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Optimizing alfalfa silage with rumen-focused tannin dynamics: Toward enhanced protein retention

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Abstract The effects of tannic acid (TA) and purified tannins from pistachio by-products (PB) and pomegranate pulp (PP) on the chemical composition, *in vitro* gas production and *in situ* ruminal disappearance of crude protein (CP) and tannins in alfalfa silage (AS) were investigated. The experimental treatments (on a dry matter [DM] basis) included: 1) control silage (CS), 2) alfalfa silage treated with 2% TA (TAS), 3) alfalfa silage treated with 2% PB tannin (PBS), and 4) alfalfa silage treated with 2% PP tannin (PPS). Four replicates of each treatment were prepared and ensiled for 60 days. The results showed that the treatment of AS with PB and PP tannins significantly lowered the pH of silage compared to the control ($P<0.05$). In addition, all tannin sources significantly decreased the ammonia nitrogen (NH_3-N) content in the silages ($P<0.05$). The treatment of AS with all sources of tannins decreased the A fraction of CP (non-protein nitrogen compounds; NPN), while it increased the B1 fraction (true soluble protein). Potential gas production (mL) was significantly lower in PB- and PP-treated silages compared to control and TA-treated silages ($P<0.05$); however, the rate constant of gas production was only reduced in TA-treated silage compared to control ($P<0.05$). Organic matter digestibility and metabolizable energy were also significantly reduced by all tannin sources ($P<0.05$). Compared to the control, the *in situ* degradability of the rapidly soluble fraction (a) of CP decreased in the AS treated with PB and PP tannins, while the slowly degradable fraction (b) increased ($P<0.05$). The degradability rate was higher in the PBS than in the TA-treated and control silages ($P<0.05$). No significant differences were found among treatments in ruminal tannin disappearance, with approximately 73% of tannins disappearing at the time zero in all silages. In conclusion, incorporation of 2% tannins from PB or PP into AS, compared to TA and the control, effectively reduced NH_3-N and the soluble protein fraction in the rumen without significantly altering the potential and effective degradability of protein.

Keywords: degradability, pistachio by-products, pomegranate pulp, protein fractionation, tannin

Introduction

The relatively high breakdown of alfalfa proteins into non-protein nitrogen (NPN) compounds during wilting and ensiling (Muck et al., 2003) is a key challenge in ruminant nutrition. This process often leads to inefficient nitrogen utilization by ruminants, resulting in increased nitrogen. excretion into the environment As a result, producers are compelled to add expensive protein supplements to silage-based diets to compensate for protein losses (Givens and Rulquin, 2004). Proteolysis during ensiling can reduce the true protein content by up to 80% (Winters et al., 2000). In addition, microbial activity in the rumen exacerbates this problem as protein residues in the rumen are rapidly degraded and ammonia is released (Givens and Rulquin, 2004).

For forage species containing tannins, such as Lotus spp.,

sainfoin, and red clover, protein degradation during ensiling and in the rumen is significantly lower than for legumes such as alfalfa, which do not contain tannins (Tavendale et al., 2005; Fijałkowska et al., 2015). Tannins are bioactive plant secondary metabolites that can bind proteins and other macromolecules, thereby reducing their susceptibility to microbial degradation. This unique property has generated considerable interest in the use of tannins as additives to reduce protein loss during ensiling and improve protein utilization in ruminants (Zamiri et al., 2015; Fonseca et al., 2023). By forming stable tannin-protein complexes, tannins can improve nitrogen retention, reduce urinary nitrogen excretion and reduce the environmental impact of ruminant production systems.

Given the rising cost of feed and the recurring challenges of drought in arid and semi-arid regions, there is a growing need for research into sustainable feeding strategies. Secondary metabolites such as tannins, derived from plants adapted to harsh environmental conditions, represent a promising avenue. Pistachio by-products (PB) and pomegranate pulp (PP) are abundant agricultural residues in Iran, with an annual production of over 400,000 tons of PB (Bagheripour et al., 2008) and 120,000 tons of PP (Mirzaei-Aghsaghali et al., 2011). These by-products are rich in tannins and could serve as cost-effective and sustainable additives in high-protein forage silage, such as alfalfa silage (AS).

