

The Effect of Ensiling Alfalfa with Acacia (*Robinia pseudoacacia*) Tree Leaves and Sugar Beet Molasses on Silage Fermentation Quality, In-vitro Ruminal Fermentation and Methane Production

Mahmood Ul Hassan

Ataturk University

Mehmet Gül

mehgul@atauni.edu.tr

Ataturk University

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Abstract

Agroforestry plant leaves notably woody perennials, are widely used to feed animals in many regions of the world, particularly in the tropics. This study also demonstrated a socio-economic and climate viable impact of ensiling alfalfa with agroforestry leaves. Acacia is a fast-growing leguminous tree that produces a huge mass of leaves rich in protein (30.43%) and condensed tannins (12.5%), as a waste which can be utilized as a ruminant feed source. In fact, alfalfa is difficult to ensile because it contains a low level of water-soluble carbohydrates, high buffering capacity, prone to extensive proteolysis and nutrient losses. In this study, supplementation of alfalfa silage (AAS) with acacia leaves and molasses significantly decreased $\text{NH}_3\text{-N}$ production and butyric acid content while increasing the acetic acid and dry matter contents of the silage. In the Hohenheim technique, 5% molasses (M5) increased the net gas production, improved the *in vitro* dry matter degradability and true NDF degradability; meanwhile, reduced the organic matter degradability during daisy incubation of alfalfa silage compared to the control. Moreover, the addition of acacia leaves with three different levels significantly lowered CH_4 production, total VFAs, and ruminal $\text{NH}_3\text{-N}$ (an indicator for N excretion) concentrations in a dose-dependent way. The results illustrated that lower level of acacia leaves (3%) in combination with molasses (5%) applied prior to ensiling alfalfa were more useful in reducing silage pH and ruminal $\text{NH}_3\text{-N}$ ($p < 0.001$), improving the relative feed value and quality ($p < 0.001$), metabolizable and net energy lactation of AAS ($p < 0.001$) without any adverse effect on degradability parameters compared to control.

Introduction

Alfalfa (*Medicago sativa L.*) is a perennial legume that is frequently grown in enterprises (Esen et al., 2024) and is used mainly as silage or haylage for ruminant nutrition. Alfalfa silage is an excellent legume forage because of its good palatability, high protein content (Xu et al., 2020) as well and high yielding (16–20 t/ha DM) capacity (Frame 2005). However, it is difficult to ensilage alfalfa because it contains low levels of water-soluble carbohydrates (WSC) and has a high buffering capacity (Jiang et al., 2020). Therefore, alfalfa silage is prone to extensive proteolysis (Sun et al., 2023) resulting in low protein utilization in ruminants (Soundharrajan et al., 2021). Because of these peculiarities, the inclusion of sugar sources such as molasses (Wang et al., 2020) and condensed tannins (CT) is an option to improve silage fermentation quality and reduce proteolysis. During the ensiling process, molasses (WSC) serves as a fermentable substrate in the early stages of silage fermentation (Silva et al., 2020). Condensed tannins are polyphenolic compounds that can bind proteins and protect them from microbial degradation (Loregian et al., 2023) during the ensilage process. These protein-binding characteristics of tannins suggest their potential as silage additives to limit proteolysis during ensiling (Aguerre et al., 2016; He et al., 2020) and reduce the environmental impact of the feed (e.g., decrease $\text{NH}_3\text{-N}$ or methane emissions during rumen fermentation). Recently, ruminant nutritionists have also focused on natural feed sources containing CT (Vasta et al., 2019) to improve energy and protein utilization. Acacia is a rich source of CT (Lazzari et al., 2023). Acacia or black locust is a fast-growing leguminous tree that is naturalized globally (Nicolescu et al., 2020), and produces a huge mass of leaves as a by-product. Black locust trees can provide ample herbage of approximately 2200–2400 kg DM/ha (Addlestone et al., 1998; Luginbuhl et al., 2000). Consequently, it can be used as an alternative and sustainable feeding source for ruminants or mixed with alfalfa silage to improve forage quality, limit proteolysis, and decrease $\text{NH}_3\text{-N}$ excretion.

