets enhances feed intake and overall animal performance when compared to rations comprising grasses or other legumes (Hetta *et al*., 2003). Red clover contains polyphenol oxidase (PPO), which is involved in reducing proteolysis during ensiling and can inhibit the activity of proteolytic microbes. The utilisation of RC can be used to reduce the need for soybean meal and other protein concentrates in animal diets (Van Den Pol-Van Dasselaar *et al*., 2019). The nutritional value of the forage can vary between cultivars, genotypes and ecotypes within the same species (Tavlas *et al*., 2009). Therefore, understanding the PPO activity, substrate composition and concentrations in different RC accessions could make it possible to select accessions that can optimize protein utilisation for milk production in dairy cattle.

The objective of this research study is to evaluate and compare the ruminal protein degradability and dietary nitrogen utilisation of diploid and tetraploid red clover (*Trifolium pretense L*.) accessions across two years and to identify accessions that potentially enhance performance for livestock dietary management.

1.1 Background

1.1.1 Protein utilisation in ruminants

The global dairy industry continuously searches for enhancement in nutritional science to increase milk production efficiency and sustainability. Optimization of cattle diets through the management of protein intake is one of the essential elements in the dairy industry because protein is the priciest ingredient in animal feed and it has a significant influence on milk production. Therefore, a good balance of the protein content in the diet is required to prevent overload or deficiency which can affect nitrogen emissions and animal health.

The process of protein metabolism in ruminants mainly depends on the activity of rumen microorganisms (Bach *et al*., 2005). In the rumen, a diverse community of microbes including bacteria, protozoa and fungi break down digestible carbohydrates and certain rumen-degradable proteins into simpler molecules (Forbes and France, 1993). Volatile fatty acids that are produced from carbohydrate fermentation, serve as the primary source of energy for the host animal. Dietary protein contains two components, rumen-degradable and undegradable dietary protein (Bach *et al*., 2005). Protein and non-protein nitrogen sources are degraded to ammonia providing building blocks, along with carbon skeletons, for the synthesis of microbial protein (Gidlund, 2017). Excess ammonia-N that is not incorporated into microbial biomass is absorbed through the rumen wall into the bloodstream and transformed into urea by the liver (Bach *et al*., 2005). Rumen microbes can absorb both dietary and liver-recycled urea and convert it into microbial protein. This microbial protein is an excellent source for the production of milk or muscle protein (Tadele and Amha, 2015). A different rumen microbial community generates amino acids, nucleic acids, and a wide range of other organic compounds from both dietary and recycled internal sources (Storm and Ørskov, 1983). Depending on the source of origin of rumen undegradable feed crude protein (RUP), different amounts of proteins are digested and absorbed in the small intestine. According to the comparison of different forages by Edmunds *et al*. (2013), 10% - 20% of the CP in the forage is calculated as RUP. Rumen undegradable crude protein is a valuable source of protein because it contains soluble protein fractions that escape rumen degradation and can be directly absorbed from the small intestine (Ahvenjärvi *et al*., 1999; Choi *et al*., 2002). Additionally, it is a source of peptides and amino acids that contain additional protein value to increase milk protein production, but only after fulfilling the microbial protein requirement (Wright *et al*., 1998).

Undegradable dietary protein and microbial protein containing digesta, pass from the rumen to the small intestine. In the abomasum, those proteins are started to break down into amino acids and small peptides by animal dietary enzymes. These amino acids and peptides are then absorbed through the intestinal wall into the blood system and utilised for different physiological functions such as growth, maintenance and milk production.

1.1.2 Limitations in protein degradability in ruminal diet

Ruminants can convert low-quality protein in the diet into high-quality animal proteins such as meat and milk (Schroeder *et al*., 2008). Nonetheless, as described by Bach *et al*. (2005), ruminants' utilisation efficiency of nitrogen is relatively poor and causes unnecessary expenses for feeds and additionally, excretion of nitrogen into the environment. To ensure production efficiency, the animal has to prevent overload or deficiency of the protein within the diet. While increasing nitrogen input in the basal diet can lead to an increase in milk protein yield, the efficiency of converting dietary nitrogen into milk protein will decline (Huhtanen and Hristov, 2009). Ruminants can utilise different protein sources as forages but the conversion of protein into milk or meat is relatively inefficient, ranging from 16.4% - to 40.2% (Huhtanen and Hristov, 2009) due to high degradability and fermentation losses in silage (McDonald, 1981; Van Soest, 1994). A primary cause contributing to the comparatively low nitrogen use efficiency in ruminants is the animals' low marginal response to increasing the CP level in their diets (Huhtanen and Hristov, 2009). As described by Huhtanen *et al*. (2011), only 10% of the additional CP from soybean concentrate is converted into milk protein. One of the challenging strategies to enhance nitrogen use efficiency is improving mechanisms of utilisation of forage protein (Bakken *et al*., 2017). One alternative is modifying the botanical composition of the forage, for example by incorporating legume species which contain bioactive substances such as tannins and PPO which can affect protein degradation and availability. Another option is to speed up the rate of the wilting process during the preservation process so that more forage protein can be conserved by avoiding the typical proteolysis that happens in plant cells upon harvest (Bakken *et al*., 2017).

1.1.3 Red clover (*Trifolium pretense L.*)

Red Clover (*Trifolium pretense L.*) is an important forage legume, that is particularly popular in Northern Europe and Nothern America. Due to its high CP content has the potential to support milk production (Westreicher-Kristen *et al*.,

2018). Additionally, RC can effectively be used to produce silage (Westreicher-Kristen *et al*., 2017). When compared with lucerne, RC prefers to grow in less fertile, humid and acidic soils, but it requires more frequent replanting or rotation to maintain high levels of productivity (Vasiljević *et al*., 2005). As a legume, RC provides environmentally sustainable alternatives by reducing the need for synthetic nitrogen fertilizers and can serve as both protein and energy sources for ruminants (Van Dorland *et al*., 2006). Due to atmospheric nitrogen fixation ability, RC is highly beneficial for organic farming (Wilkins and Jones, 2000). Several variables including stages of plant growth and development, environmental conditions, frequency of harvesting, fertilization techniques and the particular cultivar used for the cultivation can affect the quality of the fodder crops (Markovic *et al*., 2022). The natural ploidy form of RC is diploid (2n=2x=14), but since the 1940s, tetraploid (2n=4x=28) RC has been produced through various breeding techniques to increase forage yield, disease resistance and persistence (Jing *et al*., 2021).

