

# Chestnut tannin supplementation at ensiling: Effects on composition, degradability, and fermentation of forage silages

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Tannins are plant secondary compounds capable of forming pH-dependent reversible bonds with proteins, thereby reducing protein degradation in the silo and rumen. This study evaluated the effect of adding chestnut hydrolysable tannin (HYT) at ensiling on the chemical composition, protein degradability, and rumen fermentation of grass, whole-crop pea, and bean silages. Forages were harvested, wilted (48 h), chopped (5 cm), and ensiled in mini silos (~25 kg) with one of four treatments: 40 g kg<sup>-1</sup> FW HYT (HT), 20 g kg<sup>-1</sup> HYT (LT), an inoculant (IN), or water (W, control). Tannin addition reduced ( $p < 0.01$ ) ammonia-N during ensiling by 31% (HT), 19% (LT), and 7% (IN) compared to W. Tannin treatments decreased ( $p < 0.01$ ) CNCPS fraction A and increased ( $p < 0.01$ ) fractions B1, B2, and C, as well as undegradable protein at a 0.05 h<sup>-1</sup> outflow rate (HT = 282; LT = 273; IN = 222; W = 216 g kg<sup>-1</sup> CP). *In situ* degradation showed reductions ( $p < 0.01$ ) in both the immediately soluble fraction “a” and total degradable fraction “a+b” with tannin inclusion. Gas production was also reduced ( $p < 0.01$ ) with HT (254 ml) and LT (281 ml) compared to IN (310 ml) and W (337 ml). Furthermore, dry matter (DM) and organic matter (OM) disappearance declined ( $p < 0.01$ ) with tannin treatment (DM: HT = 532, LT = 583; OM: HT = 588, LT = 605 g kg<sup>-1</sup> DM) relative to control (DM = 641; OM = 661 g kg<sup>-1</sup> DM). In conclusion, ensiling with chestnut tannins improved protein preservation and reduced the rumen degradation and fermentability of protein, suggesting potential benefits for enhancing forage utilization in ruminant diets.

**Key words:** forage preservation, hydrolysable tannin, protein protection, silage additives

## Introduction

Microbial protein synthesised in the rumen is the main protein source in ruminant nutrition and can contribute over 60 % of the amino acid (AA) supply to the small intestine (Hackmann and Firkins 2015). However, additional AA supply from undegradable protein is necessary to meet the requirements of high-producing animals (Loregian et al. 2023).

Owing to human population growth and increasing affluence in developing countries, the demand for animal products is expected to rise by 85% by 2050 (OECD and FAO 2024). This will increase the demand for animal feeds, resulting in higher raw material prices and increased price volatility. Oilseed meals are the most important non-forage protein sources in ruminant diets (Ahmed and Taha 2022) owing to their high crude protein (CP) content, typically ranging from 300 to 700 g kg<sup>-1</sup> DM and relatively high biological value (Hackmann and Firkins 2015). However, the UK is currently only 30% self-sufficient in oilseed meals, with the rest being imported from overseas. Consequently, reliance on imported oilseed meals, particularly soybean meal (SBM), which carries a high environmental burden of 0.72 kg CO<sub>2</sub> kg<sup>-1</sup>. This value is often considered substantial compared to many locally produced feed ingredients and a key contributor to the overall carbon footprint of ruminant diets (Dalgaard et al. 2008), posing a serious risk to UK agriculture. Replacing imported protein sources with high-protein forages could reduce feed costs, enhance food security and reduce the environmental impact of ruminant production systems (Hart 2005).

In theory, forages can satisfy the protein requirements of ruminants (Wilkins and Jones 2000). Hart et al. (2012) reported a significant reduction in feed costs when grass and whole-crop pea silages replaced SBM in dairy cow diets, without compromising performance. However, forage protein is highly rumen degradable (AFRC 1993) and can result in an oversupply of rumen degradable protein, without satisfying the metabolizable protein requirements of high producing animals. In addition, the energy cost associated with excreting excess ammonia (NH<sub>3</sub>) of 0.8 MJ 100g<sup>-1</sup> NH<sub>3</sub> (Sinclair et al. 2014) may reduce the efficiency of energy utilisation. Reducing the protein degradability of ensiled high protein forages could improve the efficiency of protein utilisation. Plant secondary compounds, such as tannins, are one of several techniques shown to reduce protein degradability in the rumen (Wang et al. 2020, Taha et al. 2022).

Tannins are water-soluble polyphenolic compounds of high molecular weight found in a variety of plants (Piluzza et al. 2014). Tannins can be divided into two main groups, condensed tannins (CT) and hydrolysable tannins (HYT) (Makkar 2003). At a pH of 6.0–7.0, tannins have been shown to react with proteins, carbohydrates, minerals, alkaloids and nucleic acids (Taha et al. 2022) to form stable complexes that dissociate at a lower pH (Makkar 2003, Huang et al. 2022). At normal rumen pH values tannin-protein complex form, protecting protein from rumen degradation. These subsequently dissociate at the lower pH (2.3–4.1) found in the abomasum (Makkar 2003), releasing protein for digestion and absorption in the abomasum and small intestine. Thus, enhancing undegradable protein supply (Herremans et al. 2020). Tannins have been reported to reduce the protein degradability of ensiled forages, reducing silage  $\text{NH}_3$  concentration and increasing silage quality (Loregian et al. 2023).

The mechanism by which tannin reduces protein degradation in ensiled forages is unclear. However, it has been suggested that tannins might bind with plant protease enzymes, inhibiting their activity (Lorenz 2011), or they might inhibit the activity of protein-degrading microbes (Tabacco et al. 2006). Alternatively, tannins might bind with plant cell wall materials reducing proteolysis (Makkar 2003). Several studies have reported a reduction in silage  $\text{NH}_3$  concentration due to either endogenous or supplemental tannin (Salawu et al. 1999, Tabacco et al. 2006, De Oliveira et al. 2009, Herremans et al. 2020). However, there is a paucity of data on the use of chestnut hydrolysable tannin in addition to different forages at the point of ensiling. The objective of the study was to investigate the effects of chestnut hydrolysable tannin addition at ensiling on the chemical composition and rumen fermentation characteristics of grass, whole crop pea and bean silages.