Animal feeding based on legume forage diets in ruminant production systems can provide additional advantages, as mentioned above. Legume-based forage encourages more feed intake, elevates animal production (Lee et al., 2004) and alters the ruminal microflora (Lüscher et al., 2014). Moreover, the implementation of legume forage in ruminant diets decreases the amount of energy lost directly owing to a reduction in CH_4 production compared to typical grass-based diets (Lee et al., 2004). The observed phenomenon is caused by different ruminal breakdowns of legumes compared with grass materials. Legume particles contain more cuboidal-shaped fragments that appear to pass through the rumen faster than elongated fragments of grass particles (Kammes et al., 2012). Ruminants are responsible for 16–25% of the methane in global GHG emissions (Chen et al., 2021) introducing legumes such as alfalfa can be a strategy to reduce CH_4 production from the ruminant sector. Additionally, CH_4 emissions from ruminants represent a loss of 2–15% of the gross energy consumed (Eckard et al., 2010). Therefore, it is essential to improve the quality of alfalfa silage and create long-term mitigation plans for nitrogen excretion and CH_4 emissions to increase the forage conversion efficiency.

Several studies have been conducted on the separate inclusion of tannins from different sources or molasses in alfalfa silage to improve fermentation quality and reduce $\text{NH}_3\text{-N}$ excretion and CH_4 production (Coblentz et al., 2013; Chen et al., 2021; Luo et al., 2021; Ke et al., 2022). To the best of our knowledge, the potential of agroforestry tree (acacia) leaves alone or in combination with sugar beet molasses to improve alfalfa silage fermentation quality, ruminal $\text{NH}_3\text{-N}$ excretion, and CH_4 abatement has not yet been explored.

Therefore, this study aimed to explore the effects of molasses and acacia leaves on alfalfa silage fermentation quality, *in vitro* ruminal fermentation, and nutrient degradability and to reduce the environmental impact of livestock production. We hypothesized that the addition of acacia leaves with molasses (WSC) to alfalfa silage may improve silage fermentation quality and nutrient degradability. We also hypothesized that the combination of acacia leaves and molasses would be more potent in limiting extensive proteolysis and reducing $\text{NH}_3\text{-N}$ excretion and CH_4 production without any adverse effects on *in vitro* ruminal fermentation.

Materials and Methods

Plant material collection and experimental design for ensiling of alfalfa

Alfalfa (*Medicago sativa* L.) at 3rd cut and mid-flowering stage was harvested in July 2022 from the Erzincan (39° 44' 47" N and 39° 29' 29" E) region of Turkey. Leaves of acacia trees (*A*; *Robinia pseudoacacia*) were collected from at least 10 trees at the start of June in the Erzurum (39° 54' 31" N and 41° 16' 37" E) region of Turkey. Fresh leaves were air-dried for one week in a laboratory at room temperature. Sugar beet molasses (Brix value 85%) was obtained from a local sugar factory. Fresh alfalfa was chopped (20 mm dimensions) with a manual chopper. After chopping, the alfalfa piles were randomly divided into eight groups: without additive control (C), 5% molasses (M5), 3% acacia leaves (A3), 6% acacia leaves (A6), 12% acacia leaves (A12), and a mixture of acacia leaves and molasses (M5A3, M5A6, and M5A12) ensiled for 90 days in 1 L glass jars. Ensiling was performed such that each trial group had four replications and an average of 700 g of silage in each jar. The glass jars were tightly sealed and maintained at room temperature in an animal feed laboratory.

