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Comparative Evaluation of Enzymatic Crude Protein Degradation in Selected Legume Forages

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ABSTRACT

For protein evaluation of feedstuffs for ruminants, the *Streptomyces griseus* protease test offers a purely enzymatic approach to estimate ruminal protein degradation. This study was conducted to determine the enzymatic crude protein (CP) degradability of alfalfa, sainfoin, and common vetch hays, which are commonly used in ruminant nutrition. To estimate CP degradation, fifteen samples from each type of hay were incubated *in vitro* with a commercial protease extracted from *Streptomyces griseus*. The incubation was carried out for 1, 4, 24, and 48 hours in a borate-phosphate buffer at pH 8. Significant differences in CP degradability values were found among all three types of hay across all incubation periods. For all incubation periods, sainfoin had the lowest CP degradability values ($P < 0.05$), due to its high content of cell wall components and condensed tannins (CTs). For incubation periods longer than 1 hour, common vetch had the highest CP degradability values, followed by alfalfa and sainfoin, respectively ($P < 0.05$). As a result, the use of the protease enzyme extracted from Streptomyces *griseus* was confirmed as an effective method for estimating the CP degradability of selected legume forages in the laboratory, eliminating the need for animal testing. However, since plant proteins are often embedded within carbohydrate complexes, it is recommended that future tests consider the combined use of protease and carbohydrase, particularly for sainfoin, which is rich in cell wall components and condensed tannins.

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Introduction

Efficient protein utilization in ruminant nutrition is crucial for sustainable animal production, given the limited availability and high cost of feed protein. One of the critical aspects of optimizing feed efficiency is the accurate estimation of ruminal crude protein (CP) degradation. This process significantly influences nitrogen utilization, impacting both animal health and environmental sustainability (Hristov et al., 2019; Okon et al., 2023). The degradation of CP measured *in vivo* is often considered the reference method for assessing ruminal protein degradation. However, it is labor-intensive, time-consuming, and prone to errors due to individual animal variations and the use of markers (Karlsson et al., 2009; Edmunds et al., 2012; Liu et al., 2019; Titze et al., 2024). Therefore, several methodologies have been developed to estimate ruminal CP degradation, including *in situ*, *in vitro*, and enzymatic techniques.

The *in situ* method, despite its widespread use as a reference (Hvelplund & Weisbjerg, 2000), presents several challenges. At the forefront of these challenges is the necessity of using cannulated animals. Rumen cannulation is a surgical intervention on the animal's digestive system and, as such, it is criticized by animal welfare advocates for potentially causing pain and discomfort, raising ethical concerns (Castillo & Hernández, 2021). Additionally, significant challenges include increased costs, variability in results due to differences in animal physiology, and the physical characteristics of the rumen environment (Pagella et al., 2018). The accuracy of the *in situ* method can also be compromised by factors such as bag pore size, sample size, and incubation time, leading to potential inaccuracies in estimating the true rate of CP degradation (Vanzant et al., 1998). Furthermore, criticisms have been directed at this method due to the difficulty of microorganisms reaching the feed inside the bag (Meyer & Mackie, 1986), contamination of indigestible feed by microorganisms in the bag (Nocek & Grant, 1987), and the loss of feed through the bag pores (Aufrère, 1999).

Enzymatic systems utilizing proteolytic enzymes offer several advantages over live microbial cultures, including lower cost, reduced time, less contamination of feed residue, and no need for cannulated animals (Nocek, 1988; Vinyard & Faciola, 2022; Okon et al., 2023). This approach is particularly relevant for CP degradability in the rumen, as it can improve efficiency and reduce ethical concerns (Okon et al., 2023). Researchers can also utilize this method to align with the three R's of animal research: reduce, refine, and replace (Curzer et al., 2016). Although various proteases have been utilized, the protease from *Streptomyces griseus* has been the most commonly used (Licitra et al., 1998). *Streptomyces griseus* is a commercially available protease with a broad spectrum of activity, featuring both exopeptidase and endopeptidase functions, similar to those found in the rumen (Roe et al., 1991; Licitra et al., 1998). It can hydrolyze proteins, such as oligopeptides, up to 90% (Licitra et al., 1998). In comparison, ficin has been found to cause faster degradation rates of feed proteins, which may render it less suitable for accurately measuring ruminal protein degradation dynamics (Roe et al., 1991; Kosmala et al., 1996; Coblentz et al., 1997). Additionally, neutral proteases (Roe et al., 1991), bacterial proteases, pepsin, and trypsin have shown inconsistencies when compared to *in situ* degradation curves (Lynch et al., 1988). Several studies have demonstrated a close correlation between CP degradation estimated *in situ* and that obtained using the *Streptomyces griseus* protease test for both concentrates and roughages (Aufrère et al., 1991; Cone et al., 1996; Coblentz et al., 1999; Mathis et al., 2001; Edmunds et al., 2012). Studies that utilize the *Streptomyces griseus* protease test to rapidly and cost-effectively analyze CP fractions in various forages can provide valuable insights for optimizing protein utilization in dairy cattle.

In vitro enzyme methodologies offer advantages in animal nutrition research but also have certain limitations. These laboratory-based methods cannot fully replicate the natural processes in the digestive systems of animals or the complex interactions between microorganisms (Vinyard & Faciola, 2022). Pure enzyme environments can limit the scope of experiments, and the lack of interaction with other microorganisms may affect the growth of target microorganisms (Vartoukian et al., 2010). Furthermore, in the rumen, the production and constant removal of nutrients can lead to pH fluctuations, which further complicate the situation. In pure culture environments, stable pH and other controlled conditions cannot fully replicate the constantly changing environmental factors of the natural setting, thereby limiting the accurate representation of microbial growth patterns (Russell & Dombrowski, 1980). These factors necessitate careful interpretation of results obtained from *in vitro* methodologies.

The hypothesis of this study was that specific characteristics of legume forages, such as cell wall components and condensed tannins (CTs), lead to variability in the susceptibility of feed proteins to protease. As a result, a significant portion of feed protein may become linked to carbohydrates, like starch and fiber, forming a matrix that affects the ability of proteases to degrade proteins. These matrix effects might prevent the protease from completely degrading the feed protein (Abdelgadir et al., 1997; Pedersen et al., 2015). This effects may cause proteins to become trapped within plant cell walls and compartments that proteases find difficult to reach (Santra & Karim 2002; Colombatto et al., 2007), which can affect the speed and extent of protein breakdown.

The objective of the present study was to determine the degradation of CP in legume forages, specifically alfalfa, sainfoin, and common vetch hays, using the *Streptomyces griseus* protease test.

Materials and Methods

Hay Samples and Chemical Analyses

In the present study, fifteen samples each of alfalfa (*Medicago sativa* L.) hay, sainfoin (*Onobrychis viciifolia* L.) hay, and common vetch (*Vicia sativa* L.) hay, harvested as green forage from research institutes affiliated with the General Directorate of Agricultural Research and Policies (TAGEM) in various regions of Türkiye, were used. Alfalfa samples at 10% blossoming during the third mowing, sainfoin samples at 50-100% blossoming, and common vetch samples at the pod formation stage when a few beans had matured near the soil were harvested. Forage samples, initially wilted under shade, were dried in a forced-air oven at 60°C for at least 48 hours until reaching a constant weight. The temperature and moisture levels were regularly controlled to shorten drying time and prevent excessive heating. The samples were then ground to pass through a 1-mm screen using a Retsch mill (Retsch GmbH, Haan, Germany) and analyzed for dry matter (DM, 930.15), crude ash (CA, 942.05), ether extract (EE, 920.39), and crude protein (CP, 954.01) according to AOAC (2003) methods. Determinations of neutral detergent fiber (NDF) and acid detergent fiber (ADF) followed the methods described by Van Soest et al. (1991), using an Ankom200 Fiber Analyzer (Ankom Technology, Fairport, NY, USA). The acid detergent lignin (ADL) content in ADF samples was determined by immersing them in 72% sulfuric acid for 3 hours in beakers. The NDF analysis included the use of heat-stable amylase and sodium sulfite, and both NDF and ADF measurements included residual ash. Hemicellulose (HC) was calculated as the difference between NDF and ADF and cellulose (C) as the difference between ADF and ADL. The CTs content was determined using the hydrochloric acid (HCl)-butanol assay (Reed, 1986).