This study is one of the first to utilize purified tannins from PB and PP in animal feed research, expanding the knowledge of non-conventional tannin sources. By utilizing agricultural residues, this research contributes to sustainability while reducing dependence on commercial tannic acid. The main objective of this study was to evaluate the effects of tannic acid, PB tannins and PP tannins as silage additives on the chemical composition, crude protein fractions, *in vitro* gas production and *in situ* disappearance of crude protein and tannins in alfalfa silage.

Materials and methods

Preparation of tannins from pistachio and pomegranate by-products

Pistachio by-products and pomegranate pulp were obtained from a pistachio dehulling factory in Feizabad and pomegranate juice factory in Mashhad (Khorasan Razavi Province, Iran), respectively. Extraction and purification of tannin polymers were performed by water extraction combined with macroporous resin adsorption according to the protocol of Zhang et al. (2014) with slight modifications. In brief, 100 g of the raw material were ground to pass through a 2-mm sieve and soaked in distilled water at a ratio of 1:5 (w/v) at room temperature for 12 hours. The mixture was then filtered through four layers of cheesecloth. The filtrate was concentrated under reduced pressure in a rotary evaporator at 55°C to a quarter of its initial volume. The crude tannin extract was then purified using a D101 macroporous resin column (2.5 cm × 30 cm). The column was eluted sequentially with 500 mL distilled water and 500 mL 10% (v/v) methanol to remove impurities and oligomeric tannins. Subsequent elution with 500 mL pure methanol yielded the fraction of polymeric tannins. The final eluent was concentrated to remove the solvent and then freeze-dried in a freeze dryer (Beta 2-8 LD plus Martin Christ, Germany). The result was a purified tannin fraction with a yield of 4.62 g for PB and 7.83 g for PP.

Preparation of alfalfa silage treated with tannins

The second growth of alfalfa was harvested at early bloom stage and cut to a theoretical length of 3 cm using a cutter. The chopped material was packed in plastic buckets with a capacity of 500 g. Commercial tannic acid (LESEN, Xi'an, China; 72% purity), tannins from pistachio by-products (PB; 81% purity as tannic acid equivalent) and tannins from pomegranate peel (PP; 86% purity as tannic acid equivalent) were added to fresh alfalfa at a final tannin concentration of about 2%. The experimental treatments were: 1) alfalfa silage without additive (CS; control), 2) TA-treated silage: alfalfa silage supplemented with 2% tannic acid (TAS), 3) PB-treated silage: alfalfa silage supplemented with 2% PB tannin (PBS), and 4) PP-treated silage: alfalfa silage supplemented with 2% PP tannin (PPS). Four replicates were prepared for each treatment, and the silages were stored in a dark environment at room temperature (25°C) for 60 days.

In vitro gas production

Gas production (GP) was evaluated using the gas pressure transducer technique described by Theodorou et al. (1994). Approximately 200 mg of the dried sample was weighed in quadruplicate and placed in 120 mL gas-tight culture bottles. Each bottle was then filled with 30 mL of buffered rumen fluid. Preparation of the buffer and collection of the rumen fluid followed the protocol described by Menke and Steingass (1988). The rumen fluid was collected before morning feeding from three cannulated steers that had been on a fixed diet for two weeks. The diet consisted of a 70:30 ratio of forage to concentrate offered twice daily and free access to fresh water. The culture bottles containing the sample and the buffered rumen fluid mixture were incubated in a water bath set at 39°C. Gas pressure in the headspace of each bottle was measured after 2, 4, 8, 12, 24, 48, 72 and 96 hours of incubation using a digital indicator. The corresponding gas volume was calculated by recording the volume of gas displaced into a syringe barrel. After each measurement, the bottles were carefully shaken to ensure uniform mixing without removing them from the water bath. This procedure was repeated in two independent runs. Gas production values were corrected for blank incubations to account for gas production. Cumulative gas production was analyzed using the model described by Ørskov and McDonald (1979):

$Y = b (1 – e^{-ct})$

where, Y= gas produced at time t; b = gas production from the insoluble but fermentable fraction (mL); c = gas production rate constant for fraction b, and t = incubation time (h).