Determination of silage physical characteristics, pH, ammonia nitrogen concentration and organic acids

After 90 days of ensiling, the jars were opened, and approximately 250 g of silage was stored at -20°C for further analysis. The physical characteristics (odor, color, and structure) of each silage group were determined using a scale recommended by the German Agriculture Organization (DLG-1987). For pH and $\text{NH}_3\text{-N}$ concentration determination, 20 g of silage sample was mixed with 180 ml distilled water, homogenized in a grinder (IKA multidrive control, China) for 2–3 minutes and then filtered with a 4-layer sterile cloth. The pH of the filtrate was measured using a digital pH meter (HI 2211 PH/ORP METER, China). To determine the nitrogen (N) concentration for the quantification of $\text{NH}_3\text{-N}$ concentration, 100 ml of the filtrate was added to a Kjeldahl tube, and then 50 ml of 32% NaOH was added to it and placed in the distillation unit. Fifty milliliters of 2% boric acid were added to the collection chamber. After the procedure, 3–4 drops of the indicator were added and the amount of 0.1 N HCl used during titration was placed in the formula, and the $\text{NH}_3\text{-N}$ concentration was calculated (Preston 1986).

$\text{NH}_3\text{-N}$ (mg/dl) =

$$((\text{HCL}40.114)/((40\text{DM}/100)((\text{CP}/100)((\text{CP}/100)/6.25)))/100$$

For organic acids, 20 g of silage sample was added to 100 ml of distilled water and thoroughly mixed using a grinder (IKA multidrive control, China). Filtration was performed through a 4-layer sterile cloth, and 10 ml of the filtrate was centrifuged at 10,000 rpm for 25 min. Then, 5 ml of the supernatant filtrate was placed in vials. The concentrations of organic acids (lactic acid, acetic acid, propionic acid, and butyric acid) were determined using high-performance liquid chromatography (Shimadzu Nexera XR, CBM-20A, oven temperature 30°C; flow rate 0.35 ml/min; SPD 210 nm, Japan).

Determination of the chemical composition of alfalfa silage and condensed tannins in acacia leaves

Fresh alfalfa, acacia leaves, and silage were dried in a forced-air oven at 65°C for 48 h to estimate dry matter (DM). Then, all samples were ground (1 mm size) using a Wiley mill (ZM200, Retsch GmbH) for nutrient composition analysis (Helrich 1990). Nitrogen (N), ether extract (EE), and ash content were determined using the standard methods of AOAC (Helrich 1990). Nitrogen (N) was analyzed using the Kjeldahl method and then 6.25xN to calculate the crude protein. The EE was determined using diethyl ether in a Soxhlet system. Crude fiber (CF), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were quantified using an Ankom 2000 fiber analyzer (Ankom Technology, Macedon, NY, USA) (Van Soest et al., 1991). The condensed tannin content in the acacia tree leaves was determined using the butanol-HCl method (Makkar et al., 1995).

Determination of in vitro degradability, relative feed value, relative feed quality, metabolizable energy and net energy lactation of silage

In vitro degradability parameters were analyzed using an ANKOM Daisy incubator. Rumen fluid was collected from two Holstein rumen-fistulated cows after morning feeding (60% forage and 40% concentrate). The rumen fluid sample was homogenously mixed and filtered with four layers of gauze into an anaerobically maintained thermos flask for use as an inoculum medium in the daisy and Hohenheim techniques. The pH of rumen fluid was adjusted to 6.35. Each digestion unit of the daisy incubator was filled with 1600 ml of buffer solution and 400 ml of the inoculum medium. The ANKOM fiber bags containing the samples were placed into the digestion units of the incubator and incubated at 39°C for 48 h. After incubation, the incubation medium from the digestion units was poured out, and the bags were washed under running tap water (KILIÇ et al., 2016). An ANKOM 2000 fiber analyzer was used to perform the NDF procedure. True OM degradability, true NDF degradability, DMI, and TDN of the samples were determined based on the difference between the amount incubated initially and the amount after the NDF procedure.