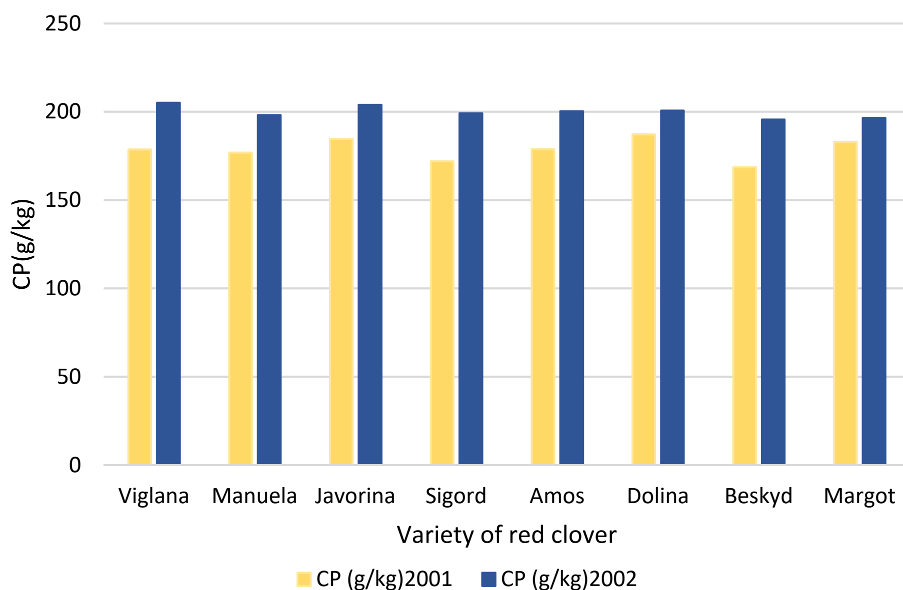

Figure 1. Crude protein concentration in RC varieties in the first and the second production years (Drobna and Jancovic, 2006)

Red clover tetraploids differ from their diploids by having a higher concentration of protein, WSC, potassium and phosphorous and lower levels of fibre (Drobna and Jancovic, 2006; Markovic *et al*., 2022). As described by Markovic *et al*. (2022), at the first, second and third stages of development the tetraploid cultivars' forage CP concentration was 12.5%, 8.7% and 10.4% higher than that of the diploid cultivars.

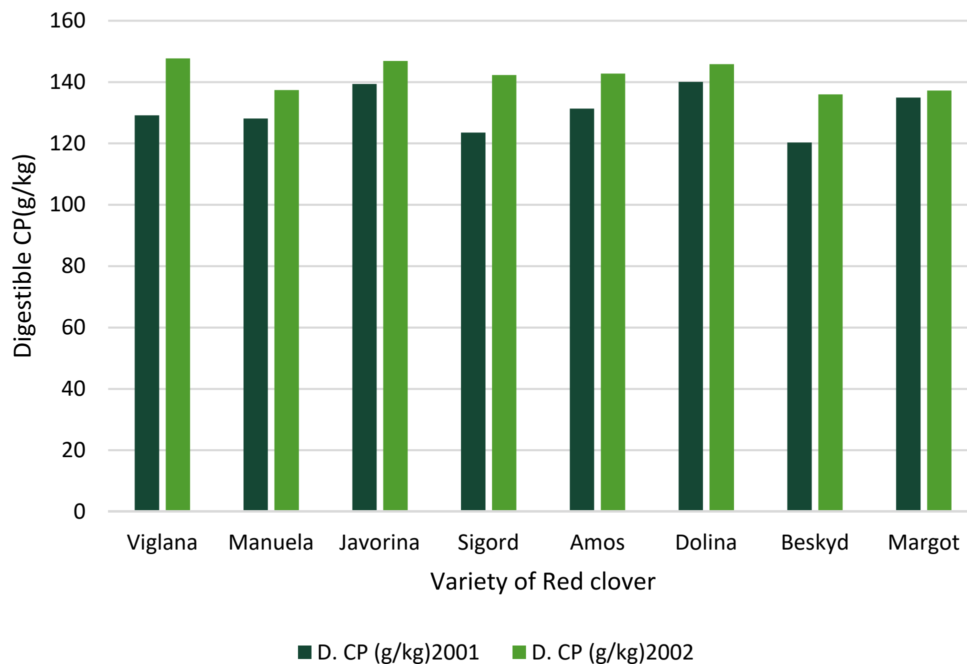

Figure 2.Digestible Crude Protein of RC varieties in the first and the second production years (Drobna and Jancovic, 2006)

Comparatively, CP in tetraploid RC cultivars has shown higher rumen degradability due to the higher leaf-to-stem ratio (Markovic *et al*., 2022). According to the study described by Drobna and Jancovic (2006), there was also a significant impact of the growing year on CP and the digestible CP in different RC varieties (Figures 1 and 2).

1.1.4 Ensiling of Red Clover

An anaerobic fermentation procedure known as ensiling is used to preserve forage crops like maize, legumes and grasses. Lactic acid bacteria which is naturally present in forage start to ferment sugars into lactic acid under anaerobic conditions. Production of lactic acid in the silage decreases the inside pH. Due to low pH conditions during storage, forage is preserved and inhibits the growth of spoiling organisms. The ensiling process enables farmers to store extra feeds for use in times of scarcity or low pasture availability. Silage-making is the most cost-effective way to preserve feeds. Legume silages are becoming more and more appealing to farmers throughout Europe due to developments in plant breeding and reducing costs for inputs like fertilizers (Dewhurst *et al*., 2003). Previous research has shown that utilising legume silages leads to higher feed consumption and milk yield when compared to grass silages (Dewhurst *et al*., 2003). In northern Europe, the most popular forage legume available for silage production is RC (Vanhatalo *et al*., 2009). Moreover, the RC silage causes an increase in the α-linolenic acid in the

milk. There are several physicochemical characteristics of legumes that could be involved in these processes (Dewhurst *et al*., 2003). Red clover contains PPOs, enzymes that inhibit the activity of plant proteases (Jones *et al*., 1995; Lee, 2014), which can help prevent protein degradation during the ensiling process and potentially increase the true protein content in the silage (Vanhatalo *et al*., 2009).

In contrast to the natural ensiling process, utilising silage additives can improve the preservation and overall quality of RC silage. Additives can increase the nutritional value of silage by reducing storage losses by enhancing the fermentation process, controlling the fermentation rate, and extending silage shelf life by increasing aerobic stability (Yitbarek and Tamir, 2014). Additives based on formic acid and propionic acid were primarily used to inhibit undesirable fermentation. Formic acid-based additives rapidly reduce the pH of silage and inhibit the activity of harmful microbes. Propionic acid has the primary advantage of suppressing yeast and moulds and enhancing the silage's aerobic stability. It guarantees that a population of LAB predominates during the fermentation process, generating lactic acid in quantities high enough to ensure a good silage. Also, LAB as an additive, has higher demand due to the efficient production of biological metabolites. It causes to production of higher lactic acid content with marginal levels of acetic acid and other organic acids. The silage pH becomes more acidic as a result of LAB's ability to use WSC and transform it into beneficial organic acids (Soundharrajan *et al*., 2021).

1.1.5 Polyphenol oxidase and phenolic compounds in forages and their effect on nitrogen utilisation

Polyphenol oxidases are enzymes found in many forages, as well as fruits and vegetable plants (Parveen *et al*., 2010). Red clover and cocksfoot (*Dactylis glomerata*) are two forages that contain significant PPO activity and high concentrations of PPO substrates when compared with other forages (Lee, 2014). Polyphenol oxidase activity has been identified in a variety of temperate grass species, with particularly high levels of the enzyme observed in cocksfoot and Italian (*Lolium multiflorum*), perennial (*Lolium perenne*), and hybrid ryegrass. Tall fescue (*Lolium arundinaceum*) and timothy (*Phleum pratense*) grasses have been found to have lower levels of PPO activity (Parveen *et al*., 2010). Polyphenol oxidases cause the browning reaction of fresh fruits and vegetables after bruising, cutting, or other types of cell-damaging processes. This browning process is associated with a decrease in protein breakdown in RC, both during ensiling and in the rumen (Lee *et al*., 2004). When natural phenolic compounds are exposed to oxygen, PPO facilitates their oxidation into quinones. These quinones are extremely reactive molecules that covalently alter and connect various components of cells, such as proteins (Figure 3), amines, and amides, resulting in the formation of

melanin pigments (Lee *et al*., 2004). Protein loss from forage legumes during ensiling is a common and significant issue in agriculture (Parveen *et al*., 2010).