Metabolizable energy (ME) and organic matter digestibility (OMD) values were estimated using the following equations (Menke and Steingass, 1988):

OMD (%) = 14.88 + 0.8893 GP + 0.448 CP + 0.651 Ash
ME (MJ/kg DM) = 2.20 + 0.1357 GP + 0.057 CP

where, GP = net production in 24 h in mL/200 mg of DM; CP = % of DM, and ash is % of DM.

In situ procedure

Silage samples were dried in an oven at 40°C to reach a constant weight and then ground through a 2-mm sieve. Approximately 5 g of each sample were weighed in triplicate into polyester bags (12×17 cm; 53±10 µm pore size). The bags were heat sealed and incubated in the rumen of three mature Holstein steers. The bags were placed in the rumen before morning feeding and removed after 2, 4, 8, 16, 24, 48 and 72 hours of incubation. Steers were housed in a covered barn and fed *ad libitum* as described in the section on *in vitro* gas production. All animal treatments were in accordance with the guidelines of the Iranian Council of Animal Care (1995). After incubation, the bags were rinsed in a top-loading washing machine to remove the loosely bound residues. Additional bags, referred to as 0-hour incubation, were rinsed without ruminal incubation and served as the control. After rinsing, the residues were dried and analyzed for crude protein (CP) and total tannin (TT) content. Rumen degradation data for CP and TT were analyzed using the first-order exponential model described by Ørskov and McDonald (1979). The analysis was performed using the nonlinear regression (NLIN) procedure (SAS, 2001) using the following model:

$P = a + b (1 – e^{-ct})$

where, P = potential degradability at time t; a = soluble fraction; b = potentially rumen degradable fraction; c = degradation rate constant (h^{-1}); and t = incubation time (h).

Effective degradability (ED) was determined using the following equations, considering rumen outflow rates of 6%/h (Orskov and McDonald, 1979):

ED (%) = a + b (c/c+k)

where, a, b, and c are the same potential degradability parameters and k is the rumen outflow rate (%/h).

Laboratory analyses

To determine the pH value, the silage samples were mixed with distilled water at a ratio of 30:270 using a blender (Bernardes et al., 2019) and the pH value was measured using a portable digital pH meter (METROHM 691). Dry matter (DM) was determined by drying the samples in an oven at 100°C until a constant weight was reached (AOAC 2005, method 934.01). Crude protein (CP; Kjeldahl N × 6.25) was analyzed by the Block Digestion Method with a copper catalyst followed by steam distillation to boric acid (Method 2001.11) using a 2100 Kjeltec distillation unit. Ash content (method 942.05) and the acid detergent fiber (ADF; method 973.18) were also determined. Neutral detergent fiber (NDF) was analyzed according to the method of Van Soest et al. (1991) in the absence of sodium sulfite and α-amylase. The NDF and ADF values were reported without residual ash. Crude protein fractions (A, B1, B2, B3 and C) were determined based on the Cornell Net Carbohydrate and Protein System (CNCPS) according to the method described by Licitra et al. (1996). Ammonia-nitrogen (NH_3-N) was quantified using the phenol-hypochlorite reaction (Weatherburn, 1967).

For the analysis of organic acids, silage extracts were filtered through a 0.22µm membrane filter and analyzed for lactic acid and butyric acids using high performance liquid chromatography (HPLC) with a diode array detector (DAD, 210 nm, SPD-20A, Shimadzu Co., Ltd., Kyoto, Japan) (Xu et al., 2022).

For tannin analysis, silage samples were dried at 40°C to a constant weight to minimize changes in tannin content and activity (Makkar, 2000). The dried samples were ground to pass through 2-mm and then 0.5-mm sieves. Approximately 200 mg of each sample were extracted in four replicates with 70% (v/v) aqueous acetone overnight at 4°C. Total phenolic compounds (TP) and total tannins (TT) were measured using the Folin-Ciocalteu reagent, with tannic acid as the standard (Makkar et al., 2000). Concentrations of TP and TT were expressed as tannic acid equivalents.

Statistical analysis

The data was analyzed using SAS statistical software (SAS, 2001) for a completely randomized design. The statistical model was as follows:

$Y_{ij} = \mu + T_i + e_{ij}$

where, Y_{ij} is the j_{th} observation in the i_{th} treatment, µ is the general mean, T_i is the effect of the i_{th} treatment and e_{ij} is the experimental error.

Differences among the least squares means (LSMEANS) were tested using the PDIFF option when P value of <0.05 was detected, with trends set at P<0.10. The results are reported as the least squares means.