The relative feed value (RFV = $\text{DMD \%} \times \text{DMI \%} \times 0.775$) was calculated as described (Van Dyke 1994); and dry matter degradability ($\text{DMD \%} = 88.9 - (0.779 \times \text{ADF\%})$) and dry matter intake ($\text{DMI \%} = 120/\text{NDF}$) were calculated as described (Güney et al., 2016). The relative feed quality ($\text{RFQ} = (\text{DMI, DM\%}) \times (\text{TDN, DM\%})/1.23$) of the alfalfa silage was calculated as described (Undersander 2003; Ward et al., 2008). The metabolizable energy in MJ/kg DM ($\text{ME} = 2.20 + 0.1357 \times \text{GP} + 0.057 \times \text{CP} + 0.002859 \times \text{EE}^2$) and net energy lactation in MJ/kg DM ($\text{NE}_L = 0.101 \times \text{GP} + 0.051 \times \text{CP} + 0.112 \times \text{EE}$) of the silage were calculated using the equation reported (Menke 1988). Where GP is net gas production (ml/200 mg of feed) at the end of 24 h incubation, CP: crude protein (%), EE: ether extract (%), and CA is crude ash content (%).

Determination of in vitro gas and CH₄ production

In the Hohenheim technique, approximately 500 mg of the silage samples were weighed in glass syringes and then 40 ml of inoculum medium (buffer solution and rumen fluid) was added to 100 ml glass syringes and incubated at 39° °C for 24 h (Menke et al., 1979). To standardize the samples and correct the total gas production with ruminal fluid origin, six glass syringes without feed samples containing only a mixture of buffer solution and ruminal fluid were considered blank. Gas production from the feed samples after 6 and 24 h of incubation was measured. Then, the total gas production from the feed samples was measured after the correction (49.61 mL per 0.2 g/DM) of the estimated gas production, as described by Hohenheim University (Palangi et al., 2021). After 24 h of fermentation, an Infrared Methane Analyzer (Sensors Europe GmbH, Erkrath, Germany) was used to quantify the amount of methane produced (Goel et al., 2008).

Determination of total VFAs and true dry matter degradability

After the 24 h ruminal fermentation in glass syringes, 10 ml of the ruminal fluid was collected and centrifuged at 3000 rpm for 10 min. After centrifugation, 2 ml of the fluid was taken from the upper part, mixed with 2 ml of solution (MgSO_4 + saturated 10 N solution of H_2SO_4) and placed in the Markham Steam Distillation unit. Then, 50 ml of the distillate was collected in a flask, and 1% phenolphthalein as an indicator (1 g phenolphthalein + 60 ml of 99% alcohol + 40 ml of distilled water) was added. After this process, titration was performed with 0.002 N NaOH until a permanent pink color appeared using a digital burette, and the amount of NaOH used was recorded. The same procedures were performed for blind trials. The total VFAs content of each sample was calculated as follows:

Equivalent to 1ml of N/100 NaOH = 10moles of VFA

Total VFAs (mmol/L) = $(A - B)/20.011000$

After 24 h of incubation, the residual contents of the syringes were digested with NDF solution, and the leftovers were collected in crucibles, washed, and dried at 90°C for 16 h. Then, true dry matter degradability ($\text{TDMD (mg)} = \text{Incubated DM (mg)} - \text{Remaining DM (mg)}$), partitioning factor ($\text{PF} = \text{TDMD}/\text{GP ml}$), microbial protein synthesis ($\text{MP} = \text{TDMD} - (\text{GP} \times 2.2 \text{ mg/ml})$), and microbial protein synthesis efficiency ($\text{MPSE} = \text{TDMD} (\text{GP} \times 2.2 \text{ mg/ml})/\text{TDMD}$) were calculated using a previously reported method (Blümmel et al., 1997; Goel et al., 2008) while the truly degraded substrate was calculated using the formula ($\text{TDS} = (\text{TDMD}/\text{Incubated DM}) \times 100$) as described (Goel et al., 2008).