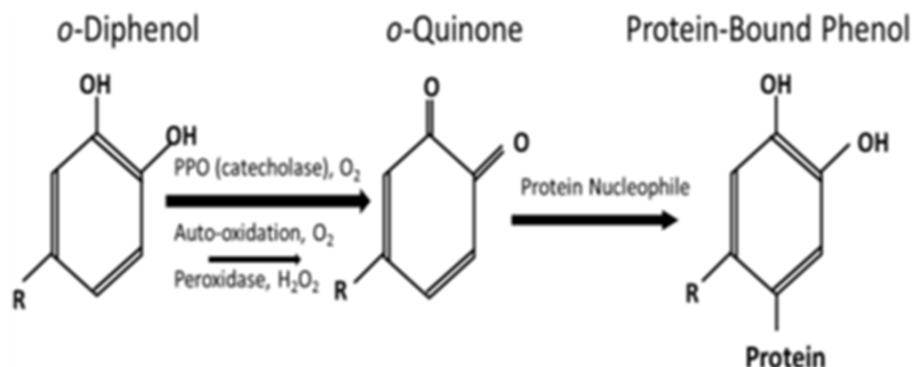

Figure 3. Protein-bound phenol is produced by the polyphenol oxidase process (Lee et al., 2013).

Due to this reduction process of protein breakdown, 80% of protein is retained as true protein in the RC silage compared with grass silage, leading to improved nitrogen use efficiency and product quality (Lee, 2004; Parveen *et al*., 2010).

Phenolic compounds are vital phytochemicals found in most plant species and encompass simple phenols, benzoic and cinnamic acids, tannins, lignins, lignans and flavonoids (Khoddami *et al*., 2013). Legumes are high in bioactive phenolic compounds, which are essential for several physiological and metabolic processes. The main phenolic compounds found in legume seeds are phenolic acids, flavonoids, and condensed tannins (Singh *et al*., 2017). Plant phenols have multiple functions, and serve as hydrogen-donating antioxidants, reducing agents and quenchers of singlet oxygen (Parveen *et al*., 2010). Many PPOs can oxidise smaller compounds like hydroxyl benzoic acids, hydroxycinnamic acids, and their derivatives. Phenols with higher antioxidant properties can also play a crucial part in the browning of forage plant material by going through secondary reactions with quinones produced by PPO (Parveen *et al*., 2010). Red clover contains a high concentration of phenolic compounds that can create indigestible phenol-protein complexes that may restrict the utilisation of protein in the animal.

1.1.6 Validity of the measurement of nitrogen in feed and protein evaluating systems

The nutritional value of feed is determined by its nutrient content, and how much it is broken down in the rumen, emphasizing nutrients transferred to the small intestine. To maintain and create microbial biomass, ruminants require a diet rich in proteins, sugars, starch, nonstructural and structural polysaccharides (Mohamed

et al., 2008). However, the fermentation process in silage modifies the composition of the forage, especially the levels of easily fermentable substances such as soluble proteins and carbohydrates (McDonald *et al*., 1991). A dairy cow diet must be nutritionally balanced to fulfil the amino acid requirements of the animal and should not create extra nitrogen to the environment as well as a negative impact on the farmer. If the diet is not nutritionally balanced it can lead to poor health and less productivity in the animal. Finally, it could show up as low milk production, poor reproduction or increased susceptibility to diseases which can affect the farmer's economy.

Ruminant protein evaluation systems are constantly evolving around the world, becoming more sophisticated over time (Edmunds *et al*., 2012). However, the complicated microbial process that occurs in the rumen, and their impact on subsequent activities in the intestines and body tissues, make the study of nitrogen metabolism in ruminants more complex than in non-ruminants (Hristov *et al*., 2019). As described by Dijkstra *et al*. (2018) the considerable variability in nitrogen excretion through urine compared to faeces allows for diet improvements to reduce urinary nitrogen output. In today's context, precision feeding of animals requires accurate evaluation systems and prediction models to enhance nutrient utilisation. Three scientific methods for evaluating feed protein quality and metabolism in the rumen are the *in situ* or nylon bag technique, *in vitro* rumen simulation, and *in vivo* whole-animal experimentation (Weiss, 1994). Accurate measurement of the degradability of feed is a significant challenge when establishing a new feeding technique for ruminants (Mohamed and Chaudhry, 2008).

1.1.7 *In vivo* methods

In vivo, protein evaluation methods are used to assess protein utilisation efficiency and metabolism within the ruminants through experimental methods. These techniques provide insights into the nutritional value of feeds and the efficiency of protein use in the animal's body and provide critical information for maximizing diets for improving animal health and productivity. Information obtained from the *in vivo* is considered the most accurate and should be utilised when assessing other methods since it reflects an animal's true response to a dietary intervention (López, 2005). Correct feed utilisation and output measurements, cannulated animals and reliable markers, are required for the successful implementation of this method. By surgically inserting cannulas into the digestive tract, researchers can collect samples straight from the animal and analyse the nutrient utilisation in different parts of the digestion tract. However, *in vivo* digestion studies are expensive, labor-intensive and difficult to apply to a wide range of feeds, especially when small amounts of each feed type are available (López, 2005; Vaga, 2017).

1.1.8 *In situ* method (*In sacco* technique / Nylon bag method)

Quin *et al*. (1938) described the first *in sacco* technique by using sheep, which has since become commonly utilised to estimate the utilisation of forages, concentrates and high protein feeds (Mohamed and Chaudhry, 2008). It is the most frequently used strategy for estimating the degradability of ruminal feed protein (Hvelplund and Weisbjerg, 2000). This method is based on incubating small feed samples in the rumen within nylon bags. These bags contain pores that are small enough to hold the feed sample in, but large enough to allow bacteria to enter. Because only small amounts of feeds are incubated, it is assumed that the feed being tested has no significant influence on rumen fermentation. Furthermore, it is assumed that the conditions inside the bags are similar to those in the surrounding rumen contents (Hvelplund and Weisbjerg, 2000). The disappearance of the feed ingredients is measured after incubation with several time points (Nozière *et al*., 2000). Following the necessary time, the samples are removed, rinsed, dried and then weighed. Several factors affect the degradation process of this method, such as the inserting method of the nylon bag into the rumen, the pore size of the bag, the type of feed material etc. (Zewdie, 2019). As described by Nozière *et al*. (2000) drawbacks of this method are low repeatability and inadequate reproducibility. In addition to these limitations, this method also faces challenges when measuring the degradation of soluble protein. During the *in situ* method, potential leakage of soluble materials and feed particles from the bag before complete degradation can happen and it can significantly impact when estimating the degradation of soluble proteins (Hvelplund and Weisbjerg, 2000). Several factors influence solubility, including extraction time, pH levels, ionic strength, and rumen temperature. These factors can affect the degradation of soluble proteins, making it difficult to isolate and accurately measure soluble protein degradation (Hedqvist *et al*., 2006). However, the *in sacco* method is laborious and needs surgically modified animals, causing animal welfare, and cost-related difficulties (Chaudhry, 2007).