Enzymatic Assay

The enzymatic degradation of CP in alfalfa, sainfoin, and common vetch hays was measured by enzymatic hydrolysis for 1, 4, 24, and 48 hours using protease extracted from *Streptomyces griseus* in a borate-phosphate buffer (BPB) at pH 8, as described by Aufrère & Cartailler (1988) and Aufrère (1999). Time points commonly used in previous studies (Aufrère & Cartailler, 1988; Janicki & Stallings, 1988; Abdelgadir et al., 1997; Coblentz et al., 1999) were selected to assess how the kinetics of protein degradation change over time and to evaluate both the short-term and long-term effects of proteolytic activity. The enzyme solution was prepared by mixing 2 g of *Streptomyces griseus* protease Type XIV, (Sigma Chemical, Catalogue No. P5147, 4.6 units/mg) with 1000 mL of BPB. The BPB was prepared by dissolving 12.20 g

of sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) and 8.91 g of sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) in distilled water. The pH of the solution was adjusted to 8 with 1 N NaOH, and the final volume was made up to 1000 mL. The BPB was freshly prepared, and the solution was periodically checked to prevent pH changes.

The enzymatic activity was tested on sodium caseinate (Sigma C 8654) (Aufrere & Cartailler, 1988). A 2% sodium caseinate suspension was prepared in a pH 8 BPB. Each tube was filled with 5 ml of this suspension, followed by the addition of 0.5 ml of enzyme solution (0.1 mg/ml for the enzyme). The tubes were incubated in a water bath at 40°C for 10 minutes. The reaction was stopped by adding 2% trichloroacetic acid to precipitate the proteins, and the mixture was filtered after being kept at 4°C for 30 minutes. The optical density of the filtrate was measured at 280 nm, and the results were expressed in terms of tyrosine equivalents. The enzymatic activity for the enzyme was calculated as 2.66 U/mg.

The duplicated 0.5 g hay samples were weighed and transferred into 80 mL pyrex tubes. The tubes were filled with 50 mL of BPB and placed in a shaking water bath (Heto SBD 50) at an adjusted temperature of 39°C for one hour. Then, 0.5 mL of the freshly prepared enzyme solution, 0.5 mg of nystatin (Sigma Chemical, Catalogue No. N3503), and 0.5 mL of tetracycline solution (Sigma Chemical, Catalogue No. T3258, 10 mg/100 mL BPB) was added and shaken gently. Duplicate blank samples and an internal standard reference feed were included in the batch. Following incubation, all tubes were centrifuged for 5 minutes at 3000 rpm, and samples were filtered through filter paper (Whatman no. 41). The residue on the filter paper was washed with 250 mL deionized water, and nitrogen in the 10 mL supernatant was analyzed by the Kjeldahl method. The enzymatic CP degradability of the samples based on incubation times was calculated using the following equation 1, as specified by Aufrère (1999).

$$\text{CP Degradability; \%} = \text{degraded CP/CP} \times 100 \quad (1)$$

Statistical Analysis

Each sample was tested in triplicate under similar conditions at different times to ensure consistency across replicates. In the statistical analysis, assumptions of normality and homogeneity of variances were assessed before applying ANOVA.

The Shapiro-Wilk test was used to confirm normality within each group, while Levene's test ensured the equality of variances across groups. Following these validations, ANOVA was conducted, and Duncan's Multiple Range Test was employed for post-hoc comparisons to identify significant differences between groups. All analyses were performed using SAS 9.4 (SAS Institute Inc., 2014).

Results and Discussion

Chemical Composition of Hays

The chemical compositions of the hays are presented in Table 1. There were no significant differences in the organic matter (OM) and ash contents between alfalfa and sainfoin, but common vetch had the highest ash content and consequently the lowest OM content ($P < 0.05$). The CP contents of hays showed significant differences among different types of legume forages ($P < 0.05$). On average, common vetch had the highest CP contents at 225.3, followed by alfalfa at 195.0, and then sainfoin at 144.5 g kg^{-1} DM. There was no significant difference between the hays for EE contents. The cell wall components HC and C showed no significant differences across the legume forage types, whereas sainfoin had the highest values for NDF, ADF, and ADL ($P < 0.05$). Additionally, the CTs contents of sainfoin were significantly higher compared to alfalfa and common vetch ($P < 0.05$).

The chemical composition of different legume hays, such as alfalfa, sainfoin, and common vetch demonstrate significant variability that can influence their nutritional value and enzymatic degradability rate. The sainfoin and alfalfa had comparable OM and ash contents, while common vetch has higher mineral content, which aligns with its higher ash content, consistent with findings by Karabulut et al. (2007) and Maxin et al. (2020).

This study revealed significant differences in CP content among different legume forages, with common vetch having the highest CP content, followed by alfalfa and sainfoin. In line with this finding, several studies in the literature have reported that alfalfa has a higher CP content than sainfoin (Parker & Moss, 1981; Moharrery & Toghyani, 2013; Delgado et al., 2014; Lobón Ascaso et al., 2015). Furthermore, Turgut & Yanar (2004) and Du et al. (2019) determined that common vetch has the highest CP content among these three legume forages.

Table 1. Chemical composition of hays (g kg^{-1} DM)

Item	Alfalfa	Sainfoin	Common vetch	SEM
OM	912.9^{a}	921.1^{a}	902.8^{b}	3.52
Ash	87.1^{b}	78.9^{b}	97.2^{a}	3.52
CP	195.0^{b}	144.5^{c}	225.3^{a}	5.45
EE	18.5	16.6	16.3	0.69
NDF	446.3^{c}	514.4^{a}	472.1^{b}	8.74
ADF	331.7^{b}	383.3^{a}	343.7^{b}	7.13
ADL	87.7^{b}	114.6^{a}	89.9^{b}	3.30
HC	114.7	131.2	128.3	6.90
C	243.9	268.7	253.8	7.12
CTs	1.7^{b}	35.4^{a}	3.5^{b}	0.88

DM, dry matter; OM, organic matter; CP, crude protein; EE: ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; HC, hemicellulose; C, cellulose; CTs; condensed tannins; SEM, standard error mean; The results in the table are presented as least square means; a,b,c, the difference between the mean values expressed with different letters in the same row is statistically significant.

The CP contents of the hays in this study were consistent with the values reported for alfalfa, sainfoin, and common vetch in previous research by Haj Ayed et al. (2001), Faría-Mármol et al. (2002), and Aufrère et al. (2008) respectively. However, the CP content of alfalfa exceeded the levels noted by González et al. (2006), common vetch was higher than the values found by Arieli et al. (1999), and sainfoin had higher CP content than reported by Khalilvandi-Behroozyar et al. (2010). The nutrient composition of legumes, especially protein content, varies based on several factors, including species, maturity, fertilization practices, soil fertility, the growing environment, and harvesting conditions (Paulson et al., 2008). These factors account for why the findings of this research are consistent with some studies while differing from others.