Results

Chemical composition of alfalfa silage

The chemical composition of the experimental alfalfa silages is summarized in Table 1. The addition of tannins had no significant effect on the DM, ash, CP or NDF content of the silages. However, the acid detergent fiber (ADF) content tended to decrease significantly in the PP-treated silage (P<0.10). Silages treated with PP and PB tannins had lower pH values than the control and those

treated with TA. Higher lactic acid concentrations were observed in PBS and PPS compared to the control and TA-treated silages. All tannin treatments reduced the silage NH_3-N content.

Table 1. Chemical composition and phenolic compounds of alfalfa silages treated with different tannin sources

Item	Silage[1]					
	CS	TAS	PBS	PPS	SEM	P values
Chemical composition (% DM)						
pH	5.03[a]	4.84[ab]	4.72[b]	4.66[b]	0.052	0.02
NH_3-N (mg/dl)	15.51[a]	11.11[b]	8.71[c]	8.82[c]	0.737	<0.001
Lactic acid	2.98[c]	3.61[b]	4.13[a]	4.08[a]	0.211	<0.01
Butyric acid	0.02	0	0	0	-	
Dry matter	24.93	25.71	25.35	25.63	0.395	
Crude protein	18.89	18.18	18.01	17.94	0.164	0.25
Neutral detergent fiber	43.31	42.22	41.62	40.91	0.540	0.11
Acid detergent fiber	30.08	28.72	27.82	26.95	0.494	0.08
Ash	9.75	10.03	10.10	10.15	0.203	0.56
Total phenolics	0.79[d]	2.98[b]	3.47[a]	2.36[c]	0.466	<0.001
Total tannins	0.36[b]	2.25[a]	2.10[a]	2.12[a]	0.247	<0.001
Crude protein fractions (% in CP)						
A	65.34[a]	62.27[b]	59.76[b]	60.24[b]	0.871	0.01
B1	0.31[b]	2.32[a]	1.92[a]	2.06[a]	0.298	0.002
B2	25.73	27.02	29.31	29.11	0.642	0.09
B3	6.20	6.12	5.88	5.94	0.143	0.93
C	2.42	2.23	3.13	2.56	0.142	0.08

[1]CS: control silage; TAS: tannic acid silage; PBS: pistachio by-products silage; PPS: pomegranate pulp silage.
SEM: standard error of the mean.
a, b: Means in a row with common superscript(s) do not differ (P>0.05).

The effects of tannin treatment on CP fractions are shown in Table 2. The proportion of NPN in the control and tannin-treated silages was 65% and 61% (average value) of CP, respectively. The PBS treatment significantly reduced the A fraction of CP (NPN) and increased the B1 fraction (true soluble protein). A tendency to increase the B2 fraction was observed in the PBS and PPS.

In vitro gas production

The cumulative GP and kinetic parameters of GP from alfalfa silage are shown in Figure 1 and Table 2. Supplementation with PB and PP tannins significantly reduced the total GP and gas volume produced from the insoluble but fermentable fraction (b) compared to TA and the control (P<0.05). The lowest GP rate constant (c) was observed in TAS (P<0.05), while PPS had a lower GP rate compared to PBS. Treatment of alfalfa silage with all tannin sources resulted in a significant reduction in estimated OMD and ME compared to the control silage.

Table 2. Gas production kinetics and *in vitro* digestibility of alfalfa silages treated with different tannin sources

Item	Silage[1]					
	CS	TAS	PBS	PPS	SEM	P values
Total gas production	81.1[a]	77.9[ab]	73.5[b]	69.1[c]	1.48	0.001
Estimated parameters						
b	78.3[a]	75.4[a]	69.6[b]	66.3[b]	1.97	0.02
c	0.048[ab]	0.039[c]	0.050[a]	0.044[bc]	0.0016	0.01
Organic matter digestibility	75.5[a]	70.3[b]	69.2[bc]	66.6[c]	0.95	<0.001
Metabolizable energy	10.27[a]	9.45[b]	9.18[b]	8.93[b]	0.147	<0.001

[1]CS: control silage; TAS: tannic acid silage; PBS: pistachio by-products silage; PPS: pomegranate pulp silage.
b: gas production from the insoluble but fermentable fraction (mL/200 mg DM); c: gas production rate constant (h^{-1}).
SEM: standard error of the mean.
[a, b]: Means in a row with common superscript(s) do not differ (P>0.05).