1.1.9 *In vitro* method

To assess the degradability of feed protein, several *in vitro* methods have been established (Hristov *et al*., 2019). The *in vitro* method is based on incubating the desired substrate in a biological medium that stimulates rumen conditions. These techniques are broadly classified into two types: those that estimate parameters by analysing the composition of the final substrate and those that focus on measuring and analysing degradation end products such as gasses and ammonia production (Vaga, 2017). Since the first implementation of Tilley and Terry (1963), the practice of using rumen fluid for the *in vitro* incubation of feeds has been well established (Mohamed and Chaudhry, 2008). In this method, to simulate rumen

fermentation, rumen fluid is collected from ruminants and incubated in anaerobic conditions with substrate samples like feedstuffs and supplements. The *in vitro* gas production technique aims to reduce the complications associated with *de novo* protein synthesis during fermentation (Karlsson *et al*., 2009). Since the first description by Raab *et al*. (1983), this methodology has been modified several times to improve the estimation of protein degradation parameters. Raab *et al*. (1983) described a method in which various quantities of carbohydrates are introduced into rumen fluid, and the subsequent gas and ammonia production is used to evaluate microbial protein after a 24-hour incubation period. Researchers like Broderick (1987), Krishnamoorthy *et al*. (1990), and Lebzien and Voigt (1999) have proposed a lot of modifications over time (Vaga, 2017; Karlsson *et al*., 2009). Gas production systems have the advantage of automation, which reduces labor requirements. *In vitro*, techniques are considered more cost-effective than *in vivo* and *in situ* methods, and they provide data for the evaluation of both remains and metabolites produced by microbial degradation.

However, the main drawback of this technique is that it requires rumen fluid, which is generally collected from fistulated animals and is frequently considered undesirable (Mohamed and Chaudhry, 2008). Maintaining anaerobic conditions, difficulty of filtration due to the high viscosity of digesta, unpleasant odors, and the need for strict hygiene protocols to reduce the risk of pathogen infection are some of the challenges of using rumen fluid in this method (Jones and Theodorou, 2000). The *in vitro* method only simulates the ruminal fermentation of feed; it does not account for the emissions and digestion of the entire animal (Storm *et al*., 2012). Using enzymatic methods instead of rumen fluid can exclude the need for fistulated animals and anaerobic procedures (Nocek, 1988; Jones and Theodorou, 2000).

Steingass and Südekum (2013) proposed an effective method for determining utilisable crude protein (uCP) based on ammonia N production by using an in vitro technique, which can be used for feed ranking. This uCP evaluation method is more labor-efficient, substrate-specific, simple and highly repeatable when compared with other methods (Edmunds *et al*., 2012; Vaga *et al*., 2014). The estimated uCP is the sum of the feed protein that remains undegraded in the rumen and the microbial protein. The method described by Calsamiglia and Stern, (1993) by using uCP values can estimate the intestinal protein digestibility, with a large number of samples. This is a two-step *in vitro* procedure, that can estimate the total tract digestibility. Further, this method also can be used to measure the effect of PPO-induced protein complexing on protein degradability in different RC varieties.

Material and Method

2.1.1 Preparation of plant material

Both diploid and tetraploid varieties of RC from the genetic selection experiment were cultivated on the experimental field at 63.1628°N, 17.6506° E, in Lännäs, Västernorrland. A total of 45 accessions (23 diploid, 22 tetraploid) in 2022 and 44 accessions (22 diploid, 22 tetraploid) in 2023 were selected for the experiment. In this study there were four fields and each field contained 40 red clover varieties including four control red clover varieties. Control varieties were chosen as two diploid varieties, designated as SW ARES and SW YNGVE, and two tetraploid varieties designated as Peggy and VICKY to correct the effect of plot variation. Harvesting was done on the 22nd of July in 2022 and the 27th of July in 2023, in both years the herbage was collected from the second harvest. Including control varieties, a total of 70 samples were obtained in 2022, while 66 samples were collected in 2023 due to heavy weed growth in some plots. Plant materials were harvested with a haldrup plot harvester (Haldrup F-55, Haldrup, USA). Obtained material was cut and left outside to wilt until it reached 25% - 30% of DM. An initial determination of the DM for each sample was done by using a microwave oven. In 2023, wilting the herbage was insufficient due to frequent rain showers. The wilted forage was chopped with a garden shredder (Bosch AXT Rapid 2000, Robert Bosch Power Tools GmbH) and a formic-acid-based additive (ProMyr XR801, Perstorp AB, Perstorp, Sweden) was added at a rate of 6 mL/kg fresh matter. Samples were then vacuum-packed, with each variety packed in three replicate bags, with approximately 800 g of herbage per bag and sealed for ensiling purposes. These bags were allowed to ensile for 100 days at room temperature. After 100 days of ensiling time, about 150g of silage from each replicate silage bag was taken out, pooled and placed into polythene grip-seal bags, frozen at -20˚C and sent for silage quality analysis. The rest of the silages were placed in polythene bags frozen at -20˚C until they were freeze-dried to prevent further fermentation or degradation. The dried silage material from replicates was pooled and then ground through 1 mm sieves to take uniform particle size. Representative samples were taken for the nitrogen and DM analysis.

2.1.2 Silage analyses

The DM concentration of preserved forages was evaluated by drying at 105°C for 16 h and ash concentration was determined by incinerating at 500°C for 4 h. Crude protein was determined in forages (Nordic Committee on Food Analysis,

1979) by using a 2020 Digestor and a 2400 Kjeltec Analyzer Unit (Foss Analytical A/S, HilleroÈd, Denmark). Once the frozen silage samples were thawed and compressed, a pH meter (Metrohm, Herisau, Switzerland) was used to determine the pH of the liquid. As described by Ericson and André (2010), volatile fatty acids (VFA) and lactic acid were further analysed. After adding MgO, silage ammonium-N was analysed using a Kjeltec 2100 Distillation Unit (Foss Analytical A/S) by direct distillation. Neutral detergent fibre was analysed using heat stable α-amylase and sodium sulfite in an Ankom 200 Fiber analyzer (Ankom Technology Corp., Macedon, NY) utilizing the filter bag approach (Mertens, 2002).

2.1.3 *In vitro* procedures

The Swedish Ethics Committee on Animal Research (Permit A 6-2021) approved the experimental protocols, which were followed in the registration and conduct of the study. The Court of Appeal for Northern Norrland in Umeå gave its approval, guaranteeing compliance with Swedish legislation and rules enforcing EU Directive 2010/63/EU on animal research.

Rumen fluid was collected after two hours of morning feeding from two fistulated lactating Swedish red cows fed a diet containing grass silage and commercial concentrate (60:40 on DM basis) twice a day. Four layers of cheesecloth were used to filter an 800 mL volume of rumen fluid into an Erlenmeyer flask. All rumen fluid treatments were carried out under a constant flow of CO_2 to maintain anaerobic conditions. The pH of the rumen fluid of each cow was measured, and 0.2 mL was collected into an Eppendorf tube filled with 0.024 mL of H_2SO_4 before being frozen for ammonia-N analysis. The rumen fluid in the Erlenmeyer flask was mixed with 10.8 g of carbohydrates (maltose, starch, xylose, and pectin in a 2:1:1:1) and 2.8 g of $NaHCO_3$. Carbohydrates were added to promote microbial activity and reduce the background ammonia-N levels (Karlsson *et al*., 2009). The mixture was preincubated in a water bath and maintained at a temperature of 39°C for three hours. The mixture was stirred for a few minutes and then the stirrer was turned off. The buoyant layer was removed after 30 minutes on the rumen fluid with a vacuum pump (15-20% of V). After that, the stirrer was turned on again and the entire rumen fluid was continuously stirred for another 2.5 h. Every hour, 2*0.6 mL of rumen fluid was taken out for ammonia-N analysis during the pre-incubation period. Samples that were taken for ammonia analysis were always put in an Eppendorf tube filled with 0.024 mL of H_2SO_4.