In this study, there were no significant differences in EE content among the hays, suggesting that the fat content of these forages is relatively stable regardless of the legume forage type, consistent with the findings of Parker & Moss (1981). However, there are also studies that show differences in EE content among these species (Karabulut et al., 2007; Moharrery & Toghyani, 2013; Rufino-Moya et al., 2019). Fat content in plants varies according to species, genotypes, different organs of the plants, and seasons (Pallardy, 2008), but remains consistent in this study across legume types.

The results indicated that sainfoin exhibited the highest values for NDF, ADF, and ADL among the cell wall components in the legume forages studied. This was consistent with findings from previous research, which showed that sainfoin generally had a higher fiber content compared to other legumes like alfalfa and common vetch (Kraiem et al., 1990; Delgado et al., 2014; Lobón Ascaso et al., 2015; Du et al., 2019). In this study, the cell wall contents of the hays were consistent with the values reported by Faría-Mármol et al. (2002) for alfalfa, Aufrère et al. (2008) for sainfoin, and Haj Ayed et al. (2001) for common vetch. However, the cell wall components of alfalfa and sainfoin exceeded those reported by Moharrery & Toghyani (2013), and those of common vetch were higher than Arieli et al. (1999). These differences may be due to the specific characteristics such as plant species, growth stages, and environmental factors, which influence nutrient composition and have been noted in previous research (Paulson et al., 2008).

The concentrations of nutrients in legume forage plants depend on the interaction of several factors, such as plant species, soil type, plant age, climate (McDowell et al., 1983; Paulson et al., 2008), and additional agronomic practices including fertilization, irrigation, harvest timing, and maturity stage (Turgut et al., 2006). The observed differences in CP and fiber content among alfalfa, sainfoin, and common vetch hays can be attributed to these key factors.

In this study, the CTs content in alfalfa and common vetch was found to be very low, with values of 1.7 g kg^{-1} DM and 3.5 kg^{-1} DM, respectively, whereas it was significantly higher in sainfoin, at 35.4 g kg^{-1} DM. Sainfoin contains CTs at levels ranging from 32-80 g kg^{-1} DM (Waghorn et al., 1998; Min & Hart, 2003; Lorenz et al., 2012). In contrast, alfalfa (Julier et al., 2003; Theodoridou et al., 2011; Kelln et al., 2020) and common vetch (Parissi et al., 2022) have low total polyphenol content and are considered legumes that do not contain CTs, which are polyphenols able to bind proteins. Berard et al. (2011) and Coblentz & Grabber (2013) reported a CTs content of zero for alfalfa, whereas Maxin et al. (2020) reported 1.6 g kg^{-1} DM, which aligns with the findings of this study. The CTs value determined for sainfoin in this study was closer to the value reported by Berard et al. (2011), but lower than that reported by Maxin et al. (2020). For vetch, the determined CTs value was comparable to that reported by Maxin et al. (2020).

Tannins in forages can form complexes with proteins, thereby impacting the availability of protein to ruminants (McSweeney et al., 1999). These complexes help protect dietary proteins from microbial degradation in the rumen, allowing a greater proportion of these proteins and amino acids to bypass the rumen and reach the small intestine for absorption (Preston, 1995; Barry & McNabb, 1999; Min et al., 2002; Zhou et al., 2019; Majewska et al., 2022). Additionally, CTs have the potential to improve ruminant health and nutritional sustainability by preventing bloat, mitigating parasitism, and reducing environmental footprints (Tedeschi et al., 2014). However, high levels of CTs (more than 5% of DM) can decrease protein utilization efficiency due to the excessive formation of tannin-protein complexes (Kumar & Singh, 1984). Therefore, it is crucial to consider the presence of CTs when evaluating the utilization of CP in legume forages. The CTs content in sainfoin, which is below the 5% of DM threshold (3.54% DM), contributes to the protection of proteins in the rumen, allowing more protein and amino acids to pass into the small intestine. Therefore, including sainfoin in ruminant diets can be beneficial for both optimizing protein absorption and improving overall health.

Enzymatic degradation of hays

The CP degradability of hays after different times of incubation with *Streptomyces griseus* protease is given in Table 2. At 1 hour of incubation, there were significant differences in CP degradability among the hays ($P < 0.05$). Alfalfa had the highest degradability, followed by common vetch and sainfoin, which showed similar degradability values. After longer incubation times of 4, 24, and 48 hours, common vetch consistently showed the highest CP degradability, significantly higher than both alfalfa and sainfoin ($P < 0.05$). Among the latter two, alfalfa exhibited higher degradability than sainfoin, which consistently had the lowest values ($P < 0.05$).

Table 2. The crude protein degradability of hays after different times of incubation with *Streptomyces griseus* protease (g kg^{-1} DM)

Incubation time (h)	Alfalfa	Sainfoin	Common vetch	SEM
1	550.8^{a}	535.3^{b}	541.9^{b}	8.42
4	601.1^{b}	592.2^{c}	611.0^{a}	9.66
24	690.3^{b}	659.3^{c}	721.9^{a}	8.94
48	730.0^{b}	692.5^{c}	770.1^{a}	8.22

SEM, standard error mean; The results in the table are presented as least square means; a,b,c, the difference between the mean values expressed with different letters in the same row is statistically significant.

Alfalfa's protein composition includes a substantial amount of soluble protein (Cherney et al., 1992; Tremblay et al., 2003), which is more susceptible to enzymatic attack than the more complex and less soluble protein fractions found in other hays. According to Marković et al. (2015), the rapid degradability of alfalfa's protein can be attributed to its lower fiber-bound protein content and higher levels of soluble proteins. This makes alfalfa's protein more accessible to proteolytic enzymes, resulting in higher degradability within the initial hours of incubation. In this study, alfalfa showed moderate degradability after the 1-hour point. (Table 2). Despite alfalfa's lower NDF content (446.3 g kg^{-1} DM) compared to common vetch, its CP degradability was lower than that of common vetch after the 1 hour of incubation. Both alfalfa and common vetch had similar ADF and ADL contents, yet common vetch consistently showed higher degradability values at all-time points. This suggests that the cell wall components may not be the sole factors influencing protein degradability. Julier et al. (2003) reported that there was only a weak correlation between protein degradability and NDF content. In addition, several studies have shown that alfalfa is a rich source of biologically active compounds, particularly secondary metabolites, with saponins and flavonoids being the well-characterized classes (Butkutė et al., 2017; Rafińska et al., 2017). Flavonoids can form complexes with proteins, influencing the degradation behavior of CP in the rumen (Stafford, 1990; Wang et al., 2006), whereas saponins have not been shown to inhibit feed protein degradation in the rumen under *in vitro* conditions (Muetzel, et al., 2005). In present study, alfalfa had a negligible CTs content (1.7 g kg^{-1} DM), which minimizes the formation of tannin-protein complexes that could otherwise inhibit degradability (Barry & McNabb, 1999).

Common vetch demonstrated the highest degradability after the initial hour, which can be attributed to its high crude protein content (225.3 g kg^{-1} DM) and relatively moderate fiber content (Table 2). According to Cassida et al. (2000) hays with higher crude protein levels and lower fiber content tend to be more accessible to protein degradation. Common vetch's protein is less bound by fibrous structures, allowing proteolytic enzymes to access and degrade the protein more efficiently over time. In their comparison of legume species, Julier et al. (2003) found that protein degradability was strongly correlated with CP content. Additionally, the lower levels of ADL (89.9 g kg^{-1} DM) in common vetch further facilitate protein accessibility, as lignin can inhibit enzymatic hydrolysis (Li et al., 2018). Notably, common vetch had a very low CTs content (3.5 g kg^{-1} DM), which means that the protein is less likely to form indigestible complexes with tannins, further enhancing its degradability (Makkar, 2003; Scharenberg et al., 2007; Gea et al., 2011). Moreover, common vetch contains flavonoids, which are a group of polyphenols (Megías et al., 2009), and the inhibitory activities of flavonoids in relation to proteolytic enzymes have been reported (Gonzales et al., 2015).