Statistical Analysis

The normality of the distribution of all 45 observed traits was verified using the Shapiro-Wilk normality test (Shapiro et al., 1965) to check whether the assumption of the residuals' normal distribution was followed for the analysis of variance (ANOVA). Bartlett's test

was used to test for the homogeneity of variance. Homogeneity of variance-covariance matrices and multivariate normality were tested using Box's M test. One-way analysis of variance (ANOVA) was carried out to determine the main effects of the group on the variability of the observed 45 traits. Mean values and standard deviations (s.d.) were calculated for each group. Fisher's least significant differences (LSDs) were estimated for individual traits at a 0.05 level of, and homogeneous groups were designated. The relationships between the 45 observed traits were estimated using Pearson's linear correlation coefficient. The relationships between the 45 observed traits are presented in a heat map. Comparisons between particular levels of the analyzed groups were calculated as appropriate contrasts using the two-sample *t*-test for all traits of the study. The results were analyzed using multivariate methods. Canonical variance analysis (CVA) was used to show a multi-trait assessment of the similarity of the tested groups in a lower number of dimensions with the least possible loss of information. The Mahalanobis distances were suggested as a measure of "poly-trait" group differentiation (Bocianowski et al., 2019), the significance of which was verified through critical value *D*_α called "the least significant distance" (Mahalanobis 1936). Statistical analysis was performed using the GenStat 22nd edition (Hempstead 2022).

Results

The nutritional compositions of the raw alfalfa and acacia tree leaves are presented in Table 1. The empirical distribution of the observations for all observed traits was normal. The results of the MANOVA indicated that the groups were significantly (*p* < 0.001) different when investigated jointly in terms of all traits. The ANOVA results indicated that the main effects of the groups were significant for all observed traits, except for silage physical characteristics (smell, color, and structure: Table 3), crude ash (Table 2), and pH (Table 4). In the present study, we used three different levels of dried acacia leaves 3% (CT; 3.75 g/kg of silage), 6% (CT; 7.5 g/kg of silage), and 12% (CT; 15 g/kg of silage).

Table 1
Nutritional composition of raw alfalfa and acacia tree leaves (mean ± standard deviation).

Parameters	Feed	
	Alfalfa	Acacia leaves
Dry Matter (DM) %	94.57 ± 0.18	96.18 ± 1.27
Crude Protein (CP) %	27.07 ± 0.72	30.43 ± 0.97
Neutral Detergent Fiber (NDF) %	57.48 ± 1.50	42.91 ± 1.11
Acid Detergent Fiber (ADF) %	36.86 ± 2.16	23.71 ± 2.78
Crude Fiber (CF) %	33.75 ± 2.65	11.88 ± 0.32
Ether Extract (EE) %	1.26 ± 0.49	1.83 ± 0.06
Crude Ash (CA) %	7.49 ± 0.12	6.45 ± 0.49
Condensed Tannins (CT) %		12.5 ± 0.5
Dry Matter of Fresh Forage	27.1 ± 1.21	32.2 ± 0.65

Table 2

Mean values and standard deviations (s.d.) as well as least significant differences (LSDs) and *F* statistics from one-way analysis of variance (ANOVA) for silage chemical compositions.