As described by Menke and Steingaß (1988), the buffered mineral solution was mixed with the pre-incubated rumen fluid at a volume ratio of 1:4 rumen fluid to buffer. For the ammonia-N analysis, 2*0.6 mL of mixed rumen fluid was collected. Substrates (RC silage) of 1 ± 0.00x g were incubated in 60 mL of buffered rumen fluid in 36 serum bottles (250 mL) (Schott, Mainz, Germany). Serum bottles were filled with rumen fluid by using an auto pipette. After the first bottle in each bath,

two tubes were connected from the recording box to the computer to initiate the gas recording of the corresponding box in the computer. These steps were repeated for the remaining 35 serum bottles. All the bottles were placed in a water bath and continuously agitated for 48 hours at 39°C. Thirteen *in vitro* incubations (7 runs for 2022 and 6 runs for 2023) were conducted for the experiment with each sample replicated three times. In each run three blank bottles were included without substrate. According to Cone *et al*. (1996), the *in vitro* procedures were carried out using a fully automated system with continuous gas recordings. Fluid samples of 0.6 mL were collected from the incubation bottles at 4, 9, 20, and 30 hours of incubation to determine the concentration of ammonia-N in the liquid phase. Fluid samples were collected according to the procedure described in detail by Karlsson *et al*. (2009).

The fluid samples were stored at -20°C after being transferred to Eppendorf tubes with 0.024 mL of 18 M H_2SO_4 for preservation. Before ammonia-N analysis, Eppendorf tubes were thawed in lukewarm water and centrifuged at 12500 × g for 5 minutes. A supernatant of 0.2 mL was transferred to test tubes and diluted with milli-Q water. Following the manufacturer´s instructions (Method No. G-102-93 Rev 7 (multitest MT7)), the concentration of ammonia-N was measured by using a continuous flow analyser (Autoanalyzer 3 HR, SEAL Analytical Ltd). The same procedure was conducted for both the 2022 and 2023 samples.

2.1.4 Calculations

The following formula was used to calculate the concentration of uCP at 4, 9, 20 and 30 hours during incubation (Edmunds *et al*., 2012).

$$\text{uCP (g/kg of DM)} = \frac{NH_3\text{-N blank (mg)} + \text{N sample (mg)} - NH_3\text{-N sample (mg)}}{\text{Sample weight (mg of DM)}} \times 6.25 \times 1000$$

NH_3 -N blank (mg)	: Average amount (mg) of ammonia -N in the three bottles of blanks
N sample (mg)	: Amount (mg) of N in the sample at the start of the incubation
NH_3 -N sample (mg)	: Amount (mg) of ammonia -N in the incubation bottles with samples

As mentioned in Vaga *et al*. (2016), the natural logarithm of uCP values was plotted against time, and the intercept and slope were then used to calculate uCP_{16}, which represents an assumed rumen retention time (RT) as 16 hours or a rumen passage rate of 0.06.

2.1.5 Statistical Analysis

All statistical analysis was performed in R (R Core Team, 2023, R Foundation for Statistical Computing, Vienna, Austria). Analyses of the variance of data for the whole range of constituents in the silages were derived from linear mixed-effects models using the 'lmer' procedure in the 'lme4' package (Bates *et al.*, 2015). RC accession, harvest year and ploidy were fixed variables while field within a year was the random variable. Due to the augmented design, the effect of the field was only assessed by the control varieties replicated in random locations within the fields.

The mixed model for uCP included accession, CP concentration, harvest year and ploidy as fixed variables and field within a year and *in vitro* run as a random factor. The effects of silage constituents and interaction between year and ploidy were assessed for the uCP model but were found insignificant and excluded from the final model. The difference between the means of harvest year and ploidy was determined by Tukey's HSD test. The model-predicted uCP_{16} concentration in RC silages predicted by CP concentration was plotted against the means to assess the differences in accessions. Unless otherwise specified, a probability of $P < 0.05$ was considered significant.

Correlations between pairs of silage parameters were analysed by the procedures from the 'correlation' package (Makowski *et al.*, 2022) and expressed as Pearson's correlation coefficients.

Results

3.1.1 Chemical and nutritional characteristics of red clover silage

In 2022 and 2023, differences are observed in several fermentation characters of silage in diploid and tetraploid accessions (Table 1). All silages maintained a pH lower than 4.06 in 2023, but the pH for 2022 was not analysed. The mean value of silage DM was comparatively higher in 2022 (P = 0.007) at 226.0 g/kg (SD = 31.97) than in 2023 at 180.5 g/kg (SD = 16.08). The mean silage ammonia N content in 2022 at 0.26 g/kg DM (SD = 0.07) was comparatively lower (P = 0.019) than in 2023, being 0.96 g/kg DM (SD = 0.213). In both years the silage ammonia N was below 1g/kg DM. All silage samples had a high lactic acid content compared with other fermentation acids.

Table 1. Digestive data on RC silage characteristics between 2022 and 2023 (values expressed as g/kg DM, unless otherwise stated)

	2022 Red clover silage			**2023 Red clover silage**		
	Diploid	Tetraploid	SD	Diploid	Tetraploid	SD
No. of samples	34	36		30	36	
pH	NA	NA	NA	3.92	3.96	0.055
Silage DM (g/kg)	249.1	204.0	31.97	188.1	174.1	16.08
Ash	93.02	89.31	4.465	89.27	80.94	5.833
Ammonia N	0.31	0.20	0.076	0.99	0.93	0.213
lactic acid	35.85	34.48	5.490	86.87	76.52	13.846
Acetic acid	6.51	5.94	1.183	11.74	15.39	4.326
Propionic acid	1.62	1.94	0.362	3.57	3.88	0.752
Butyric acid	0.28	0.36	0.273	0.43	0.48	0.045
Ethanol	0.91	0.62	0.228	1.78	1.98	1.056
Formic acid	5.85	7.74	1.438	18.30	18.83	3.232
Butanediol	0.28	0.36	0.050	0.43	0.48	0.045

NA – not available; SD – standard deviation

However, silages of 2022 had a lower amount of lactic acid than in 2023, being 35.15 (SD = 5.49) and 81.22 g/kg DM (SD=13.84), respectively. All the silages

contained a very low amount of butyric acid and ethanol. There were no significant differences (P = 0.639) in ethanol content in the silage.

Table 2, shows variations in NDF, CP content and uCP_{16} in diploid and tetraploid silages over the two years. CP content was constantly higher (P = 0.008) in diploid silages across both years, while the year also affected (P = 0.002) the silage CP content. In contrast, the NDF content was higher (P = 0.044) in tetraploid silages than in diploids in both years. However, the silage NDF content did not differ (P = 0.214) between years. According to the analysis of the data, only the year had a significant effect (P = 0.048) on the uCP_{16} values of silages.

Table 2.Comparative analysis of CP, NDF content and uCP_{16} content in RC silage across ploidy and, growing year. P values show significance for the effect of ploidy and year.