In situ ruminal disappearance

The *in situ* crude protein (CP) degradability of alfalfa silages is shown in Table 3. Treatment with PB and PP tannins significantly (P<0.05) decreased the rapidly degradable CP fraction (a) while increasing the slowly degradable fraction (b). Notably, despite the reduction in the rapidly degradable fraction, the rate of protein degradation in PBS was increased. The potential degradability (PD) and effective degradability (ED) of CP were not significantly affected by tannin treatment.

The percentage of total tannins that disappeared from the rumen over time is shown in Table 4. At the beginning of incubation (zero time), 76%, 70% and 73% of the total tannins disappeared in TAS, PBS and PPS, respectively. After 2 hours of incubation, 87.5%, 83.6% and 87.1% of the tannins in the TAS, PBS and PPS had disappeared, respectively. This means that the rumen

microorganisms degraded 48.3%, 44.1% and 51.7% of the remaining tannins in the TAS, PBS and PPS within 2 hours. No significant differences in tannin degradation were observed between silage treatments at any incubation time. These results indicate that the rate and extent of tannin degradation in the rumen were consistent for different tannin sources.

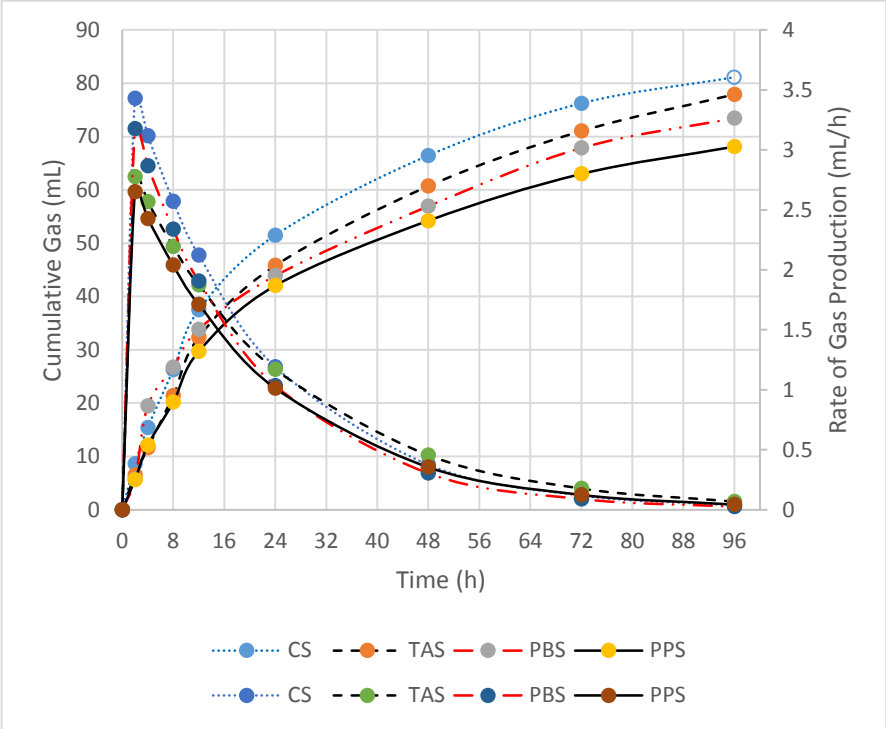

Figure 1. Cumulative and rate of gas production over time from alfalfa silage treated with different tannin sources.
[1]CS: control silage; TAS: tannic acid silage; PBS: pistachio by-products silage; PPS: pomegranate pulp silage.

Table 3. *In situ* ruminal disappearance of crude protein from alfalfa silages treated with different tannin sources

Item	Silage[1]					
	CS	TAS	PBS	PPS	SEM	P values
Crude protein disappearance						
a	66.3[a]	63.8[ab]	59.4[b]	60.0[b]	1.13	0.03
b	26.2[b]	26.4[b]	30.3[a]	29.1[a]	0.72	0.04
c	0.096[b]	0.101[b]	0.116[a]	0.113[ab]	0.002	0.049
Potential degradability (a + b)	92.3	90.2	89.7	89.2	0.73	0.21
Effective degradability	82.3	80.4	79.4	79.0	0.60	0.24

[1]CS: control silage; TAS: tannic acid silage; PBS: pistachio by-products silage; PPS: pomegranate pulp silage.
a = rapidly soluble fraction (%); b = slowly degradable fraction (%); c = degradation rate constant (%/h).
SEM: standard error of the mean.
[a, b]: Means in a row with common superscript(s) do not differ (P>0.05).