## Materials and methods

### Experimental design and forage conservation

Grass from a mixed sward, mainly consisting of perennial grass (*Lolium perenne*), and whole crop pea (*Pisum sativum*) and bean (*Vicia faba*) crops were grown at Harper Adams University and harvested in the beginning of July. The pea and bean crops were common, white-flowered varieties cultivated in the UK (i.e., Pea ‘Ambassador’ and Faba Bean ‘Tundra’), chosen for their significantly reduced or absent condensed tannin content. They were harvested at the early to mid-pod fill stage to optimize biomass yield and nutritional quality for ensiling (Fraser et al. 2008). After cutting, all forages were wilted for 48 hours prior to chopping using a forage harvester (Jaguar 870-840) and ensiling in 25 kg experimental silos. Prior to ensiling each forage was treated with one of four additives: Water (W) (1.0 litre  $\text{tonne}^{-1}$ ); an inoculant (IN) (1.0 litre  $\text{tonne}^{-1}$ ) with an application of  $10^6$  colony forming units  $\text{gram}^{-1}$  FW (fresh weight) of *Lactobacillus plantarum* (Genus Plc, Nantwich Cheshire); Chestnut low tannin (LT) (20 g  $\text{kg}^{-1}$  FW) and Chestnut high tannin (HT) (40g  $\text{kg}^{-1}$  FW). The Chestnut tannin was obtained from Thomas Ware and Sons Ltd, Bristol, UK, had a dry matter content of 750 g  $\text{kg}^{-1}$  and contained castalagin, vescalagin, castalin and vescalin at 530, 350, 30 and 80 g  $\text{kg}^{-1}$  tannin respectively. Both tannin treatments were dissolved in water and applied at 1.0 litre  $\text{tonne}^{-1}$ . Prior to treatment, a sample of each fresh forage was stored at  $-20^\circ\text{C}$  prior to further analysis. The experimental silos consisted of lengths of plastic drainpipe with a diameter of 35 cm, sealed at one end. Each silo was lined with a plastic bag, filled with 25 kg of treated forage and compacted well. The top of the plastic bag was sealed with silage tape, and approximately 4.0 kg of sand was placed on top to maintain compaction. Three replicates of each treatment were ensiled giving 36 silos. Each silo was ensiled for 100 days prior to opening. Samples of the ensiled forage were stored at  $-20^\circ\text{C}$  prior to the *in situ* rumen degradability and *in vitro* fermentation studies.

### *In situ* rumen degradability

The dry matter (DM) and nitrogen (N) degradability characteristics of the treated silages were determined using four mature castrate sheep (80 kg) fitted with permanent rumen cannula. The sheep were offered straw *ad-libitum*, together with 500 g  $\text{day}^{-1}$  of a concentrate in two equal meals at 09:00 and 16:00 h to supply 1.1 times their maintenance energy requirement (AFRC 1993). The concentrate contained barley, sugar beet pulp, rapeseed meal, soybean meal and molasses at 485, 220, 150, 110, and 35 g  $\text{kg}^{-1}$  DM, respectively. Water was freely available. Approximately 20 g of fresh silage from each treatment was weighed into synthetic fibre bags (5 × 10 cm, 50  $\mu\text{m}$  pore size) conforming to AFRC (1992) specifications. Each of the twelve unique forages × treatment combinations (3 forage types: grass, whole-crop pea, and bean; 4 treatments: HT, LT, IN, or W) was incubated in the rumen of each sheep for 4, 8, 16, 24, 48, and 72 hours using the complete exchange method (Paine et al. 1982). To obtain two technical replicates for each forage × treatment × time point combination per sheep, the incubations were performed across two separate runs for each sheep, with adequate washout periods between runs. In each run,

one bag of each of the twelve forages × treatment combinations were inserted into the rumen of each sheep simultaneously for a specific time point. Therefore, at any single incubation time, a maximum of 12 bags were present in the rumen of each sheep. On removal from the sheep, bags were immediately washed under cold running tap water until the rinse water was clear to remove adhering digesta and stop microbial activity. Subsequently, bags were washed in an automatic washing machine set to a 45-minute cold rinse cycle followed by a slow spin (600 rpm). The bags were then dried to a constant weight at 60 °C. The dried residue was subsequently weighed, transferred to individually labelled plastic bags, and stored at room temperature in a cupboard prior to further analysis.

### *In vitro* rumen fermentation kinetics

The rumen fermentation kinetics of the treated silages were assessed using the *in vitro* gas production technique (Theodorou et al. 1994, Huntington and Givens 1998). Rumen fluid was collected 4 hours post-feeding from the four cannulated sheep, which were raised under the same feeding regime and were the same sheep used in the *in situ* rumen degradability studies described above. Following collection, the rumen fluid of the four sheep were pooled and transported to the laboratory in pre-warmed thermos flasks prior to straining through four layers of muslin into pre-warmed conical (39 °C) flasks under a constant stream of gaseous CO<sub>2</sub>.

A nitrogen-sufficient (Huntington and Given 1998) fermentation buffer was used to dilute the strained rumen fluid. Exactly 750 ml of strained rumen fluid was added to 4250 ml of the fermentation media and stored at 39 °C under anaerobic conditions prior to use. Approximately 2.0 g of each oven-dried (60 °C) and milled (1.0 mm screen) treated silage was weighed into duplicate 250 ml glass bottles and mixed with 200 ml of mixed rumen fluid buffer solution. The bottles were then sealed using a plastic cap fitted with a three-way gas valve. The bottles were then incubated at 39 °C for 72 hours, with the gas pressure being measured using a manual pressure transducer (T443; Bailey and MacKay Ltd, Birmingham UK) at 2, 4, 6, 8, 12, 16, 20, 32, 40, 48, 60 and 72 hours post-inoculation. Four blank bottles were also included. After 72 h, the fermentation was terminated by placing it in a freezer at –20 °C for 30 minutes to inhibit microbial activity. After chilling, the pH was recorded, and a 20 ml sample was taken and stored at –20 °C for NH<sub>3</sub>-N analysis. The solid residue was collected by filtering under vacuum through 50 ml crucibles fitted with a porous glass filter (Pyrex P1). The residue was then dried to a constant weight at 60 °C for 48 hours, prior to ashing at 550 °C for 12 hours and the organic matter (OM) loss was calculated. The *in vitro* dry matter (DM) and OM disappearance were calculated after correcting for the blanks. The experiment was repeated four times to provide four replicates per treatment.

### Chemical analysis

The DM content of both the fresh and ensiled forages was determined by oven drying at 100 °C (MAFF 1986), prior to grinding through a 1.0-millimetre screen. The dried ground samples were then analysed for ash and ether extract (EE) using the Soxtec system (FOSS 1987, Warrington, UK), according to MAFF (1986), and crude protein (CP) using the Dumas method (AOAC 2000, method 992.15). The gross energy (GE) was determined using an adiabatic bomb calorimeter (Parr 620, USA), and both the neutral detergent fibre (aNDFom) and acid detergent fibre (ADFom) content were determined according to Van Soest et al. (1991) using alpha amylase enzyme and sodium sulphite. The NH<sub>3</sub>-N and pH of the fresh silages were also determined (MAFF 1986). In addition, the CP content of the residue samples obtained from the *in situ* degradability study, and the DM and ash content of the residue samples obtained from the *in vitro* rumen fermentation study were determined as above. The hydrolysable tannin content of the ensiled forages treated with tannin was determined using the method of Makkar et al. (2007).

Samples of the ensiled forages were also freeze-dried to a constant weight and milled through a 1.0 mm screen, prior to *in vitro* protein fractionation according to the Cornell Net Carbohydrate and Protein System (CNCPS, Sniffen et al. 1992) as described by Licitra et al. (1996). Following extraction with aNDFom and ADFom reagents, the CNCPS subdivides dietary crude protein into non-protein nitrogen (Fraction A), soluble protein (Fraction B1), NDF-soluble protein (Fraction B2), ADF-soluble protein (Fraction B3) and ADF-insoluble protein (Fraction C). Fractions A and B1 were assumed to be rapidly available in the rumen, whereas fractions B2 and B3 were assumed to be insoluble but potentially degradable in the rumen. Fraction C was assumed to represent protein that was undegradable and indigestible. In addition, the undegradable protein content at a rumen outflow rate of 0.05 h<sup>-1</sup> (RUP5) was calculated as described by Shannak et al. (2000) and Kirchhof (2007).