Sainfoin exhibited the lowest degradability beyond the initial hour, primarily due to its high fiber content (NDF: 514.4 g kg^{-1} DM, ADF: 383.3 g kg^{-1} DM) and the highest ADL content (114.6 g kg^{-1} DM) among the three hays (Table 2). High fiber and lignin content are known to restrict the accessibility of proteolytic enzymes to the protein fractions within plant material (Li et al., 2018). Fiber-bound proteins are generally embedded within the structural components of plant cell walls, such as cellulose, hemicellulose, and lignin, making them less accessible to enzymes (Terrett & Dupree, 2019). The presence of lignin, a complex and indigestible component of plant cell walls, further limits the accessibility of these proteins (Thorstensson et al., 1992). Lignin, depending on its concentration and structural composition, can limit the extent (Buxton, 1989) and rate (Jung, 1989) of degradability. Moreover, the degradability of protein is strongly influenced by the quality of fiber in the plant, including its concentration, type, and especially the presence of lignin. Proteins bound to cellulose and hemicellulose can still be partially degraded, whereas lignin-bound proteins are far more resistant, resulting in significantly lower protein degradability (Jung & Dietz, 1993). In addition, sainfoin contains high amounts of secondary metabolites, with CTs being the predominant, followed by phenolic compounds and flavonoids (Butkutė et al., 2017). Indeed, the present study has shown that sainfoin contained a high level of CTs (35.4 g kg^{-1} DM), which can bind to proteins and inhibit their degradation by forming tannin-protein complexes that are resistant to microbial and enzymatic breakdown (Broderick & Albrecht, 1997; Makkar, 2003). This combination of high fiber, lignin, and tannin content, which is known to protect proteins from rapid degradation (Getachew et al., 2008), significantly reduces the enzymatic degradability of sainfoin's protein.

The CP degradability of alfalfa hay after 24 hours of incubation was consistent with the values recorded in previous studies (Aufrère & Cartailler, 1988; Janicki & Stallings, 1988; Coblentz et al., 1999), although was higher than those reported by Licitra et al. (1998) and lower than those determined by Abdelgadir et al. (1997). At 48 hours of incubation, the CP degradability corresponded with the values found by Janicki & Stallings (1988), Coblentz et al. (1999), and Mathis et al. (2001), but remained lower than those reported by Abdelgadir et al. (1997). For the 48 hours of incubation, the CP degradability of common vetch hay aligned with the values reported by Alzueta et al. (1995). For the first time, the enzymatic CP degradability of sainfoin hay was investigated in this study, making comparisons with previous studies impossible. The differences in CP degradability values observed in this study compared to previous research could be attributed to variations in the enzyme concentrations used (Coblentz et al., 1999; Mathis et al., 2001), since enzyme/substrate concentration affects the estimated degradability. Notably, previous studies (Aufrère & Cartailler, 1988; Roe et al., 1990; Licitra et al., 1999; Cone et al., 2004; Inal et al., 2018) have demonstrated a wide variation in the amount of enzyme used to represent ruminal activity.

The degradability of CP with *Streptomyces griseus* protease showed dynamic changes, exhibiting a consistent increase over various incubation times (Figure 1). The degradability of CP levels in all three hays showed a significant increase in the first 24 hours, but the increase slowed down after 24 hours. This indicated that a 24-hour incubation period was largely sufficient for enzymatic protein degradability. However, it is suggested that longer incubation periods would better reflect the complex structure of forages, thereby providing more accurate results.

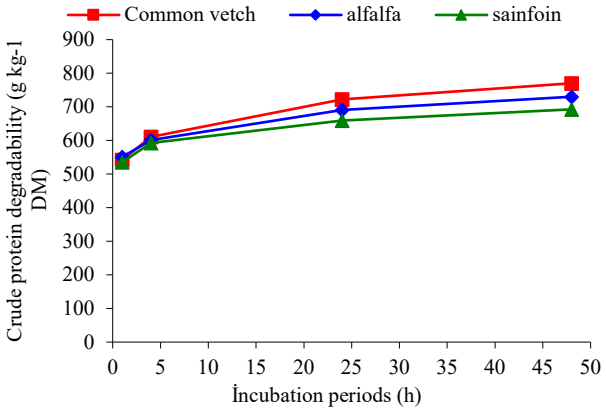

Figure 1. The time-dependent crude protein degradability of hays

In forages, there are protein fractions that degrade at different times, with the proteins initially degrading rapidly and then slowing down over time, necessitating a longer treatment with the enzyme (Inal et al., 2018).

Extending the incubation period in the *in vitro* protease method leads to results that are more similar to those of the *in situ* method, as demonstrated by Abdelgadir et al. (1997), Antoniewicz & Kosmala (1998) and Okon et al. (2023). This can be explained by the fact that a longer incubation period allows proteases to break down proteins more effectively, thereby better simulating rumen conditions. Roe et al. (1990) indicated that a 48-hour incubation period using *Streptomyces griseus* protease in *in vitro* experiments is sufficient. This period is considered adequate for accurately measuring the degree of protein degradation. Further support comes from Skinner et al. (1995), Coblentz et al. (1999), and Mathis et al. (2001), who also found that a 48-hour duration provides optimal results.

Conclusion

The study highlights the distinctive chemical and degradability characteristics of alfalfa, sainfoin, and common vetch hays. The enzymatic degradability of these hays is significantly influenced by their chemical compositions. The use of proteolytic enzymes to estimate CP degradability proves to be an efficient and practical method, as it can be conducted in laboratory settings without the need for live animals. However, the structural complexity of plant proteins, often embedded within carbohydrate complexes, necessitates a more comprehensive approach for accurate assessment. This is particularly relevant for sainfoin, where the inclusion of both protease and carbohydrase enzymes during testing can significantly improve the accuracy of CP degradability estimates. By incorporating carbohydrase to break down carbohydrate complexes, the accessibility of plant proteins to proteolytic enzymes is enhanced, leading to a more precise measurement of protein degradability. Future research should focus on empirically testing this dual-enzyme method to provide concrete evidence of its benefits, particularly in ensuring a better representation of the degradability potential of plant proteins. Such studies would help optimize feed evaluation and formulation for ruminants.

Declarations

Author Contribution Statement

Hülya Hanoğlu Oral was responsible for the conception and design of the experiments, execution of the experiments, data analysis, preparation of figures and tables, drafting or reviewing of the article, and approval of the final version.

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Conflict of Interest

The authors state that there are no conflicts of interest.

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References

Abdelgadir, I. E. O., Cochran, R. C., Titgemeyer, E. C., & Vanzant, E. S. (1997). In vitro determination of ruminal protein degradability of alfalfa and prairie hay via a commercial protease in the presence or absence of cellulase or driselase. *Journal of Animal Science*, 75(8), 2215-2222. https://doi.org/10.2527/1997.7582215x

Alzueta, C., Rebolé, A., Barro, C., Treviño, J., & Caballero, R. (1995). Changes in nitrogen and carbohydrate fractions associated with the field drying of vetch (*Vicia sativa* L.). *Animal Feed Science and Technology*, 52(3-4), 249-255. https://doi.org/10.1016/0377-8401(94)00727-Q

Antoniewicz, A.M., & Kosmala, I. (1998). Use of *in vitro* digestion by protease from *Streptomyces griseus*, ficin or pancreatin for the estimation of ruminal effective degradability of dried lucerne forage. In: *In vitro* techniques for measuring nutrient supply to ruminants, *British Society of Animal Science* (pp. 118-119). https://doi.org/10.1017/S0263967X00032377

AOAC. (2003). *Official methods of analysis of the association of official's analytical chemists*, 17th edn. Association of official analytical chemists, Arlington, Virginia.