Table 4. *In situ* disappearance of tannin from alfalfa silages treated with different tannin sources

Time of incubation (h)	Silage[1]					
	CS	TAS	PBS	PPS	SEM	P values[2]
0	nd	75.8	70.1	73.3	2.03	0.22
2	nd	87.5	83.6	87.1	2.73	0.45
4	nd	88.1	88.5	90.2	1.88	0.43
8	nd	96.4	95.4	96.1	1.34	0.87
16	nd	97.8	98.0	98.6	1.58	0.95
24	nd	98.8	98.2	99.2	0.97	0.71
48	nd	99.0	98.4	99.2	0.85	0.97
72	nd	99.4	98.7	99.2	0.77	0.86

[1]CS: control silage; TAS: tannic acid silage; PBS: pistachio by-products silage; PPS: pomegranate pulp silage.
[2]Comparison between treatments was performed for treating silages (TAS, PBS, and PPS).
nd: not detected.
SEM: standard error of the mean.

Discussion

Effect of tannin treatment on chemical composition of alfalfa silage

To the best of our knowledge, this study is the first to use purified tannins derived from PB and PP in animal research. The lower pH values observed in tannin-treated silages are consistent with the findings of Chen et al. (2021a), who reported that alfalfa silage treated with 2% and 5% hydrolyzable tannin (chestnut tannin) on a DM basis had a lower pH compared to control silage. Conversely, Ke et al. (2022) observed that alfalfa silage treated with 4% hydrolyzable tannin had a higher pH than the control silage. The increased lactic acid concentrations in PBS and PPS probably contributed to the lower pH values in these silages. The minimum butyric acid concentration in all treatments indicates high fermentation quality, which is consistent with the criteria proposed by Guo et al. (2024). Wang et al. (2020) similarly reported that the addition of 1% or 2% tannic acid lowered pH and increased lactic and acetic acid concentrations in stylo (*Stylosanthes guianensis*) silage. However, Li et al. (2018) found that although 2% tannic acid had no significant effect on the lactic acid concentration in alfalfa silage, it reduced the butyric acid content after 35 days of ensiling.

Effect of tannin on protein fractionation

The reduction in the A fraction of CP and NH_3-N could be due to the binding of tannins to alfalfa proteins, which inhibits the enzymatic hydrolysis of tannin-protein complexes (He et al., 2020, Ke et al., 2022). This could also be due to the interaction of tannins with proteolytic enzymes (Wang et al., 2020). Similar reductions in NPN and NH_3-N were reported by Jayanegara et al. (2019) and Van den Bossche et al. (2024). Li et al. (2018) demonstrated that the tannic acid supplementation reduced proteolysis in alfalfa silage by inhibiting key enzymatic activities, including acid proteinase, carboxypeptidase and aminopeptidase. A meta-analysis conducted by Jayanegara et al. (2019) revealed that tannins have the ability to limit extensive proteolysis which may occur during ensiling and thus may improve the fermentative quality of silages. The reduction in NPN concentration in the tannin-treated silages was accompanied by a significant increase in the B1 fraction and a numerical increase in the B2 fraction, with no change in the B3 fraction. Our results align with Dentinho et al. (2019), who observed that the inclusion of *Cistus ladanifer* tannin in alfalfa silages the reduced soluble nitrogen with a large increase in true protein content. However, PBS showed a trend towards a higher content of the C fraction compared to the control and TA treatments. The C fraction of CP represents proteins that are damaged by heat (*via* the Maillard reaction), proteins that are complexed with lignin, and proteins that are bound to tannins. This fraction is resistant to degradation in the rumen and is also indigestible in the intestine (Licitra et al., 1996). It is noteworthy that the interaction between tannins and proteins is pH dependent. Tannins bind effectively to proteins at neutral to slightly acidic pH values, but at pH values below 3.5, the tannin-protein complex dissociates and releases the protein (Mueller-Harvey, 2006). This pH sensitivity underscores the role of environmental conditions in influencing tannin-protein interactions and their subsequent effects on protein availability in the rumen.