Trait	Group	C	M5	A3	A6	A12	M5A3	M5A6	M5A12	LSD _{0.05}	<i>F</i> -ANOVA
CP %	Mean	26.22 ^{bc}	25.57 ^{cd}	25.46 ^{cd}	25.01 ^d	25.41 ^{cd}	26.91 ^{ab}	26.07 ^{bc}	27.59 ^a	1.042	5.83 ^{***}
	s.d.	0.188	0.282	1.339	0.628	0.512	1.070	0.329	0.508		
NDF %	Mean	47.9 ^c	41.35 ^e	52.27 ^b	53.17 ^b	55.75 ^a	43.99 ^d	47.11 ^c	46.55 ^c	2.046	48.28 ^{***}
	s.d.	0.371	1.172	0.532	1.339	0.561	1.484	2.768	1.398		
ADF %	Mean	37.34 ^c	30.14 ^f	40.52 ^a	38.66 ^b	39.44 ^{ab}	31.16 ^{ef}	34.23 ^d	32.35 ^e	1.22	92.41 ^{***}
	s.d.	0.559	0.912	1.114	0.398	0.743	0.863	1.015	0.850		
CF %	Mean	35.85 ^a	28.03 ^c	36.66 ^a	31.7 ^b	31.96 ^b	27.57 ^c	28.93 ^c	25.14 ^d	1.598	55.12 ^{***}
	s.d.	0.422	0.705	2.037	0.624	0.904	0.147	0.566	1.794		
EE %	Mean	2.082 ^d	2.72 ^{ab}	2.39 ^c	2.119 ^d	2.443 ^c	2.441 ^c	2.548 ^{bc}	2.769 ^a	0.21	11.98 ^{***}
	s.d.	0.091	0.126	0.084	0.224	0.183	0.135	0.035	0.177		
CA %	Mean	8.545 ^{ab}	7.049 ^c	8.395 ^{ab}	7.837 ^{abc}	7.468 ^{bc}	8.656 ^a	8.728 ^a	8.263 ^{ab}	1.15	2.38
	s.d.	0.417	1.142	1.794	0.112	0.086	0.261	0.246	0.347		

CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; CF, crude fiber; EE, ether extract; CA, crude ash. *** $p < 0.001$; In rows, mean values marked with the same letter are not statistically different.

Table 3
Alfalfa silage physical (smell, color and structure) and fermentation parameters

Trait Group		C	M5	A3	A6	A12	M5A3	M5A6	M5A12	LSD _{0.05}	F-ANOVA
pH	Mean	5.793 ^c	4.348 ^e	5.97 ^{bc}	6.133 ^a	6.047 ^{ab}	4.357 ^e	4.675 ^d	4.77 ^{cd}	0.172	178.27***
	s.d.	0.061	0.039	0.237	0.139	0.081	0.064	0.125	0.062		
NH ₃ -N (mg/dl)	Mean	7.112 ^a	1.573 ^c	6.89 ^a	3.625 ^b	3.458 ^b	4.41 ^b	3.493 ^b	3.567 ^b	1.287	17.93***
	s.d.	1.423	0.317	0.245	1.048	1.160	0.677	0.864	0.625		
DM %	Mean	23.29 ^e	29.09 ^d	24.89 ^e	29.03 ^d	32.99 ^b	30.59 ^{cd}	31.5b ^c	34.98 ^a	1.943	34.63***
	s.d.	1.248	1.695	1.561	0.532	1.192	1.938	0.618	1.211		
LA %	Mean	90.61 ^a	90.17 ^a	88.4 ^a	89.81 ^a	89.69 ^a	90.44 ^a	89.61 ^a	91.27 ^a	1.825	20.36***
	s.d.	0.574	0.682	2.372	0.878	1.218	1.826	0.435	0.553		
AA %	Mean	3.491 ^e	6.677 ^c	5.032 ^d	5.538 ^d	6.724 ^c	7.866 ^b	7.218b ^c	7.409b ^c	0.783	38.15***
	s.d.	0.299	0.221	1.080	0.593	0.534	0.503	0.318	0.074		
PA %	Mean	1.69 ^{bc}	0.654 ^d	3.511 ^a	1.828b ^c	1.786 ^{bc}	1.291 ^{bcd}	2.074 ^b	1.125 ^{cd}	0.9	7.55***
	s.d.	0.255	0.374	0.750	0.367	0.531	1.183	0.550	0.392		
BA %	Mean	4.211 ^a	2.499 ^b	5.053 ^a	2.82 ^b	1.799 ^c	0.401 ^d	1.099 ^{cd}	0.201 ^d	0.952	28.51***
	s.d.	0.365	1.232	0.669	0.208	0.252	0.803	0.730	0.149		
Smell	Mean	11 ^{ab}	12.25 ^a	8.67 ^b	9.5 ^{ab}	10.25 ^{ab}	10.75 ^{ab}	10.5 ^{ab}	10 ^{ab}	3.137	0.98
	s.d.	1.155	2.062	1.886	3.416	1.708	2.217	2.380	1.633		
Color	Mean	1.25 ^a	1.75 ^a	1.25 ^a	1.25 ^a	1 ^a	1.5 ^a	1.25 ^a	1.25 ^a	1.246	0.27
	s.d.	0.957	0.500	0.957	0.957	0.817	1.000	0.957	0.500		
Structure	Mean	3.25 ^a	3 ^a	2.667 ^a	2.75 ^a	3.25 ^a	3.25 ^a	2.5 ^a	2.75 ^a	1.616	0.29
	s.d.	0.957	1.414	1.247	0.957	0.957	0.957	1.291	0.957		