### Statistical analysis

All data were analysed by ANOVA as a 3 × 4 factorial experiment with the main effects being forage type and additive treatment using Genstat 15 (VSN International Ltd, UK). The rate and extend of *in situ* CP degradability was

determined by fitting the model of McDonald (1981), which incorporates a lag phase prior to the commencement of degradation as:

$$p = a + b (1 - e^{-c(t-t_0)})$$

Where:  $p$  = disappearance at time  $t$ ,  $a$  is the immediately soluble fraction,  $b$  is the insoluble but potentially degradable fraction,  $c$  is the rate of degradation of fraction  $b$ , and  $t_0$  is the lag time. The effective degradability, assuming a rumen outflow rate ( $k$ ) of  $0.05 \text{ h}^{-1}$ , was calculated according to Orskov and McDonald (1979) as:

$$P = a + (b \times c) / (c + k)$$

The undegradable protein (UDP) supply at a rumen outflow rate of  $0.05/\text{h}$  (ED5) was calculated by difference. The rate ( $c$ ) and extent ( $b$ ) of *in vitro* gas production were also determined by fitting the model of McDonald (1981) with the ' $a$ ' term being zero.

## Results

### The chemical competition of fresh forages

The chemical composition of the fresh forages used in this study are presented in Table 1. The grass sample was found to have the highest DM (345, 318 and  $235 \text{ g kg}^{-1}$  for grass, pea and bean respectively), CP (174, 148 and  $150 \text{ g kg}^{-1}$  DM for grass, pea and bean respectively) and aNDFom ( $496, 351$  and  $365 \text{ g kg}^{-1}$  DM for grass, pea and bean respectively), whereas the pea and bean samples were found to be broadly similar in their chemical composition.

Table 1. Chemical composition of fresh forages used in the experiment

Parameters	Ryegrass	Pea	Bean
Dry matter ( $\text{g kg}^{-1}$ )	345	318	235
Organic matter	888	922	899
Crude protein	174	148	150
aNDFom <sup>1</sup>	496	351	365
ADFom <sup>2</sup>	254	271	276
Ether extract	21	11	9
Gross energy	17.2	17.1	16.8

<sup>1</sup> aNDFom = Neutral detergent fibre analysed with a heat stable amylase and expressed exclusive of residual ash; <sup>2</sup> ADFom = Acid detergent fibre expressed exclusive of residual.

### Fermentation parameters and chemical analysis of silage

The fermentation characteristics and chemical composition of the ensiled forages are presented in Tables 2 and 3, respectively. All forages were well fermented as indicated by their low pH (3.9 – 4.3) and  $\text{NH}_3\text{-N}$  ( $37 - 66 \text{ g kg}^{-1}$  total nitrogen [TN]) values. However, there was a significant effect ( $p < 0.01$ ) of forage type on silage DM content and pH with the DM and pH values for the grass silage ( $303 \text{ g kg}^{-1}$  and 4.27) being higher than those of the pea silage ( $255 \text{ g kg}^{-1}$  and 4.01), which were higher than those of the bean silage ( $209 \text{ g kg}^{-1}$  and 3.90). Forage type also had a significant effect ( $p < 0.01$ ) on silage  $\text{NH}_3\text{-N}$  content, with the  $\text{NH}_3\text{-N}$  content of the bean silage ( $56 \text{ g kg}^{-1}$  TN) being higher than that of the pea silage ( $51 \text{ g kg}^{-1}$  TN), which had a higher value than grass silage ( $42 \text{ g kg}^{-1}$  TN) as shown in Table 2.

The chemical composition of the ensiled forages reflected that of the original forages (Table 3). The CP and aNDFom contents of the grass silage (174 and  $477 \text{ g kg}^{-1}$  DM) were significantly higher ( $p < 0.01$ ) than those of the bean silage (154 and  $396 \text{ g kg}^{-1}$  DM), which had higher values than those of the pea silage (146 and  $368 \text{ g kg}^{-1}$  DM). However, the ADFom content of grass silage was significantly lower ( $p < 0.01$ ) than that of the bean silage, which was lower than that of the pea silage (279 vs 329 vs  $334 \text{ g kg}^{-1}$  DM, respectively).

Table 2. Effect of supplementary chestnut tannin or inoculate at ensiling on the fermentation quality of bean, pea and rye-grass silage

Forages	Additives	pH	NH <sub>3</sub> -N g kg <sup>-1</sup> TN	DM g kg <sup>-1</sup> FW	OM g kg <sup>-1</sup> DM	GE MJ kg <sup>-1</sup> DM
Grass	W	4.3	48.4 <sup>c</sup>	302	879	17.0
	IN	4.2	42.6 <sup>d</sup>	303	877	17.4
	LT	4.3	40.2 <sup>e</sup>	302	878	18.1
	HT	4.2	37.1 <sup>e</sup>	303	880	17.4
Pea	W	4.0	65.7 <sup>a</sup>	257	919	17.1
	IN	4.0	62.8 <sup>b</sup>	255	913	17.0
	LT	4.0	55.1 <sup>c</sup>	256	915	16.9
	HT	4.0	39.6 <sup>d</sup>	253	921	17.7
Bean	W	3.9	65.8 <sup>a</sup>	221	898	16.8
	IN	3.9	61.5 <sup>b</sup>	206	892	16.9
	LT	3.9	50.9 <sup>c</sup>	205	891	16.9
	HT	3.9	48.1 <sup>c</sup>	203	895	16.8
Additives						
	SED	0.022	1.71	4.0	3.0	0.27
	<i>p</i> -value	0.697	<0.001	0.387	0.270	0.083
Forages						
	SED	0.025	1.97	3.5	2.6	0.31
	<i>p</i> -value	<0.001	<0.001	<0.001	<0.001	0.681
Interaction (Additives X Forages)						
	SED	0.044	3.42	7.0	5.3	0.53
	<i>p</i> -value	0.329	0.024	0.540	0.976	0.510

Means in the same column with a different superscript differ  $p < 0.05$ ; W = water; IN = inoculate; LT = low tannin; HT = high tannin; NH<sub>3</sub>-N = ammonia N; DM = dry matter; OM = organic matter

Conversely, the EE content of the grass silage was significantly higher ( $p < 0.01$ ) than that of the pea silage, which was higher than that of the bean silage (29 vs 12 vs 9 g kg<sup>-1</sup> DM, respectively). There was no significant effect of forage type on silage GE content. Additive treatment had no significant effects on the fermentation quality or chemical composition of the ensiled forages, except NH<sub>3</sub>-N content. Forages treated with HT had a significantly lower ( $p < 0.01$ ) NH<sub>3</sub>-N content than those treated with LT, which had lower values than those treated with W or IN (41.6 vs 48.7 vs 53.3 and 55.6 g kg<sup>-1</sup> TN, respectively). As expected, the tannin content of forages treated with HT was approximately double that of forages treated with LT (138 vs 69 g kg<sup>-1</sup> DM, Table 3).