Arieli, A., Shahar, K., Mabjeesh, S. J., Zamwel, S., & Sklan, D. (1999). Estimation of the digestible energy of ruminant feedstuffs by the combined bag technique. *Journal of Dairy Science*, 82(3), 566-573. https://doi.org/10.3168/jds.S0022-0302(99)75268-9

Aufrère, J. (1999). Estimation of *in situ* degradability of feed proteins in the rumen by a laboratory method using a protease. In: Tisserand J.-L. (ed.). *Evaluation of the Nutritive Value of Mediterranean Roughages* (pp. 73-78), Zaragoza : CIHEAM.

Aufrère, J., & Cartailler, D. (1988). Mise au point d'une méthode de laboratoire de révision de la dégradabilité des protéines alimentaires des aliments concentrés dans le rumen. *Annales de Zootechnie*, 37(4), 255-270.

Aufrère, J., Dudilieu, M., & Poncet, C. (2008). *In vivo* and *in situ* measurements of the digestive characteristics of sainfoin in comparison with lucerne fed to sheep as fresh forages at two growth stages and as hay. *Animal,* 2(9), 1331–1339. https://doi.org/10.1017/S1751731108002450

Aufrère, J., Graviou, D., Demarquilly, C., Verite, R., Michalet-Doreau, B., & Chapoutot, P. (1991). Predicting *in situ* degradability of feed proteins in the rumen by two laboratory methods (solubility and enzymatic degradation). *Animal Feed Science and Technology*, 33(1-2), 97-116. https://doi.org/10.1016/0377-8401(91)90049-X

Barry, T. N., & McNabb, W. C. (1999). The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. *British Journal of Nutrition*, 81(4), 263-272. https://doi.org/10.1017/S0007114599000501

Berard, N. C., Wang, Y., Wittenberg, K. M., Krause, D. O., Coulman, B. E., McAllister, T. A., & Ominski, K. H. (2011). Condensed tannin concentrations found in vegetative and mature forage legumes grown in western Canada. *Canadian Journal of Plant Science,* 91(4), 669-675. https://doi.org/10.4141/cjps10153

Broderick, G. A., & Albrecht, K. A. (1997). Ruminal *in vitro* degradation of protein in tannin-free and tannin-containing forage legume species. *Crop Science*, 37(6), 1884-1891. https://doi.org/10.2135/cropsci1997.0011183X003700060037x

Butkutė, B., Benetis, R., Padarauskas, A., Cesevičienė, J., Dagilytė, A., Taujenis, L., & Lemežienė, N. (2017). Young herbaceous legumes-a natural reserve of bioactive compounds and antioxidants for healthy food and supplements. *Journal of Applied Botany & Food Quality*, 90, 346–353. https://doi.org/10.5073/JABFQ.2017.090.043

Buxton, D. R. (1989). *In vitro* digestion kinetics of temperate perennial forage legume and grass stems. *Crop Science*, 29(1), 213-219. https://doi.org/10.2135/cropsci1989.0011183X002900010046x

Cassida, K. A., Griffin, T. S., Rodriguez, J., Patching, S. C., Hesterman, O. B., & Rust, S. R. (2000). Protein degradability and forage quality in maturing alfalfa, red clover, and birdsfoot trefoil. *Crop Science*, 40(1), 209-215. https://doi.org/10.2135/cropsci2000.401209x

Castillo, C., & Hernández, J. (2021). Ruminal fistulation and cannulation: a necessary procedure for the advancement of biotechnological research in ruminants. *Animals*, 11(7), 1870. https://doi.org/10.3390/ani11071870

Cherney, D. J. R., Volenec, J. J., & Cherney, J. H. (1992). Protein solubility and degradation in vitro as influenced by buffer and maturity of alfalfa. *Animal Feed Science And Technology*, 37(1-2), 9-20. https://doi.org/10.1016/0377-8401(92)90116-N

Coblentz, W.K., Abdelgadir, I.E.O., Cochran, R.C., Fritz, J.O., Fick, W.H., Olson, K.C., & Turner, J.E. (1999). Degradability of forage proteins by *in situ* and *in vitro* enzymatic methods. *Journal of Dairy Science*, 82(2), 343-354. https://doi.org/10.3168/jds.S0022-0302(99)75241-0

Coblentz, W. K., Fritz, J. O., Cochran, R. C., Rooney, W. L., & Bolsen, K. K. (1997). Protein degradation in response to spontaneous heating in alfalfa hay by in situ and ficin methods. *Journal of Dairy Science*, 80(4), 700-713. https://doi.org/10.3168/jds.S0022-0302(97)75989-7

Coblentz, W. K., & Grabber, J. H. (2013). In situ protein degradation of alfalfa and birdsfoot trefoil hays and silages as influenced by condensed tannin concentration. *Journal of Dairy Science*, 96(5), 3120–3137. https://doi.org/10.3168/jds.2012-6098

Colombatto, D., Mould, F. L., Bhat, M. K., & Owen, E. (2007). Influence of exogenous fibrolytic enzyme level and incubation pH on the *in vitro* ruminal fermentation of alfalfa stems. *Animal Feed Science and Technology*, 137(1-2), 150-162. https://doi.org/10.1016/j.anifeedsci.2006.10.001

Cone, J. W., Gelder, A. H. V., Steg, A., & Vuuren, A. M. V. (1996). Prediction of *in situ* rumen escape protein from in vitro incubation with protease from *Streptomyces griseus. Journal of the Science of Food and Agriculture*, 72(1), 120-126. https://doi.org/10.1002/(SICI)1097-0010(199609)72:1<120: AID-JSFA630>3.0.CO;2-3

Cone, J. W., Gelder, A. H. V., Mathijssen-Kamman, A. A., & Hindle, V. A. (2004). Rumen escape protein in grass and grass silage determined with a nylon bag and an enzymatic technique. *Animal Feed Science and Technology*, 111, 1-9. https://doi.org/10.1016/j.anifeedsci.2003.08.004

Curzer, H. J., Perry, G., Wallace, M. C., & Perry, D. (2016). The three Rs of animal research: what they mean for the institutional animal care and use committee and why. *Science and Engineering Ethics*, 22, 549-565. https://doi.org/10.1007/s11948-015-9659-8

Delgado, I., Muñoz, F., & Andueza, D. (2014). Evaluation of the competition between alfalfa and sainfoin sown in mixture. In: Baumont, R., Carrère, P., Jouven, M., Lombardi, G., López-Francos, A., Martin, B., Peeters, A., Porqueddu, C. (Eds.) *Joint Meeting of the "Mountain Pastures, Mediterranean Forage Resources* (pp. 139–142), Zaragoza: CIHEAM / INRA / FAO.