Effect of tannin on in vitro gas production

The gas production method is commonly used to evaluate the feed fermentation parameters and provides insight into rumen fermentation patterns (Esen, 2023). In agreement with the current results, Chen et al. (2021a, b) reported a decrease in GP after 48 hours of incubation when alfalfa silage was treated with 2% and 5% hydrolyzable tannins from chestnut or different doses of tannic acid (1-6% of DM). Similarly, Mokhtarpour et al (2015) observed that the addition of PB extract reduced the *in vitro* gas volume in alfalfa silage. In the present study, reductions in the gas volume of 11.1%, 14.8% and 18.4% were observed in the TAS, PBS and PPS treatments, respectively. Lagrange et al. (2019) also reported that partial replacement of alfalfa (30–50%) with tannin-rich forages such as sainfoin and birdsfoot trefoil or a mixture of these forages reduced GP. The observed reduction in GP after 24 hours of incubation, together with the decreased OMD in the tannin-treated silages, can be attributed to the inhibitory effects of tannins on microbial activity in the rumen. Furthermore, the reduced gas production from tannin treatment in comparison to the control suggested that the addition of tannin restricted ruminal carbohydrate fermentation, as rumen gases are primarily produced along with VFA formation during carbohydrate digestion (Chen et al., 2021a).

The extent of tannin inhibition of microbial activity varies between *in vitro* and *in vivo* conditions. Bae et al. (1993) found that a concentration of 7 mg of condensed tannins in the ruminal fluid completely inhibited microbial fermentation *in vitro* but slightly reduced the digestibility of DM *in vivo*. This discrepancy could be due to the abundance of substrates available in the rumen for binding the tannins, which are often limited in *in vitro* systems. In addition, Min et al. (2003) suggested that tannin levels need to exceed 5.5% of dietary DM to adversely affect the *in vivo* digestibility.

The GP rate constant (c) was significantly increased in PBS. In contrast to the results of this study, Makkar et al. (1995) reported that purified tannins from various dietary sources decreased the GP rate constant, while the final GP amount was less affected. This discrepancy was attributed to lower microbial attachment to feed particles, suggesting that the threshold at which GP rate is affected is lower than that for digestibility. In addition, purified tannins from the leaves of different plant species showed different GP rates and true digestibility, although they were used at identical tannin concentrations (Makkar, 2003). Consistent with the present results, Mahanani et al. (2020) observed that the incorporation of *Leucaena leucocephala* leaves at 25% of the diet

decreased overall GP while it increased the GP rate constant. While it has been suggested that tannins distribute nutrients to increase microbial production or improve their efficiency *in vitro*, lower GP indicates inhibition of microbial growth and lower feed efficiency (Makkar et al., 1995). Recent studies also demonstrated the inhibitory effects of tannins on microbial attachment and enzymatic activity to plant fibers, resulting in lower fermentation rates and lower GP (Fagundes et al., 2020; Yanza et al., 2021). This mechanism is in line with the increasing emphasis on environmentally sustainable livestock practices, as lower GP indirectly reduces greenhouse gas emissions.

Among the tannin sources evaluated, those derived from PP and PB, which are rich in hydrolyzable tannins, were more effective in reducing GP compared to TA. This greater efficacy may be attributed to the diverse phenolic composition and stronger protein-binding capacity of PP and PB tannins. These findings highlight the potential of tannins from specific plant sources as functional additives to reduce environmental impacts in ruminant production systems.

Effect of tannin on in situ ruminal disappearance

Rumen degradability of crude protein

The CP in the control silage exhibited extensive ruminal degradation, with an effective degradability ($ED_{0.06}$) of 82.3%. Similar findings have been reported by Dentinho et al. (2019) for alfalfa silage harvested at early bloom stage, which showed an effective degradability ($ED_{0.08}$) of 90.5%. The extent of CP degradation in the rumen is influenced by various factors, including the forage type and maturity stage (Tamminga et al., 1991). The increased CP degradation rate observed in the present study may be attributed to the degradation of phenolic compounds by microorganisms that can utilize these compounds as an energy source (Salem et al., 2011).