### *In situ* and *in vitro* protein degradability

The *in situ* CP degradability characteristics of the ensiled forages are presented in Table 4. As expected, there was a significant effect ( $p < 0.01$ ) of forage type on *in situ* CP degradability. The immediately soluble fraction (*a-term*) for the pea silage was higher than that of the grass and bean silages (0.46 vs 0.43 and 0.43, respectively). However, the insoluble but potentially degradable fraction (*b-term*) for the grass silage was higher than that of the pea and bean silages (0.43 vs 0.34 and 0.34, respectively). Consequently, the potential degradable (*a+b*) proportion of the grass silage was higher than that of the pea silage, which was higher than that of the bean silage (0.86 vs 0.80 vs 0.77, respectively). However, the effective degradability, calculated at a rumen outflow rate of 0.05, was highest for the pea silage, which was higher than the grass silage, which was higher than the bean silage (0.58 vs 0.56 vs 0.54, respectively). There was no significant effect of forage type on the rate of degradation (*c*) or the *lag* phase.

Additive treatment had a significant effect ( $p < 0.01$ ) on the *in situ* CP degradability characteristics of the ensiled forages, and there were significant interactions ( $p < 0.01$ ) between forage type and additive treatment. Forages treated with HT had a lower *a-term* than those treated with LT, which had a lower *a-term* than those treated with W or IN (0.39 vs 0.42 vs 0.48 and 0.47, respectively). However, additive LT only reduced the *a-term* in the pea silage, with additive HT reducing the *a-term* further in both the pea and bean silages. There was no effect of additive type on the *b-term* or on the rate constant (*c*). Consequently, forages treated with HT had a lower potentially degradable (*a+b*) fraction than those treated with LT, which had a lower *a+b* fraction than those treated with W or IN.

Tannin addition reduced the a+b fraction to a greater extent in the pea and bean silages compared to the grass silage. Similarly, forages treated with HT had a lower effective degradability, calculated at a rumen outflow rate of 0.05, than those treated with LT, which had a lower value than those treated with W or IN (0.50 vs 0.54 vs 0.60 and 0.60, respectively). Additive treatment also had a significant effect ( $p < 0.01$ ) on the lag phase, with forages treated with HT having a longer lag than those treated with LT, which was longer than those treated with IN or W (1.59 vs 1.12 vs 0.84 vs 0.43 h, respectively). Tannin addition increased the lag phase to a greater extent in the bean silage compared to the pea or grass silage.

Table 3. Effect of supplementary chestnut tannin or inoculate at ensiling on the chemical composition of bean, pea and rye-grass silage

Forages	Additives	CP g kg <sup>-1</sup> DM	NDF g kg <sup>-1</sup> DM	ADF g kg <sup>-1</sup> DM	EE g kg <sup>-1</sup> DM	Tan mg kg <sup>-1</sup> DM
Grass	W	176	460	277 <sup>d</sup>	29	-
	IN	177	475	299 <sup>c</sup>	28	-
	LT	170	489	279 <sup>d</sup>	28	65
	HT	173	487	263 <sup>d</sup>	30	130
Pea	W	149	370	295 <sup>c</sup>	13	-
	IN	148	370	319 <sup>b</sup>	12	-
	LT	148	368	312 <sup>bc</sup>	12	61
	HT	141	363	310 <sup>bc</sup>	10	122
Bean	W	151	392	323 <sup>b</sup>	9	-
	IN	154	398	317 <sup>b</sup>	10	-
	LT	155	399	344 <sup>a</sup>	8	81
	HT	155	395	335 <sup>ab</sup>	8	162
Additives						
	SED	2.8	8.0	8.8	0.9	
	<i>p</i> -value	0.723	0.527	0.361	0.479	
Forages						
	SED	2.5	6.9	7.7	0.7	
	<i>p</i> -value	<0.001	<0.001	<0.001	<0.001	
Interaction (Additives X Forage)						
	SED	4.9	13.8	15.3	1.5	
	<i>p</i> -value	0.501	0.665	0.011	0.121	

Means in the same column with a different superscript differ  $p < 0.05$ ; W = water; IN = inoculate; LT = low tannin; HT = high tannin; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; EE = ether extract; GE = gross energy; Tan = hydrolysable tannin

Table 4. Effect of supplementary chestnut tannin or inoculate at ensiling on *in situ* CP degradability of bean, pea and rye-grass silage

Forages	Additives	a-term	b-term	a+b	c-term	Lag	ED <sub>5</sub>
Grass	W	0.45 <sup>d</sup>	0.45	0.91 <sup>a</sup>	0.02	0.48	0.58 <sup>a</sup>
	IN	0.44 <sup>d</sup>	0.44	0.87 <sup>b</sup>	0.03	0.85	0.61 <sup>b</sup>
	LT	0.41 <sup>f</sup>	0.43	0.84 <sup>c</sup>	0.02	0.90	0.55 <sup>b</sup>
	HT	0.41 <sup>f</sup>	0.41	0.82 <sup>cd</sup>	0.02	1.27	0.53 <sup>c</sup>
Pea	W	0.52 <sup>b</sup>	0.34	0.86 <sup>b</sup>	0.03	0.40	0.65 <sup>b</sup>
	IN	0.53 <sup>a</sup>	0.34	0.87 <sup>b</sup>	0.02	0.81	0.63 <sup>b</sup>
	LT	0.41 <sup>f</sup>	0.36	0.76 <sup>e</sup>	0.03	1.06	0.55 <sup>e</sup>
	HT	0.38 <sup>g</sup>	0.33	0.72 <sup>e</sup>	0.03	1.41	0.50 <sup>f</sup>
Bean	W	0.48 <sup>c</sup>	0.34	0.81 <sup>d</sup>	0.02	0.41	0.58 <sup>b</sup>
	IN	0.44 <sup>de</sup>	0.33	0.77 <sup>e</sup>	0.03	0.86	0.55 <sup>de</sup>
	LT	0.43 <sup>e</sup>	0.33	0.76 <sup>e</sup>	0.03	1.40	0.55 <sup>d</sup>
	HT	0.37 <sup>h</sup>	0.34	0.73 <sup>f</sup>	0.02	2.10	0.47 <sup>g</sup>

Additives							
SED	0.006	0.012	0.013	0.003	0.279	0.009	
p-value	<0.001	0.414	<0.001	0.194	0.002	<0.001	
Forages							
SED	0.005	0.010	0.011	0.003	0.241	0.007	
p-value	<0.001	<0.001	<0.001	0.134	0.379	<0.001	
Interaction (Additives X Forages)							
SED	0.011	0.020	0.022	0.006	0.482	0.016	
p-value	<0.001	0.529	0.007	0.110	0.862	0.002	

Means in the same column with a different superscript differ  $p < 0.05$ ; W = water; In = inoculate; LT = low tannin; HT = high tannin; a-term = soluble and immediately available; b-term = insoluble but potentially degradable; a+b = total degradable fraction; c-term = time dependant rate of degradation; Lag = lag time (h); ED<sub>5</sub> = effective degradability (rumen outflow 0.05 h<sup>-1</sup>)

The *in vitro* CP degradability characteristics of the ensiled forages determined using the CNCPS are presented in Table 5. There was no significant effect of forage type on the non-protein nitrogen (A), and ADF-insoluble fraction (C). However, the bean silage had the highest non-protein N fraction A ( $p = 0.06$ ) and neutral soluble protein fraction B2 ( $p < 0.001$ ), and lowest ( $p < 0.001$ ) buffer soluble N fraction B1 compared with grass and pea silages (Table 5), whereas, grass silage was found to have the highest ( $p < 0.001$ ) acid soluble protein fraction B3 and the lowest ( $p = 0.014$ ) acid insoluble protein fraction C compared with bean and pea silages (B3: 114, 60 and 58 g kg<sup>-1</sup> CP and C: 66, 86 and 89 g kg<sup>-1</sup> CP for grass, bean and pea silages, respectively).