	Red clover 2022		**Red clover 2023**		**SEM***	**P value**	
	Diploid	Tetraploid	Diploid	Tetraploid		Ploidy	Year
No of samples	34	36	30	36			
CP g/kg DM	198	184	220	206	2.20	0.008	0.002
NDF g/kg DM	304	334	289	320	9.25	0.044	0.214
No of samples for uCP_{16}	107	117	90	108			
uCP_{16} g/kg DM	186	188	183	185	2.06	0.279	0.048

*SEM – standard error of the mean

3.1.2 Relationship between utilisable crude protein, neutral detergent fibre, crude protein, and the year

There was a strong positive correlation (R^2 = 0.78) indicated between uCP_{16} and CP content in the silage across the two years. Further analysis of data presented in Figure 4 indicates the connection between CP content and uCP_{16} in 2022 (R^2 =

0.35) and 2023 (R^2 = 0.64) separately. A negative linear relationship was found between NDF and both uCP16 (R^2 = 0.47) and the silage's CP content (R^2 = 0.69).

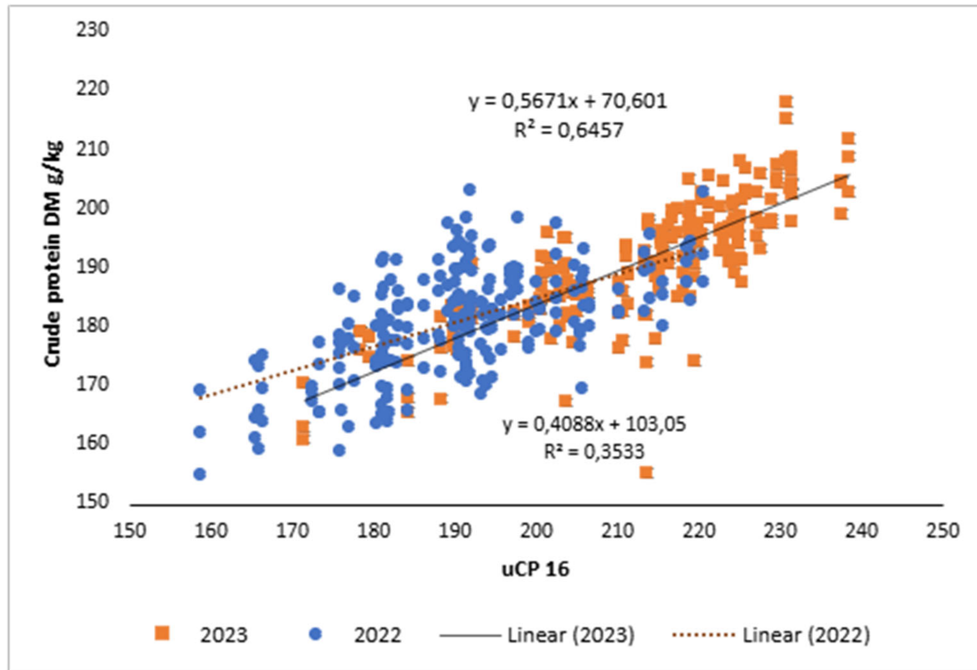

Figure 4. Relationship between CP in silages and the uCP_{16} in 2022 and 2023 silages (slope 2023:P<0.001, 2022: P<0.001)

Figure 5. shows the least squares means of ammonia-N concentration in the *in vitro* incubation medium for diploids, tetraploids and blanks at different time points following the initiation of *in vitro* incubation. Ammonia-N concentration was higher in diploids than in tetraploids at all the time points in both years but did not differ between blanks.

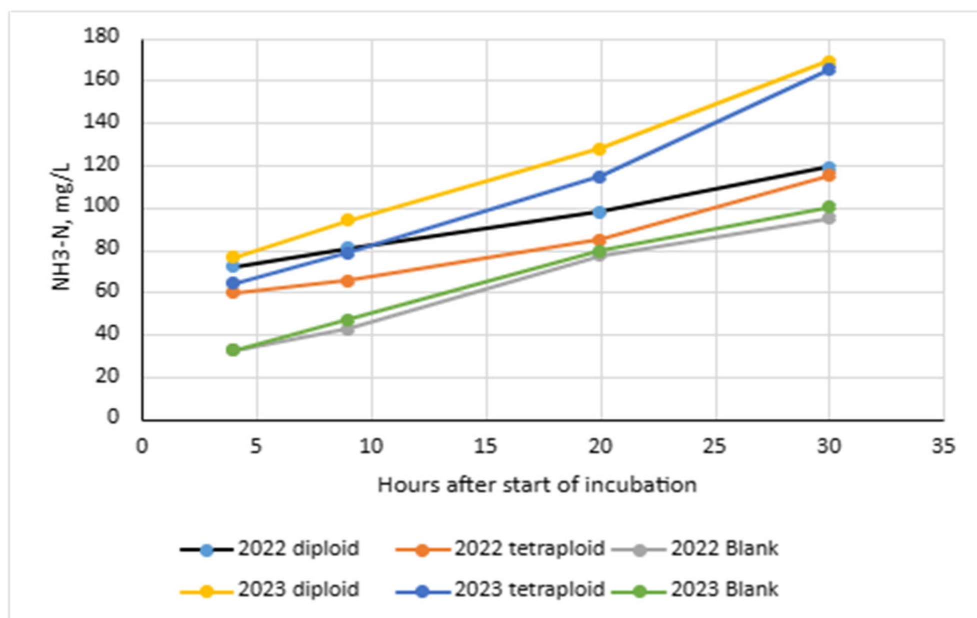

Figure 5. Means of ammonia N concentration (mg/L) in in vitro incubation medium for diploid, tetraploid and blanks.

The mean ammonia-N concentration in blanks increased linearly after the starting time point up to 30 hours. The mean ammonia-N concentration in rumen fluid at the beginning of the incubation was 30.9 mg/L (SD = 6.2) in 2022 and 30.2 mg/L (SD = 8.7) in 2023.

3.1.3 Comparison of utilisable crude protein between individual red clover varieties

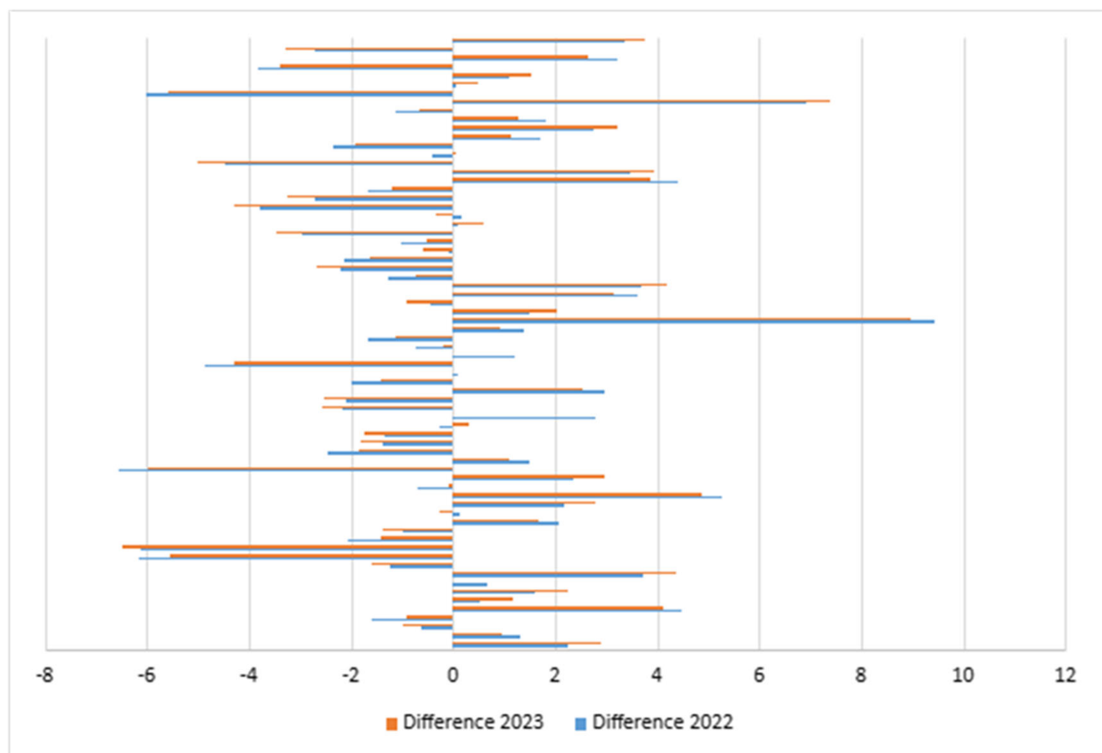

Figure 6. Calculated differences between the predicted and measured uCP_{16} values among individual RC varieties in 2022 and 2023 (0 indicates no difference between predicted and measured). Measured vs. predicted values over the incubation period, with negative values indicating that measured values being higher than predicted and positive values indicating lower than predicted.