Du, W., Hou, F., Tsunekawa, A., Kobayashi, N., Ichinohe, T., & Peng, F. (2019). Effects of the diet inclusion of common vetch hay versus alfalfa hay on the body weight gain, nitrogen utilization efficiency, energy balance, and enteric methane emissions of crossbred Simmental cattle. *Animals*, 9(11), 983. https://doi.org/10.3390/ani9110983

Edmunds, B., Südekum, K. H., Spiekers, H., & Schwarz, F. J. (2012). Estimating ruminal crude protein degradation of forages using *in situ* and *in vitro* techniques. Animal feed science and technology, 175(3-4), 95-105. https://doi.org/10.1016/J.ANIFEEDSCI.2012.04.003

Faría-Mármol, J., González, J., Rodríguez, C.A., & Alvir, M.R. (2002). Effect of diet forage to concentrate ratio on rumen degradability and post-ruminal availability of protein from fresh and dried lucerne. *Animal Science* 74, 337-345. https://doi.org/10.1017/S1357729800052498

Gea, A., Stringano, E., Brown, R. H., & Mueller-Harvey, I. (2011). *In situ* analysis and structural elucidation of sainfoin (*Onobrychis viciifolia*) tannins for high-throughput germplasm screening. *Journal of Agricultural and Food Chemistry*, 59(2), 495-503. https://doi.org/10.1021/jf103609p

Getachew, G., Pittroff, W., DePeters, E. J., Putnam, D. H., Dandekar, A., & Goyal, S. (2008). Influence of tannic acid application on alfalfa hay: *in vitro* rumen fermentation, serum metabolites and nitrogen balance in sheep. *Animal*, 2(3), 381-390. https://doi.org/10.1017/S1751731107001486

Gonzales, G., Smagghe, G., Grootaert, C., Zotti, M., Raes, K., & Camp, J. (2015). Flavonoid interactions during digestion, absorption, distribution and metabolism: a sequential structure–activity/property relationship-based approach in the study of bioavailability and bioactivity. *Drug Metabolism Reviews*, 47, 175 - 190. https://doi.org/10.3109/03602532.2014.1003649

González, J., Ouarti, M., Rodríguez, C. A., & Alvir, M. R. (2006). Effects of considering the rate of comminution of particles and microbial contamination on accuracy of in situ studies of feed protein degradability in ruminants. *Animal Feed Science and Technology*, 125(1-2), 89-98. https://doi.org/10.1016/j.anifeedsci.2005.05.013

Haj Ayed, M., González, J., Caballero, R., & Alvir, M. R. (2001). Effects of maturity on nutritive value of field-cured hays from common vetch and hairy vetch. *Animal Research*, 50(1), 31-42. https://doi.org/10.1051/animres:2001103

Hristov, A. N., Bannink, A., Crompton, L. A., Huhtanen, P., Kreuzer, M., McGee, M., & Yu, Z. (2019). Invited review: Nitrogen in ruminant nutrition: A review of measurement techniques. *Journal of Dairy Science*, 102(7), 5811-5852. https://doi.org/10.3168/jds.2018-15829

Hvelplund, T., Weisbjerg, M.R., (2000). *In situ* techniques for the estimation of protein degradability and postrumen availability. In: Givens, D.I., Owen, E., Axford, R.F.E., Omed, H.M. (Eds.), *Forage Evaluation in Ruminant Nutrition*. CAB International, Wallingford, UK, 233–258. https://doi.org/10.1079/9780851993447.00

Inal, F., Tamkoc, A., Alatas, M. S., Kahraman, O., Ozbilgin, A., & Coskun, B. (2018). Determination of protein degradability of alfalfa hay via buffer or protease. *Italian Journal of Animal Science*, 17(2), 353-358. https://doi.org/10.1080/1828051X.2017.1364986

Janicki, F. J., & Stallings, C. C. (1988). Degradation of crude protein in forages determined by *in vitro* and *in situ* procedures. *Journal of Dairy Science*, 71(9), 2440-2448. https://doi.org/10.3168/jds.S0022-0302(88)79829-X

Julier, B., Guines, F., Emile, J. C., & Huyghe, C. (2003). Variation in protein degradability in dried forage legumes. *Animal Research*, 52(5), 401-412. https://doi.org/10.1051/animres:2003029

Jung, H. G. (1989). Forage lignins and their effects on fiber digestibility. *Agronomy Journal*, 81(1), 33-38. https://doi.org/10.2134/agronj1989.00021962008100010006x

Jung, H.J.G., & Dietz, D.A. (1993). Cell wall lignification and degradability. In: Jung, H.G., Buxton, D.R., Hatfield, R.D. (Eds.), *Forage Cell Wall Structure and Digestibility* (pp. 315–346) American Society of Agronomy, Madison, WI, USA. https://doi.org/10.2134/1993.foragecellwall.c13

Karabulut, A., Canbolat, O., Kalkan, H., Gurbuzol, F., Sucu, E., & Filya, I. (2007). Comparison of *in vitro* gas production, metabolizable energy, organic matter digestibility and microbial protein production of some legume hays. *Asian-Australasian Journal of Animal Sciences*, 20(4), 517-522. https://doi.org/10.5713/ajas.2007.517

Karlsson, L., Hetta, M., Udén, P., & Martinsson, K. (2009). New methodology for estimating rumen protein degradation using the *in vitro* gas production technique. *Animal Feed Science and Technology*, 153, 193-202. https://doi.org/10.1016/J.ANIFEEDSCI.2009.06.010

Kelln, B. M., Penner, G. B., Acharya, S. N., McAllister, T. A., & Lardner, H. A. (2020). Impact of condensed tannin-containing legumes on ruminal fermentation, nutrition, and performance in ruminants: a review. *Canadian Journal of Animal Science*, 101(2), 210-223. https://doi.org/10.1139/cjas-2020-0096

Khalilvandi-Behroozyar, H., Dehghan-Banadaky, M., & Rezayazdi, K. (2010). Palatability, *in situ* and *in vitro* nutritive value of dried sainfoin (*Onobrychis viciifolia*). *The Journal of Agricultural Science*, 148(6), 723-733. https://doi.org/10.1017/S0021859610000523

Kosmala, I., Antoniewicz, A., De Boever, J., Hvelplund, T., & Kowalczyk, J. (1996). Use of enzymatic solubility with ficin (EC 3.4. 22.3) to predict in situ feed protein degradability. *Animal Feed Science and Technology*, 59(4), 245-254. https://doi.org/10.1016/0377-8401(95)00910-8

Kraiem, K., Garrett, J. E., Meiske, J. C., Goodrich, R. D., & Marten, G. C. (1990). Influence of method of forage preservation on fibre and protein digestion in cattle given lucerne, birdsfoot trefoil and sainfoin. *Animal Production*, 50(2), 221-230. https://doi.org/10.1017/S0003356100004670

Kumar, R., & Singh, M. (1984). Tannins: their adverse role in ruminant nutrition. *Journal of Agricultural and Food Chemistry*, 32(3), 447-453. https://doi.org/10.1021/jf00123a006

Li, X., Li, M., Pu, Y., Ragauskas, A. J., Klett, A. S., Thies, M., & Zheng, Y. (2018). Inhibitory effects of lignin on enzymatic hydrolysis: The role of lignin chemistry and molecular weight. *Renewable Energy*, 123, 664-674. https://doi.org/10.1016/j.renene.2018.02.079

Licitra, G., Lauria, F., Carpino, S., Schadt, I., Sniffen, C. J., & Van Soest, P. J. (1998). Improvement of the *Streptomyces griseus* method for degradable protein in ruminant feeds. *Animal Feed Science and Technology*, 72(1-2), 1-10. https://doi.org/10.1016/S0377-8401(97)00178-8

Licitra, G., Van Soest, P. J., Schadt, I., Carpino, S., & Sniffen, C. J. (1999). Influence of the concentration of the protease from *Streptomyces griseus* relative to ruminal protein degradability. *Animal Feed Science and Technology*, 77(1-2), 99-113. https://doi.org/10.1016/S0377-8401(98)00233-8

Liu, C., Li, D., Chen, W., Li, Y., Wu, H., Meng, Q., & Zhou, Z. (2019). Estimating ruminal crude protein degradation from beef cattle feedstuff. *Scientific Reports*, 9(1), 11368. https://doi.org/10.1038/s41598-019-47768-3

Lobón Ascaso, S., Blanco Alibés, M., Sanz Pascua, A., Rufino Moya, P. J., Molino Gahete, F., & Torrens, M. J. (2015). Influence of alfalfa or sainfoin grazing on productive and reproductive parameters in ewes and lambs. *Sitio Argentino de Producción Animal*. 45(1): 36-46.