Table 5. Effect of supplementary chestnut tannin or inoculate at ensiling on CNCPS protein fractions of bean, pea and rye-grass silage

Forages	Additives	A	B1	B2	B3	C	RUP <sub>5</sub>
Grass	W	0.48 <sup>c</sup>	0.14 <sup>f</sup>	0.18 <sup>e</sup>	0.131 <sup>a</sup>	0.05	0.20
	IN	0.47 <sup>c</sup>	0.19 <sup>c</sup>	0.18 <sup>e</sup>	0.13 <sup>a</sup>	0.06	0.19
	LT	0.43 <sup>d</sup>	0.22 <sup>b</sup>	0.20 <sup>d</sup>	0.09 <sup>b</sup>	0.08	0.27
	HT	0.40 <sup>e</sup>	0.23 <sup>a</sup>	0.20 <sup>d</sup>	0.09 <sup>b</sup>	0.08	0.27
Pea	W	0.56 <sup>a</sup>	0.15 <sup>e</sup>	0.17 <sup>ef</sup>	0.06 <sup>d</sup>	0.07	0.24
	IN	0.52 <sup>b</sup>	0.16 <sup>e</sup>	0.17 <sup>f</sup>	0.05 <sup>d</sup>	0.07	0.25
	LT	0.48 <sup>c</sup>	0.19 <sup>c</sup>	0.22 <sup>c</sup>	0.06 <sup>cd</sup>	0.11	0.27
	HT	0.35 <sup>f</sup>	0.22 <sup>b</sup>	0.24 <sup>b</sup>	0.07 <sup>c</sup>	0.11	0.28
Bean	W	0.56 <sup>a</sup>	0.10 <sup>h</sup>	0.21 <sup>d</sup>	0.06 <sup>cd</sup>	0.07	0.23
	IN	0.51 <sup>b</sup>	0.12 <sup>g</sup>	0.23 <sup>c</sup>	0.06 <sup>cd</sup>	0.06	0.23
	LT	0.41 <sup>e</sup>	0.19 <sup>cd</sup>	0.25 <sup>a</sup>	0.06 <sup>cd</sup>	0.10	0.28
	HT	0.40 <sup>e</sup>	0.17 <sup>e</sup>	0.26 <sup>a</sup>	0.06 <sup>c</sup>	0.11	0.29
Additives							
SED	0.010	0.006	0.007	0.005	0.009	0.014	
p-value	<0.001	<0.001	<0.001	0.048	<0.001	<0.001	
Forages							
SED	0.009	0.006	0.006	0.004	0.008	0.012	
p-value	0.062	<0.001	<0.001	<0.001	0.014	0.043	
Interaction (Additives X Forages)							
SED	0.017	0.011	0.012	0.008	0.016	0.025	
p-value	<0.001	0.027	0.002	0.004	0.895	0.731	

Means in the same column with a different superscript differ  $p < 0.05$ ; W = water; In = inoculate; LT = low tannin; HT = high tannin; A = non-protein N; B1 = soluble protein; B2 = NDF soluble protein; B3 = ADF soluble protein; C = ADF insoluble protein; RUP5 = rumen undegradable protein (g kg DM<sup>-1</sup>)

In addition, grass silage had the highest protein solubility in acid detergent reagent compared to pea and bean silages. Approximately 935 g kg<sup>-1</sup> CP of grass silage was soluble in CNCPS solutions compared to 910 and 914 g kg<sup>-1</sup> CP in pea and bean silages, respectively. Supplemented chestnut HYT (both levels of inclusion) or inoculate reduced ( $p < 0.01$ ) fraction A compared to the control silage (535, 500, 404 and 384 g kg<sup>-1</sup> CP for W, IN, LT and HT, respectively). Treating leguminous silages with both tannin levels significantly reduced fraction A compared to grass silage (Table 5). Treating forage silages with tannin or inoculating at ensiling increased fraction B1 (209, 197,

156 and 131 g kg<sup>-1</sup> CP for HT, LT, IN and W, respectively), especially in pea silage. Both tannin levels increased ( $p < 0.01$ ) in fraction B2 compared to the inoculum and control silages (232, 224, 193 and 188 g kg<sup>-1</sup> CP for HT, LT, IN and W, respectively). Both tannin levels increased ( $p < 0.05$ ) fraction B2 only in bean and pea silages, although a slight increase ( $p > 0.05$ ) was noticed in grass silage (Table 5).

Treating forages with HT or LT significantly ( $p < 0.05$ ) reduced fraction B3 compared to the IN and W (73, 72.2, 83 and 81 g kg<sup>-1</sup> CP, respectively); the increment was found only in grass silage, while a slight increase was observed in the leguminous silages (Table 5). Supplemented tannin at ensiling increased significantly the fraction-C compared to IN and W (Table 5). Leguminous silages had a higher ( $p < 0.01$ ) UDP5 compared to grass silage (Table 5). Supplemented forages with either level of chestnut tannin were found to increase UDP5 by approximately 22–55 % compared to both the inoculum and control treatments.

### *In vitro* fermentation kinetics – gas production

*In vitro* fermentation kinetics of the ensiled forages are presented in Table 6. Total gas production for each forage was found to be similar ( $p = 0.574$ ). However, fitted values for the curve parameters found differences between the forages for the asymptote (b-term; 337, 331, 391 ml g<sup>-1</sup> DM for bean, pea and grass silages respectively;  $p = 0.016$ ) and the rate of fermentation (c-term; 0.043, 0.044, 0.029 for bean, pea and grass silages respectively;  $p < 0.001$ ). Dry and organic matter degradability were also found to be different for each forage (DM degradability 0.572, 0.549, 0.663; OM degradability 0.582, 0.612 and 0.678 for bean, pea and grass silages, respectively;  $p < 0.001$ ). Culture pH and NH<sub>3</sub>-N at the end of the fermentation period were also found to be different for each forage (pH; 6.51, 6.45 and 6.46;  $p = 0.007$ ; NH<sub>3</sub>-N; 0.215, 0.218 and 0.183;  $p = 0.005$  for bean, pea and grass silages, respectively). Additive treatment reduced total gas production for all forages ( $p < 0.001$ ; 337, 297, 276 and 245 for W, IN, LT and HT, respectively). Fitted values for the curve parameters followed a similar trend for the asymptote ( $p = 0.034$ ; 373, 374, 344 and 322 for W, IN, LT and HT, respectively) and the rate of fermentation (c-term; 0.043, 0.040, 0.035 and 0.033 for W, IN, LT and HT, respectively).