Calculated differences between the model predicted and measured uCP_{16} values in both years have shown that in most of the varieties, the differences are close to zero, but some outliers can be identified (Figure 6). Positive values on the X-axis in Figure 6 indicate that the measured values were lower than the predicted values and the negative values on the X-axis indicate that the measured values were higher than the predicted values. There were a few varieties which had higher differences in uCP_{16} values among those selected RC varieties. According to Figure 6, three

varieties had 5 to 9 g/kg DM ($P<0.001$) lower uCP_{16} concentration than predicted by the model based on silage CP concentration, whereas 6 varieties had observed uCP_{16} values between 4 to 7g/kg DM higher ($P < 0.001$) than predicted values. As shown in Figure 6, the differences between measured and predicted values were similar in both years in the same varieties.

Discussion

4.1.1 Methodology

The objective of this study is to measure the variation in protein concentration in diploid and tetraploid RC accessions and evaluate ruminal nitrogen utilisation by using *in vitro* uCP_{16} values. The aim is to identify those accessions that potentially enhance performance for better livestock dietary management. Over time, various methodologies have been developed for estimating feed protein value; nevertheless, they all have various forms of methodological errors and variations. Although the *in situ* technique has long been the most popular method for determining RUP and rumen CP degradability, there are several issues with this technique (Nozière and Michalet-Doreau, 2000). For example, the *in situ* technique assumes that all the soluble protein is entirely broken down in the rumen but some of it escapes to the small intestine, which creates an error when estimating the RUP (Nozière and Michalet-Doreau, 2000). Also, this effect was observed by Ahvenjärvi *et al*, (2017) where it was shown that up to 15% of the soluble non-ammonia N escaped from rumen degradation. Tiny particles that leave the bag without breaking down are presumed to have done so and microbial colonization of the undegraded protein particles can create an error when estimating the protein degradability by using the *in situ* method (Gidlund *et al*., 2018).

The estimation of uCP in the past was based on the direct measurement or prediction of RUP and microbial protein levels (Lebzien and Voigt, 1999). Techniques based on uCP determination are more practical than the *in situ* and *in vivo* measurements of RUP and microbial protein synthesis because of the variability of the measurements (Vaga *et al*., 2014). Under an *in vitro* system, protein degradation occurs in closed systems without the intake of new feed or removal of digesta. The time required to reach a particular digestive feed from the rumen is shorter *in vitro* than *in vivo*. When estimating uCP under *in vitro* technique, RUP and microbial protein perform concurrently during incubation. However, one of the drawbacks of this procedure is microbial and feed nitrogen are difficult to separate (Gidlund *et al*., 2018). The modified Hohenheim gas test (modHGT), created by Steingaß *et al*. (2001), was employed to determine the uCP values. Steingaß and Sudekum (2013) further described this method to use ammonia production for the evaluation of uCP values. This method estimates the combined value of microbial and RUP as uCP based on the measurement of ammonia production following incubation of a substrate with rumen fluid. The effectiveness of this method in determining the protein value of feeds or diets has

been further enhanced by technical advancements that provide repeated sampling from the same incubation vessel (Karlsson *et al*., 2009).

Retention time determination of individual protein supplements in the rumen is important because it affects the amount of protein degradation by microorganisms in the rumen, which can be affected by the residence time of feed protein in the rumen (Stern *et al*., 1983). The feed digestion rate determines the RT needed for obtaining effective uCP in batch culture systems. If the digestion rate is faster, less RT is required to obtain the uCP values (Gidlund *et al*., 2018). In this study, 16 hours was chosen as the mean retention time. This was selected as the most optimal representation of the mean RT of proteins for comparison of silage proteins (Vaga *et al*., 2014). Various estimates of uCP at different passage rates were tested in the current study but they did not show any significant improvements for silage comparisons, as all silages were fairly similar and different passage rates do not change the ranking of silages. Strong correlations were identified between uCP levels calculated using various RTs by Gidlund *et al*. (2018), indicating that this is not a big issue for similar diets.

As described by Lorenz *et al*. (2011), there were variations in ammonia concentration and microbial activity in rumen fluid which can affect the accuracy and the consistency of the method. Because these factors can vary between animals and even at different sampling times from the same animal, the rumen fluid was collected from two animals at each time to mitigate the variability and enhance the reliability of the findings. Pre-incubation procedure was done to stimulate microbial activity and reduce background ammonia-N levels in the rumen fluid by using relevant carbohydrates (pectin, maltose, starch, xylose) and rumen fluid, as Lorenz *et al*., (2011) mentioned. In this current study, at the beginning of the incubation period, we observed a stable level of ammonia in the *in vitro* medium and there was little variation of ammonia - N levels between replicates and the *in vitro* runs. Those observations indicated the effectiveness of preincubation and carbohydrate utilisation in the method. To facilitate optimal microbial growth, the concentration of ammonia in rumen fluid is maintained at or above 50 mg/L (Satter and Slyter, 1974). In our study, ammonia N concentration in rumen fluid was around 30mg/L in both years which was considered not too low for the microbial activity.

The *in vivo* technique is not ideal for frequent feed evaluation since it is time-consuming, costly, requires huge amounts of feed, and is often inappropriate for individual feedstuffs (Mohamed, R. and Chaudhry., 2008). Analytical errors can happen during the differentiation of proteins that originate from endogenous, microbial and feed might complicate *in vivo* techniques (Karlsson *et al*., 2009). However, the findings of *in vitro* studies may not be directly applicable to *in vivo* conditions, but a good prediction value has been found between *in vitro* uCP value and *in vivo* measurements in the study of Gidlund *et al*. (2018). In that study, predicting milk protein yield from uCP supply showed a positive relationship

(coefficient of determination = 0.79), with no difference in model fit for different time points of incubation (16, 20, or 24 h). Those results indicate that the *in vitro* method is a useful technique for evaluating the protein value of a ruminant diet. Due to the static nature of *in vitro* environments, which are not equal to the *in vivo* conditions, *in vitro* results do not represent similar results as *in vivo* conditions. For example, microbes are unable to constantly receive feeds and new feed sources during incubation in *in vitro* studies. Furthermore, *in vivo* conditions involve a complex interplay between host physiology, immune response, and interactions with other organisms, which cannot be fully replicated in an *in vitro* system. However, *in vitro* techniques are more suitable than *in sacco* and *in vivo* methods as it has high analytical capacity, are low-cost and speedy (Bueno *et al.*, 2005; Yáñez-Ruiz *et al.*, 2016).