The reduction in protein solubility following the addition of PB and PP tannins is in agreement with the findings of Tabacco et al. (2006), who reported a decrease in the rapidly fermentable CP fraction (a) and an increase in the slowly degradable fraction (b) upon the inclusion of chestnut tannins (hydrolyzable tannins). A similar trend was observed by Dentinho et al. (2019) in alfalfa silage treated with varying levels of *Cistus ladanifer* condensed tannins. A meta-analysis by Yanza et al. (2021) further confirmed that tannin extract significantly reduced ruminal protein degradability and total tract apparent digestibility. Santos et al. (2000) found no significant difference in the effective degradability of CP between control and TA-treated alfalfa silage, which aligns with the results of the present study. Conversely, Mohamaden et al. (2020) reported no significant effect on ruminal protein degradation when 2 g of hydrolyzable tannin was included in the diet of sheep or when tannin-containing alfalfa varieties (G3 and G9, with 0.89% and 0.76% tannin, respectively) were fed. Interestingly, Mhlongo et al. (2025) found that supplementing compound feed with tannin extract at concentrations of 0.75%, 1.5%, and 3% increased the population of proteolytic bacteria, leading to an enhanced the (a) fraction and greater potential degradability, without affecting the degradation rate of the b fraction. These variations in degradability could be attributed to differences in the biological activity of tannins, which influenced their effectiveness in modulating protein breakdown (Yanza et al., 2021).

Beyond their effects on protein metabolism, tannins play a crucial role in mitigating the environmental impact of livestock production. By reducing ruminal protein degradation and ammonia volatilization, tannins can lower nitrogen excretion and thereby minimize soil and water contamination associated with nitrogen runoff. These findings highlight the potential of tannin-enriched silages as a sustainable strategy for improving nitrogen utilization in ruminants, particularly in regions where feed costs are high, and efficient nitrogen management is essential (Fonseca et al., 2023).

Rumen disappearance of polyphenolic compounds

The high degradation rates of tannins from PB and PP indicate that a significant portion of these tannins are water-soluble. This solubility allows free tannins to influence enzymatic activity in rumen microorganisms and form complexes with macromolecules, potentially reducing GP and digestibility (Battelli et al., 2023). There are few data on the disappearance of tannins in the digestive tract of ruminants. In this study, the average percentage of tannin degradation after 72 hours of rumen incubation was 96.7%, indicating extensive degradation by rumen microorganisms. Perez-Maldonado and Norton (1996) reported that total losses of infused radioactive [^{14}C] condensed tannin in the rumen were 49% in sheep and 42% in goats, while apparent losses of condensed tannin from the diet through the rumen were lower (12% in sheep and 10% in goats). Recently, Rira et al. (2022) found that free condensed tannins disappeared completely after 24 hours of incubation in the rumen in all plants tested. However, to date, there are no published studies that specifically address the disappearance of hydrolyzable tannins in the rumen. The results of this study reflect the disappearance of tannins from the alfalfa silages and not from the ruminal fluid itself and underline the need for further research in this area.

Conclusion

The results of this study showed that the treatment of alfalfa silage with several tannin sources reduced the NPN and OMD. Silages treated with PB and PP tannins showed a significant decrease in GP. Despite significant disappearance of polyphenolic compounds, tannin supplementation decreased protein solubility without affecting potential or effective degradability. The increased protein degradation rate in PBS-treated silage suggests a faster nitrogen release, which may require adequate carbohydrate sources to optimize nitrogen utilization. Overall, PB and PP tannins showed potential

as natural additives to enhance protein preservation and reduced nitrogen losses in alfalfa silage. Further research should optimize tannin selection and concentration for different livestock systems and environmental conditions. Future studies also should explore microbial and enzymatic changes in tannin-rich silages to clarify their impact on feed efficiency, nutrient utilization, and animal performance.

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Authors' contributions

This project was conducted and written by Amir Mokhtarpour. The author read and approved the final manuscript.

Conflict of interest

There is no conflict of interest associated with this publication.

Data availability statement

The data that support the findings of this study are available from the author upon reasonable request.

Ethics approval

Use of the animals was performed according to the guidelines of Iranian Council of Animal Care (1995).

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