Table 6. Effect of supplementary chestnut tannin or inoculate at ensiling on *in vitro* fermentation kinetic parameters of bean, pea and rye-grass silage

Forages	Additives	TotGP ml g DM <sup>-1</sup>	b-term ml gDM <sup>-1</sup>	c-term	DMdeg	OMdeg	pH	NH <sub>3</sub> -N mg l <sup>-1</sup>
Grass	W	323	391	0.03	0.72	0.72	6.44 <sup>e</sup>	305
	IN	289	405	0.03	0.71	0.72	6.44 <sup>e</sup>	173
	LT	268	391	0.02	0.63	0.67	6.50 <sup>cd</sup>	147
	HT	251	377	0.03	0.60	0.61	6.44 <sup>e</sup>	122
Pea	W	331	363	0.05	0.58	0.64	6.44 <sup>e</sup>	283
	IN	306	343	0.05	0.59	0.62	6.43 <sup>e</sup>	245
	LT	298	324	0.04	0.54	0.61	6.48 <sup>d</sup>	207
	HT	252	295	0.04	0.50	0.58	6.43 <sup>e</sup>	153
Bean	W	357	365	0.05	0.61	0.61	6.42 <sup>e</sup>	260
	IN	296	373	0.05	0.58	0.62	6.51 <sup>bc</sup>	253
	LT	261	316	0.04	0.59	0.54	6.56 <sup>a</sup>	216
	HT	232	294	0.04	0.51	0.54	6.53 <sup>b</sup>	141
Additives	SED	16.5	11.7	0.002	0.016	0.018	0.015	13.5
	<i>p</i> -value	<0.001	0.034	0.007	<0.001	<0.001	<0.001	<0.001
Forages	SED	14.4	10.2	0.002	0.014	0.016	0.012	12.3
	<i>p</i> -value	0.574	0.016	<0.001	<0.001	<0.001	0.007	0.005
Interaction (Additives X Forages)	SED	21.5	20.3	0.003	0.028	0.032	0.026	23.1
	<i>p</i> -value	0.410	0.972	0.770	0.188	0.641	0.001	0.792

Means in the same column with a different superscript differ  $p < 0.05$ ; W = water; In = inoculate; LT = low tannin; HT = high tannin; TotGP = total gas production; b-term = gas production asymptote; c-term = time-dependent rate of fermentation; DMdeg = *in vitro* dry matter degradability; OMdeg = organic matter degradability; NH<sub>3</sub>-N = ammonia nitrogen

Dry and organic matter degradability were also found to be reduced ( $p < 0.001$ ) by additive treatment (DM degradability: 0.636, 0.625, 0.585 and 0.533; OM degradability 0.660, 0.655, 0.607 and 0.573 for W, IN, LT and HT, respectively). An interaction between forage type and additive treatment was observed for final culture pH ( $p = 0.001$ ), however, it was noted that the LT treatment had the highest final culture pH value for all forage types.

## Discussion

### Fermentation and chemical composition of the silage

The chemical composition of the bean, pea and grass silages (Table 2) was within the normal range for the forages cultivated in the UK (Salawu et al. 1999, Hart et al. 2012, Martens et al. 2019) myrabolam and quebracho tannins. Fresh grass had the highest DM, CP, NDF and EE and lowest OM and ADF compared to leguminous forages, which could be due to species differentiation and/or forage maturity at harvesting. Fraser et al. (2008) reported that peas respond better to wilting than beans, which could be due to the differentiation of forage varieties and peas having thinner stems compared to beans, thus increasing the surface area to volume ratio.

Lactic acid bacteria inoculants are common commercial silage additives used in the UK (Salawu et al. 2001). Silage inoculants have been developed for their ability to promote a beneficial fermentation that maximizes the nutritive value of the silage for ruminant animals (Merry and Davies 1999, Taha 2019). Wilkinson and Davies (2013) reported that the addition of homofermentative lactobacillus species to forage at ensiling would cause a rapid reduction of the silage pH in order to prevent the growth of undesirable microorganisms such as clostridia, in addition to reducing DM losses. For these reasons, an inoculant containing *Lactobacillus (L.) plantarum* (a homofermentative species) was used in the present study as a positive control.

All treatments were considered to be well fermented as indicated by the low pH (3.7–4.3) and  $\text{NH}_3\text{-N}$  values ( $< 120 \text{ g kg}^{-1} \text{ TN}$ ). This is indicative of rapid anaerobic fermentation of forage carbohydrates, resulting in good-quality silage (Davies et al. 1998, Wilkinson and Davies 2013). In addition, rapid acidification of the silage would reduce protein hydrolysis, hence reducing silage  $\text{NH}_3\text{-N}$  concentration (Wilkinson and Davies 2013). Deaville et al. (2010) reported that low  $\text{NH}_3\text{-N}$  concentrations produced inside the silage are one of the indicators of a well-fermented silage. The concentration of  $\text{NH}_3\text{-N}$  in the current experiments was lower than reported by Hart (2005) who observed that  $\text{NH}_3\text{-N}$  concentration was 76.8–81.7  $\text{g kg}^{-1} \text{ TN}$  for pea and bean silages, respectively. Deaville et al. (2010) reported that low  $\text{NH}_3\text{-N}$  concentrations produced inside the silage are one of the indicators of a well-fermented silage.

Two levels (20 or 40  $\text{g kg}^{-1} \text{ FW}$ ) of chestnut hydrolysable tannin were supplemented to bean, pea and grass forages at ensiling based on forage fresh weight; however, due to variation in forage DM content, the actual tannin levels on a DM basis differed among the forages. The results from the silage proximate analysis were corrected for the additional DM from the tannin added at ensiling (HT and LT; 40 and 20  $\text{g kg}^{-1} \text{ FW}$ , respectively, which was equivalent to 162 and 81  $\text{g kg}^{-1} \text{ DM}$  in bean silage, 122 and 61  $\text{g kg}^{-1} \text{ DM}$  in pea silage, and 130 and 65  $\text{g kg}^{-1} \text{ DM}$  in grass silage). Although tannin was applied on a fresh weight basis to mimic practical on-farm use, we acknowledge that the variation in DM content resulted in differing DM-based tannin concentrations, which may have influenced the magnitude of the responses observed. A similar approach was reported by Salawu et al. (1999) and Deaville et al. (2010) myrabolam and quebracho tannins. Deaville et al. (2010) found that treating grass silage with mimosa and chestnut tannin (74.8  $\text{g kg}^{-1} \text{ DM}$ ) either at ensiling or at feeding increased the silage DM compared to untreated silage, corresponding to the addition of tannin DM. The chemical composition of the ensiled forages showed that grass silage had the highest pH, DM, CP, NDF and EE and lowest  $\text{NH}_3\text{-N}$  and ADF compared to pea and bean silages, which could perhaps be due to the differences between the species, cultivation system and the maturity at harvesting.