4.1.2 Fermentation quality

Some differences can be identified according to the analysis results of silages prepared in two years. The formation of ammonia N is a reflection of the action of proteolysis in silage. In both years the amount of ammonia N was below 1g/kg DM. This is an indication of good-quality silage (Dryden, 2008) suggesting that there might not have been considerable proteolysis. Proteolytic activity primarily occurs by microbes in low-DM silages at high pH values (pH > 4.2) due to microbial development and activity (McDonald *et al.*, 1991). In 2023 all silage samples had a pH below 4.06 which is an indication of good-quality silage (Table 1; Eurofins, 2015). Legumes have higher buffering capacity than grasses making it more difficult to reach the necessary pH to satisfactorily ensile (McDonald *et al.*, 2010). In the current study, adding formic acid and propionic acid-based additives enhances silage quality by reducing silage pH and ammonia N concentration as expected (Jones, 1991; McDonald *et al.*, 2010). Our silages maintained a higher concentration of lactic acid in both years which is an indication of good quality silage (Kung *et al.*, 2018; Wang *et al.*, 2022). Generally, 10–12 times stronger lactic acid is found in proper silages than any of the other major acids (such as acetic and propionic acid) and contributes most to the reduction of pH during the fermentation process (Kung *et al.*, 2018). Lactic acid concentrations in grass silages typically range from 80 to 120 g/kg DM, but RC silages have low soluble carbohydrates and high buffering capacity, thus acid production can be limited (McDonald *et al.*, 2010). Acetic acid concentration was the second highest acid in our silages, typically ranging between 1–3% of the DM. A moderate concentration of acetic acid can contribute to increasing the aerobic stability of the silage (Kung *et al.*, 2018). Propionic and butyric acid concentrations were low in both years of prepared silages which is a better criterion of good quality silage. Ethanol concentration, which is associated with the undesirable growth of yeast in the silage (Van Den

Pol-Van Dasselaar *et al*., 2019), was also low in all our silages. After a short wilting period of one or two days, to make a good quality silage the DM content should be increased from approximately 20% to, preferably, 25% to 30% (Van Den Pol-Van Dasselaar *et al*., 2019). Overall, in both years all the silages were well-preserved and good in fermentation quality.

4.1.3 *In vitro* protein utilisation

In this study, we have found variations in uCP_{16} content, CP and NDF content in red clover silages across ploidy levels and growing years that can influence the feed management of dairy cattle. According to previous studies, RC that is harvested at the same development stage can differ from its nutritional content due to climatic, seasonal and geographic factors (Buxton, 1996). In this study, diploids contained more CP across both years than tetraploids, indicating the influence of genetic factors on silage composition. Due to the negative linear correlation between the CP and the NDF content, tetraploids contain higher NDF values in the silage in both two years. A higher content of structural fibre in tetraploid is suggested by a higher NDF concentration, which can lead to decreased protein availability and reduced digestibility compared with the diploid silages. Seasonal and genetic variations are major variables that affect the fibre concentration in the same forage silage (Vasiljević *et al*., 2011).

In the current study, a strong relationship was identified between uCP_{16} values and CP values of herbage. According to the study by Marković *et al*. (2022), tetraploid RCs contain more rapidly degradable proteins due to lower concentrations of lignin and unavailable carbohydrate fractions than diploids. Plant cell walls contain lignin, a complex polymer highly resistant to digestion. According to Du *et al*. (2016), there was a negative correlation between the lignin content and the effective rumen degradability of CP content. Also, Purwin *et al*. (2011) have shown that the genetic form of the RC is affected by the proteolysis process of silage. They suggested that diploids and tetraploids differed in chemical properties (PPO, polyphenol content) that could affect the proteolysis. Compared with other forages like grasses, RC has an extra advantage due to containing PPO, an enzyme that deactivates plant proteases (Jones *et al*., 1995a; 1995b; Lee, 2014). PPO can reduce the activity of proteases that can prevent protein breakdown during the ensiling process and can enhance true protein content in silages (Broderick *et al*., 2001; Vanhatalo *et al*., 2009).

In our results, we identified a significant year effect on uCP_{16} values that can be due to variations in weather conditions, agronomic practices and other variables. Due to the variability between two years, the year effect always influences the results. High concentrations of ammonia N production were observed in diploids at

different time points in the *in vitro* procedure which may be due to the high content of CP content in the diploid silages. However, as described by Drobna and Jancovic, (2006) CP content alone is not enough to measure the accurate nutritional value of feed nitrogen for dairy cows because it does not represent the true value of protein absorbed from the small intestine.

4.1.4 Utilisable crude protein between individual red clover varieties

The main predictor for uCP_{16} was the silage CP concentration with a correlation of 0.78. Therefore, uCP_{16} values of selected accessions were corrected for CP concentration in the silage, year and field effect. As shown in Figure 6, most RC cultivars appear to have nearly zero variation between predicted and measured uCP_{16} levels, indicating that the model used to predict the uCP_{16} values based on the CP concentration was accurate for most of the varieties. In our study, in both years most of the varieties had the same patterns of differences between predicted and measured uCP_{16} values and this consistency indicates that these differences are not simply due to seasonal effect or a random error. According to the variance results, some accessions had higher variance and some had lower variance between the predicted and measured uCP_{16} values. This indicated that uCP_{16} values were varied according to the accessions. Selecting better RC accessions only by using CP values would not make reliable results. Because rumen degradability can depend on various other factors such as plant maturity, forage conservation method, and animal factors like passage rate, stage of production etc. As described by Drobna and Jancovic (2006) the diploid Viglana variety has high protein and high energy levels in the herbage, and the tetraploid cultivars of Jaguarina and Dolina exhibited high CP and lowest energy values. While the Manuela type of forage had the highest energy values but the lowest protein levels. These results indicate that the nutritional characteristics of the forage vary between the individual varieties.

It is necessary to enhance the quality of the forage to feed animals sufficiently during the long winter months. Lower animal productivity results from nutrient deficiencies in forages, which also have an impact on rumen fermentation and microbial development (Assefa and Ledin, 2001). Thus, selecting better genotypes in terms of quality and yield is essential for creating diets that greatly increase the production of milk and meat (Tavlas *et al*., 2009).

Conclusions

An *in vitro* technique based on ammonia- N production was used to evaluate and compare the dietary nitrogen utilisation by using uCP_{16} values of diploid and tetraploid RC accessions across two years (2022 and 2023). In both years, all silage samples exhibited good fermentation characteristics, of well preserved silage including low pH values, high concentration of lactic acid than other fermentation acids , and low concentration of butyric acid. Diploid RC silages were higher in CP and lower in NDF content than tetraploid RC silages. In both years a negative linear correlation between NDF and both uCP_{16} and CP indicates that higher content of fibre levels may compromise the ruminal protein utilisation. The CP content of RC silage was higher in 2023 than in 2022. Variations in uCP_{16} values between measurements and predictions indicated accession-specific effects in RC silage in both years. Certain accessions had higher differences in their predicted uCP_{16} values, while others exhibited lower differences compared to the measured values. From this study, we can conclude that there were numerical variations between diploids and tetraploids of RC accessions and significant differences between the years in terms of protein utilisation in the rumen. Further research should be conducted to evaluate the relationship between CP utilisation efficiency, relevant variables and RC accessions. For example, the research could focus on, genetic and environmental interactions of different accessions, nutritional quality assessments of RC with various accessions and long-term feeding trials.

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Appendix 1

Figure 7. Harvested red clover at Lännäs, Västernorrland

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