Lorenz, M. M., Hayot Carbonero, C., Smith, L., & Udén, P. (2012). In vitro protein degradation of 38 sainfoin accessions and its relationship to tannin content by different assays. *Journal of Agricultural and Food Chemistry*, 60(20), 5071-5075. https://doi.org/10.1021/jf3001179

Lynch, G.L., Van der Aar, P.J., Berger, L.L., Fahey, G.C. and Merchen, N.R. (1988) Proteolysis of alcohol-treated soybean meal proteins by *Bacteroides ruminocola*, *Bacteroides amilophilus*, pepsin, trypsin, and in the rumen of steers. *Journal of Dairy Science*, 71, 2416–2427. https://doi.org/10.3168/jds.S0022-0302(88)79827-6

Majewska, M. P., Miltko, R., Bełżecki, G., Kędzierska, A., & Kowalik, B. (2022). Comparison of the effect of synthetic (tannic acid) or natural (oak bark extract) hydrolysable tannins addition on fatty acid profile in the rumen of sheep. *Animals*, 12(6), 699. https://doi.org/10.3390/ani12060699

Makkar, H. P. (2003). Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research*, 49(3), 241-256. https://doi.org/10.1016/S0921-4488(03)00142-1

Marković, J., Dinić, B., Blagojević, M., Anđelković, B., Babić, S., Petrović, M., & Terzić, D. (2015). Effects of alfalfa and red clover cultivars on protein fractions by CNCPS system of analyzis. In: *Proceedings of the 4th International Congress New Perspectives and Challenges of Sustainable Livestock Production* (pp. 796-802), October 7-9, Belgrade, Serbia.

Mathis, C.P., Cochran, R.C., Vanzant, E.S., Abdelgadir, I.E.O., Heldt, J.S., Olson, K.C., Johnson, D.E., Caton, J., Fulkner, D., Horn, G., Paisley, S., Mass, R., Moore, K., & Halgerson, J. (2001). A collaborative study comparing an *in situ* protocol with single time-point enzyme assays for estimating ruminal protein degradability of different forages. *Animal Feed Science and Technology*, 93(1-2), 31-42. https://doi.org/10.1016/S0377-8401(01)00273-5

Maxin, G., Graulet, B., Le Morvan, A., Picard, F., Portelli, J., & Andueza, D. (2020). Cover crops as alternative forages for ruminants: nutritive characteristics, *in vitro* digestibility, methane and ammonia production. *Animal Production Science*, 60(6), 823-832. https://doi.org/10.1071/AN19091

McDowell L.R., Conrad J.H., Ellis G.L. & Loosli J.K. (1983). Minerals for Grazing Ruminants in Tropical Regions. University of Florida, Gainesville. 86 p.

McSweeney, C. S., Palmer, B., Bunch, R., & Krause, D. O. (1999). Isolation and characterization of proteolytic ruminal bacteria from sheep and goats fed the tannin-containing shrub legume *Calliandra calothyrsus*. *Applied and Environmental Microbiology*, 65(7), 3075-3083. https://doi.org/10.1128/AEM.65.7.3075-3083.1999

Megías, C., Pastor-Cavada, E., Torres-Fuentes, C., Girón-Calle, J., Alaiz, M., Juan, R., & Vioque, J. (2009). Chelating, antioxidant and antiproliferative activity of Vicia sativa polyphenol extracts. *European Food Research and Technology*, 230, 353-359. https://doi.org/10.1007/s00217-009-1178-x

Meyer, J. H., & Mackie, R. I. (1986). Microbiological evaluation of the intraruminal in sacculus digestion technique. *Applied and Environmental Microbiology*, 51(3), 622-629. https://doi.org/10.1128/aem.51.3.622-629.1986

Min, B. R., Attwood, G. T., Reilly, K., Sun, W., Peters, J. S., Barry, T. N., & McNabb, W. C. (2002). *Lotus corniculatus* condensed tannins decrease *in vivo* populations of proteolytic bacteria and affect nitrogen metabolism in the rumen of sheep. *Canadian Journal of Microbiology*, 48(10), 911-921. https://doi.org/10.1139/w02-08

Min, B. R., & Hart, S. P. (2003). Tannins for suppression of internal parasites. *Journal of Animal Science*, 81 (E Suppl. 2), E102-E109. https://doi.org/10.2527/2003.8114_suppl_2E102x

Moharrery, A., & Toghyani, E. (2013). Evaluation of nutritional properties of alfalfa and sainfoin forages by gas production techniques. *Journal of Livestock Science and Technologies*, 1(1), 1-9.

Muetzel, S., Akpagloh, R., & Becker, K. (2005). Sapindus rarak saponins do not affect rumen protein degradation in vitro. *Proceedings of the Society of Nutrition and Physiology*, 14, 17.

Nocek, J. E. (1988). *In situ* and other methods to estimate ruminal protein and energy digestibility: a review. *Journal of Dairy Science*, 71(8), 2051-2069. https://doi.org/10.3168/jds.S0022-0302(88)79781-7

Nocek, J. E., & Grant, A. L. (1987). Characterization of in situ nitrogen and fiber digestion and bacterial nitrogen contamination of hay crop forages preserved at different dry matter percentages. *Journal of Animal Science*, 64(2), 552-564. https://doi.org/10.2527/jas1987.642552x

Okon, P., Bachmann, M., Wensch-Dorendorf, M., Titze, N., Rodehutscord, M., Rupp, C., & Zeyner, A. (2023). Feed clusters according to in situ and in vitro ruminal crude protein degradation. *Animals*, 13(2), 224. https://doi.org/10.3390/ani13020224

Pagella, J. H., Mayes, R. W., Pérez-Barbería, F. J., & Ørskov, E. R. (2018). The development of an intraruminal nylon bag technique using non-fistulated animals to assess the rumen degradability of dietary plant materials. *Animal*, 12(1), 54-65. DOI: https://doi.org/10.1017/S1751731117001203

Pallardy, S.G. (2008). *Physiology of Woody Plants*. 3rd ed, Amsterdam: Elsevier, 469 p.

Parissi, Z., Irakli, M., Tigka, E., Papastylianou, P., Dordas, C., Tani, E., Abraham, E.M., Theodoropoulos, A., Kargiotidou, A., Kougiteas, L., & et al. (2022). Analysis of genotypic and environmental effects on biomass yield, nutritional and antinutritional factors in common vetch. *Agronomy*, 12(7), 1678. https://doi.org/10.3390/agronomy12071678

Parker, R. J., & Moss, B. R. (1981). Nutritional value of sainfoin hay compared with alfalfa hay. *Journal of Dairy Science*, 64(2), 206-210. https://doi.org/10.3168/jds.S0022-0302(81)82555-6

Paulson, J., Jung, H.G., Raeth-Knight, M., & Linn, J. (2008). *Grass vs legume forages for dairy cattle.* University of Minnesota: Saint Paul, MA, USA, 119–133.