Additive treatment was only found to affect the  $\text{NH}_3\text{-N}$  content of the ensiled forages. Additive treatment reduced the  $\text{NH}_3\text{-N}$  content of ensiled forage as compared to the W treatment by 7.3, 18.8 and 30.7% for IN, LT and HT treatments respectively. The effect of adding tannin at the point of ensiling was considered to be the formation of a tannin-protein complex (Makkar 2003), inhibiting plant protease (Salawu et al. 1999) and/or reducing the microbial activity in herbage (Ohshima and McDonald 1978). Tabacco et al. (2006) stated that tannins could be considered to be an anti-microbial compound, which could affect the micro-flora in the silo both chemically and biologically. Similar results have been reported by Tabacco et al. (2006) who found that additional chestnut hydrolysable tannin (20, 40 and 60  $\text{g kg}^{-1} \text{ DM}$ ) in lucerne silage reduced the  $\text{NH}_3\text{-N}$  value compared to the control. Ammonia nitrogen concentration in all forage silages treated with IN was found to be lower than W treated forage, suggesting

that the inoculant rapidly reduced forage pH during ensiling and inhibited both the activity of plant proteases and undesirable micro-organisms such as clostridia, which are associated with protein breakdown (Davies et al. 1998).

### *In situ* and *in vitro* protein degradability

For the *in situ* rumen degradability fresh un-chopped silage samples were incubated in the rumen which may have limited the homogeneity and underestimated the true rumen degradability compared to using milled (3 mm screen) dried samples according to AFRC (1992) recommendation, thus increasing the replications necessary to reduce the variation between the samples. However, drying and grinding the silage has the effect of changing the biological, chemical and physical composition of silage such that the results may not be as valid for interpretation to ruminant feeding.

Crude protein fraction a-term results for all treatments were found to range from 400–491 g kg<sup>-1</sup> DM, respectively. These values are slightly lower than the values published by AFRC (1993) (423–715 g kg<sup>-1</sup> DM) or NRC (2001) (573 g kg<sup>-1</sup> DM), for grass and leguminous silages. This might be due to using 20–25 g fresh silage per bag in this study. Huntington and Givens (1995) reported that processing feeds (chopping or grinding) before rumen incubation might affect the *in situ* rumen degradability results.

Adding tannin at ensiling reduced the CP fraction a-term (481, 467, 415 and 387 g kg<sup>-1</sup> CP for W, IN, LT and HT, respectively). Similarly, it has been reported (Tabacco et al. 2006, Sinclair et al. 2009, Coblenz and Grabber 2013) that the presence of tannin reduced *in situ* DM or CP degradability, probably due to complexing tannin with forage proteins or fibre reducing their solubility in the rumen (Makkar 2003, Jerónimo et al. 2016) and/or the negative effect of tannin on rumen microbes (Loregian et al. 2023). Loregian et al. (2023) suggested that tannin-protein complexes would reduce *in situ* CP fraction a-term and increase fraction b-term; and overall reduce ED CP and increase UDP. Salawu et al. (2001) observed that using quebracho (condensed tannin) as a silage additive reduced CP fraction a-term, ED and fraction c-term and increased CP fraction b-term. Sinclair et al. (2009) found that high tannin level pea silage had a significantly lower CP fraction a-term compared to low tannin level pea silage. Furthermore, Coblenz and Grabber (2013) showed that increasing the level of condensed tannin reduced the CP fraction a-term in different alfalfa hay and silages. However, Messman et al. (1996) found that different proportions of tannin in leguminous silage had no effect on the fraction a-term. Azuhwi et al. (2012) also found that fraction a-term was not affected by sainfoin varieties, which contained different amounts of tannin.

The insoluble but potentially degradable CP fraction b-term ranged from 332–446 g kg<sup>-1</sup> DM. These values are in agreement with those reported by AFRC (1993) (190–530 g kg<sup>-1</sup> DM) and NRC (2001) (350 g kg<sup>-1</sup> DM) for different grass and leguminous silages. Treating forage silages with either tannin or inoculate had no effect on fraction b-term compared to silages treated with water only. The rate of degradation fraction c-term was not affected by either additives or forage types. Similar results have been published in several studies (Tabacco et al. 2006, Sinclair et al. 2009). The effective rumen CP degradability at an outflow rate of 0.05 h<sup>-1</sup> ranged from 661–884 g kg<sup>-1</sup> DM; values in agreement with those reported by AFRC (1993) (750–780 g kg<sup>-1</sup> DM) for grass and leguminous silages. The addition of tannin or inoculum at ensiling reduced the CP ED<sub>5</sub> as compared to the W treatment (mean ED<sub>5</sub> were 0.848, 0.819, 0.782 and 0.725 for W, IN, LT and HT, respectively). These results agreed with the result published by Alipour and Rouzbehan (2010) who found that CP ED at an outflow rate of 0.05 of SBM CP reduced linearly (630, 573, 511, 502 and 480) when tannin level was increased (0, 15, 30, 45 and 60 g kg<sup>-1</sup> DM, respectively). In contrast, Loregian et al. (2023) observed that any tannin had no effect on fraction b-term, fraction c-term and ED when they added a level of chestnut tannin (20 and 30 g kg<sup>-1</sup> DM) to pea seeds compared with the untreated diet.

Grass silage had the lowest fractions A and C and the highest fractions B1, B2 and B3 fractions compared to leguminous silages (Table 5). These differences were considered to relate to differences between species and/or maturity stage at harvesting. Kirchof (2007) reported that fraction A was increased with increasing maturity. Hymes-Fecht et al. (2013) reported that the rate of pH decline also has an effect on fractions A and B1; with rapid fermentation, low amounts of forage protein will be converted to non-protein nitrogen during ensiling, in the current study. Grass silage had the highest silo pH compared to pea and bean silages (4.27, 4.02 and 3.91 for grass, pea and bean silages, respectively). Hart (2005) reported that leguminous forages had a higher buffering capacity compared to grass silage, and the reduction of herbage pH to reach <4.5 took approximately 96 h, compared to only 36h in grass. In addition, the DM content will also impact on rate and extent of pH decline with higher DM silages, irrespective of herbage type having a higher final pH (McDonald et al. 1991). Thus, whilst grass has the lower buffering capacity, the higher DM also gave rise to lower microbial activity and thus less proteolysis and lower NH<sub>3</sub>-N concentrations. Ammonia-N concentrations were higher in leguminous

silages compared with grass silage (421, 558, and 566 g kg<sup>-1</sup> DM for grass, pea, and bean silages, respectively). These results concur with the findings of Hart (2005), who suggested that both plant and microbial protease enzymes remain active for a longer period due to the higher buffering capacity of leguminous forages. This buffering slows the pH decline, thereby maintaining conditions that favour protease activity during the ensiling process. As a result, proteins are more extensively degraded into peptides and amino acids. The slower pH decline also allows microbial populations, including clostridia and Enterobacteria, to survive and further convert proteolytic breakdown products through deamination of amino acids, leading to increased ammonia production. The process of proteolysis resulting in an increase in amine groups which further increases the buffering capacity of the forage thus further reducing the rate of in-silo pH decline and thus extending proteolytic activity further. Furthermore, slower silage fermentations produce more acetic acid which combines with ammonia to form ammonium acetate which is a well-known buffer with optimal activity at pH 4.75. It is likely that the IN treatment by speeding up the production of lactic acid reduced proteolytic activity and the tannin treatments by binding to protein and breakdown products thereof reduced buffering and thus improved the rate of silo fermentation. Guo et al. (2008a) reported that in leguminous silages the proteolysis process converted most of B1 to A fraction during the ensiling period.

Addition of chestnut hydrolysable tannin to each forage type (LT and HT) reduced fraction A (27 and 29 %) and increased fractions B1 (79 and 69%), B2 (23 and 24%), B3 (3 and 9%) and C (43 and 53%) respectively compared to the W treatment (control silage). Guo et al. (2008b) observed a similar response when they treated lucerne with tannic acid at ensiling, except in the case of Guo et al. (2008b) the B3 fraction was not affected by treatment. This may have been related to the effects of tannin on proteolytic enzymes during the fermentation period. In addition, tannin could bind with macromolecules of feedstuff making them less soluble in chemical solutions (Suominen et al. 2023). Kirchof et al. (2010) found a negative relationship between tannin concentration and fractions A and B1. Grabber and Coblenz (2009) also found that tannin concentrations shift CP solubility from fractions A and B1 to fractions B2 and C when they used different conservation methods on lucerne, red clover and three varieties of birdsfoot trefoil (low, moderate and high tannin) silages in two seasons. In all cases (Guo et al. 2008b, Grabber and Coblenz 2009) the reduction in the A fraction was assumed to relate to reduced destruction of crop proteins during the ensiling process. Elevated concentration of the C fraction would result in reduced digestibility of dietary crude protein. This concurs with the findings of Deaville et al. (2010), who observed a mean increase in faecal N losses (g 100 g<sup>-1</sup> N intake) of 38% when tannins (mimosa or chestnut) were added at ensiling.

### *In vitro* fermentation kinetics – gas production

The results of the b-term (gas production asymptote) and total gas production of the samples used in the current study showed that all forages were considered highly fermentable. Cone et al. (1999) reported that the maximum gas production of grass silages could reach 208 ml g<sup>-1</sup> OM, while it could get up to 246 ml g<sup>-1</sup> OM in maize silages.

Grass silage had a higher asymptote gas production compared to bean and pea silages (364, 326 and 322 ml g<sup>-1</sup> DM, respectively), which might indicate biochemical differences between the forages that grass behaves differently in the rumen compared to leguminous forages, or due to the differences between forages N concentration or other proximate analysis such as NDF and EE. Getachew et al. (2000) reported that increasing the CP content of the feedstuff would affect rumen fermentation as NH<sub>3</sub>-N is the main source of microbial N in the rumen. The results observed in the current study agreed with those published by Hart (2005), who observed that the asymptote gas production was 303 and 294 ml g<sup>-1</sup> DM for pea and bean silages, respectively.

Supplementation with either inoculum or tannin level depressed gas production. An apparent reduction in gas production as a result of inoculation of the fresh forage with *L. plantarum* may reflect the homofermentative action of the inoculum, creating a higher concentration of lactic acid in the resulting silage. Elevated lactic acid fermented in the rumen would result in increased propionic acid, reduced methane and less gas evolution *in vitro*. Navarro-Villa et al. (2012) indicated non-significant lower methane production from grass silages treated with homofermentative inoculants compared to grass silage treated with mixed homo/hetero fermentative inoculants. In their study, the mixed inoculant silages had significantly higher acetic acid and lower lactic acid than the homofermentative inoculant-treated silages. Thus, lending support to this hypothesis. The study of Navarro-Villa et al. (2012), also showed that the homofermentative inoculant treated silages had a higher total VFA production compared to the mixed inoculant treated silages, further suggesting that the inoculant treatment did not reduce degradation just gas production outputs and is likely to be having a positive response when all aspects are considered. Reduced fermentation for tannin treatments may be due to tannin complexing with macromolecules of the feedstuff, making them less available or possibly unavailable to the rumen bacteria, as indicated by the in-

creased CNCPS C fraction (Table 5). In addition, tannin could have a direct effect on rumen microbes (Getachew et al. 2000), and / or an indirect effect via tannins reacting with bacterial enzymes (Huang et al. 2022). Reduced DM and OM degradability (Tables 5 and 6) support the notion that supplemental tannin had direct and indirect effects on forage fermentation/degradation. Jerónimo et al. (2016) reported that tannins bind with dietary protein in the rumen and reduce protein degradation, whilst increasing by-pass protein supplied to the small intestine. However, the results of the current experiment would suggest that the tannin supplements reduced rumen degradability but may also have reduced protein digestibility.

A high concentration of tannin was added in this study (mean addition was 69 and 138 g kg<sup>-1</sup> DM for LT and HT, respectively). Whilst these tannin concentrations are higher than would be recommended for feeding. On-farm forage sources are often mixed and then further mixed with concentrates. In this case, if the test forage were to be mixed 50:50 with another forage and then mixed 60:40 with concentrates the final tannin concentration in the diet would be 21 and 42 g kg<sup>-1</sup> DM for LT and HT treatments, respectively, which would represent recommended tannin feeding levels and may not lead to high ADIP (C fraction) values for final diet. Further research to test the labile nature of tannin when mixed post-ensiling but prior to feeding should be investigated.

The current results are in agreement with the results published by Hervas et al. (2003) and Alipour and Rouzbehan (2010). However, Getachew et al. (2008) reported that supplemented tannic and gallic acid reduced total gas production but increased potential gas production. They explained that these differences were due to the variation between tannin levels and types and their effects on rumen fermentation.

Treating forages with tannin or inoculate reduced NH<sub>3</sub>-N concentration *in vitro* which was probably due to the additives reducing proteolysis as explained previously. Furthermore, the results of the final supernatant pH showed that there was a significant difference between media, forages, additives and an interaction between them. However, the pH value range was 6.35–6.73 with a variation of only 0.38 (CV=0.7) for 288 samples suggesting that although there was a statistically significant interaction, the difference between values was unlikely to have a biological consequence.

Using chestnut hydrolysable tannin at ensiling to different forages reduced forage protein hydrolysis during the ensiling period. Moreover, supplemented tannin was found to reduce the proportion of non-protein nitrogen and increase forage true protein inside the silage clamp. These results indicate that using tannin as a silage additive could alter the quality of the final silage. The *in situ* rumen degradability of bean, pea and grass silages was reduced when forage was supplemented with chestnut HT at ensiling. In addition, tannin supplementation (especially HT) was found to reduce rumen fermentation *in vitro*. Collectively, these results suggest that feeding ruminant silage treated with chestnut HYT may increase rumen bypass protein.

## Conclusion

Supplementing chestnut hydrolysable tannin (HYT) at ensiling reduced protein degradation across bean, pea, and grass forages, as indicated by lower NH<sub>3</sub>-N and NPN and increased true protein in silage. HYT-treated forages also showed reduced *in situ* rumen degradability and *in vitro* fermentation. These findings demonstrate that HYT can improve silage protein retention and may increase the supply of rumen undegradable protein, supporting its use as a functional silage additive in ruminant diets.

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