Pedersen, M. B., Dalsgaard, S., Arent, S., Lorentsen, R., Knudsen, K. E. B., Yu, S., & Lærke, H. N. (2015). Xylanase and protease increase solubilization of non-starch polysaccharides and nutrient release of corn-and wheat distillers dried grains with solubles. *Biochemical Engineering Journal*, 98, 99-106. https://doi.org/10.1016/j.bej.2015.02.036

Preston, T.R. (1995). *Tropical Animal Feeding: A Manual for Research Workers*. FAO: Rome.

Rafińska, K., Pomastowski, P., Wrona, O., Górecki, R., & Buszewski, B. (2017). Medicago sativa as a source of secondary metabolites for agriculture and pharmaceutical industry. *Phytochemistry Letters*, 20, 520-539. https://doi.org/10.1016/j.phytol.2016.12.006

Reed, J. D. (1986). Relationships among soluble phenolics, insoluble proanthocyanidins and fiber in East African browse species. *Rangeland Ecology & Management/Journal of Range Management Archives,* 39(1), 5-7.

Roe, M.B., Chase, L.E., & Sniffen, C.J. (1991). Comparison of *in vitro* techniques to the *in situ* technique for estimation of ruminal degradation of protein. *Journal of Dairy Science*, 74, 1632–1640. https://doi.org/10.3168/jds.S0022-0302(91)78325-2

Roe M.B., Sniffen CJ., & Chase L.E. (1990). Techniques for measuring protein fractions in feedstuffs. In: *Proceedings of Cornell Nutrition Conference* (pp. 81-88), Cornell University. Ithaca. NY.

Rufino-Moya, P. J., Blanco, M., Bertolín, J. R., & Joy, M. (2019). Methane production of fresh sainfoin, with or without PEG, and fresh alfalfa at different stages of maturity is similar but the fermentation end products vary. *Animals*, 9(5), 197. https://doi.org/10.3390/ani9050197

Russell, J. B., & Dombrowski, D. B. (1980). Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. *Applied and Environmental Microbiology*, 39(3), 604-610. https://doi.org/10.1128/aem.39.3.604-610.1980

Santra, A., & Karim, S. A. (2002). Influence of ciliate protozoa on biochemical changes and hydrolytic enzyme profile in the rumen ecosystem. *Journal of Applied Microbiology*, 92(5), 801-811. https://doi.org/10.1046/j.1365-2672.2002.01583.x

SAS Institute Inc. (2014). *SAS/STAT 9.4 User's Guide*. Cary, NC: SAS Institute Inc.

Scharenberg, A., Arrigo, Y., Gutzwiller, A., Wyss, U., Hess, H. D., Kreuzer, M., & Dohme, F. (2007). Effect of feeding dehydrated and ensiled tanniferous sainfoin (*Onobrychis viciifolia*) on nitrogen and mineral digestion and metabolism of lambs. *Archives of Animal Nutrition*, 61(5), 390-405. https://doi.org/10.1080/17450390701565081

Skinner, D.Z., Abdelgadir, I.E.O., Fish, T.K., & Cochran, R.C. (1995). Influence of growth conditions on alfalfa protein degradability. In: *24th Central Alfalfa Improvement Conference* (p. 13), Spearfish, SD.

Stafford, H. A. 1990. *Flavonoid metabolism*. CRC Press, Boca Raton, FL.

Tedeschi, L. O., Ramírez-Restrepo, C. A., & Muir, J. P. (2014). Developing a conceptual model of possible benefits of condensed tannins for ruminant production. Animal, 8(7), 1095-1105. https://doi.org/10.1017/S1751731114000974

Terrett, O. M., & Dupree, P. (2019). Covalent interactions between lignin and hemicelluloses in plant secondary cell walls. *Current Opinion in Biotechnology,* 56, 97-104. https://doi.org/10.1016/j.copbio.2018.10.010

Theodoridou, K., Aufrère, J., Andueza, D., Le Morvan, A., Picard, F., Stringano, E., Pourrat, J., Mueller-Harvey, I., & Baumont, R. (2011). Effect of plant development during first and second growth cycle on chemical composition, condensed tannins and nutritive value of three sainfoin (*Onobrychis viciifolia*) varieties and lucerne. *Grass and Forage Science*, 66(3), 402–414. https://doi.org/10.1111/j.1365-2494.2011.00798.x

Thorstensson, E. M., Buxton, D. R., & Cherney, J. H. (1992). Apparent inhibition to digestion by lignin in normal and brown midrib stems. *Journal of the Science of Food and Agriculture*, 59(2), 183-188. https://doi.org/10.1002/jsfa.2740590208

Titze, N., Chi, Y. P., Haese, E., Hartung, J., & Rodehutscord, M. (2024). Linkage of in situ ruminal degradation of crude protein with ruminal degradation of amino acids and phytate from different soybean meals in dairy cows. *Journal of Dairy Science*, 107(4), 2011-2025. https://doi.org/10.3168/jds.2023-23587

Tremblay, G. F., Michaud, R., & Bélanger, G. (2003). Protein fractions and ruminal undegradable proteins in alfalfa. *Canadian Journal of Plant Science*, 83(3), 555-559. https://doi.org/10.4141/P02-148

Turgut, L., & Yanar, M. (2004). *In situ* dry matter and crude protein degradation kinetics of some forages in Eastern Turkey. *Small Ruminant Research*, 52(3), 217-222. https://doi.org/10.1016/S0921-4488(03)00261-X

Turgut, L., Yanar, M., Kaya, A., & Tan, M. (2006). Farklı olgunluk dönemlerinde hasat edilen bazı fiğ türlerinin ham besin maddeleri içeriği ve bunların *in situ* rumen parçalanabilirlikleri. *Atatürk Üniversitesi Ziraat Fakültesi Dergisi*, 37(2), 181-186.

Van Soest, P.J., Robertson, J.D., & Lewis, B.A. (1991). Methods for dietary fibre, neutral detergent fibre and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Vanzant, E. S., Cochran, R. C., & Titgemeyer, E. C. (1998). Standardization of in situ techniques for ruminant feedstuff evaluation. *Journal of Animal Science*, 76(10), 2717-2729. https://doi.org/10.2527/1998.76102717x

Vartoukian, S. R., Palmer, R. M., & Wade, W. G. (2010). Strategies for culture of 'unculturable' bacteria. *FEMS Microbiology Letters*, 309(1), 1-7. https://doi.org/10.1111/j.1574-6968.2010.02000.x

Vinyard, J. R., & Faciola, A. P. (2022). Unraveling the pros and cons of various in vitro methodologies for ruminant nutrition: a review. *Translational Animal Science*, 6(4), txac130. https://doi.org/10.1093/tas/txac130

Waghorn, G. C., Douglas, G. B., Niezen, J. H., McNabb, W. C., & Foote, A. G. (1998). Forages with condensed tannins-their management and nutritive value for ruminants. In: *Proceedings of the New Zealand Grassland Association* (pp. 89-98). https://doi.org/10.33584/jnzg.1998.60.2315

Wang, Y., Frutos, P., Gruber, M. Y., Ray, H., & McAllister, T. A. (2006). *In vitro* ruminal digestion of anthocyanidin-containing alfalfa transformed with the maize Lc regulatory gene. *Canadian Journal of Plant Science*, 86(4), 1119-1130. https://doi.org/10.4141/P06-001

Zhou, K., Bao, Y., & Zhao, G. (2019). Effects of dietary crude protein and tannic acid on rumen fermentation, rumen microbiota and nutrient digestion in beef cattle. *Archives of Animal Nutrition*, 73(1), 30-43. https://doi.org/10.1080/1745039X.2018.1545502