DM, dry matter; NH₃ -N, ammonia nitrogen concentration; AA, acetic acid; PA, propionic acid; BA, butyric acid; LA, lactic acid. *** $p < 0.001$; In rows, mean values marked with the same letter are not statistically different.

Table 4
In vitro ruminal fermentation characteristics of alfalfa silage

Trait	Group	C	M5	A3	A6	A12	M5A3	M5A6	M5A12	LSD _{0.05}	F-ANOVA
pH	Mean	6.957 ^a	6.95 ^a	6.967 ^a	6.987 ^a	6.97 ^a	6.967 ^a	6.96 ^a	6.97 ^a	0.038	0.71
	s.d.	0.029	0.014	0.025	0.038	0.014	0.017	0.008	0.042		
TVFA _s (mmol/L)	Mean	71.75 ^a	71.47 ^a	63.92 ^c	63.3 ^c	60.7 ^d	67.8 ^b	64.67 ^c	64.22 ^c	2.307	25.35 ^{***}
	s.d.	0.991	0.450	1.559	0.324	0.268	0.521	3.793	1.243		
NH ₃ -N (mg/dl)	Mean	374 ^a	353 ^{ab}	316.3 ^{cd}	315.7 ^{cd}	299.3 ^d	327 ^c	351.7 ^b	309.7 ^{cd}	21.493	12.34 ^{***}
	s.d.	8.160	5.350	3.400	4.030	16.440	3.740	36.310	3.090		
Net gas (ml/200mg)	Mean	25.05 ^f	40.31 ^a	22.68 ^g	27.27 ^e	25.34 ^f	36.9 ^b	33.35 ^c	31.86 ^d	0.943	376.59 ^{***}
	s.d.	1.167	0.756	0.363	0.555	0.000	0.961	0.000	0.210		
CH ₄ (ml/200mg)	Mean	4.852 ^{ef}	7.505 ^a	4.195 ^g	4.993 ^e	4.773 ^f	6.755 ^b	6.238 ^c	5.879 ^d	0.211	245.71 ^{***}
	s.d.	0.237	0.085	0.081	0.186	0.030	0.049	0.223	0.100		
CH ₄ (%)	Mean	19.37 ^a	18.62 ^{bc}	18.5 ^{bc}	18.3 ^c	18.83 ^b	18.32 ^c	18.71 ^{bc}	18.45 ^{bc}	0.509	4 ^{**}
	s.d.	0.092	0.142	0.251	0.313	0.119	0.443	0.670	0.355		

TVFA_s: total volatile fatty acids; NH₃-N: ammonia nitrogen; CH₄: methane. *** $p < 0.001$; In rows, mean values marked with the same letter are not statistically different.

Mean values of *in vitro* dry matter degradability after 48 h varied from 40.06% (for A3) to 59.63% (for M5). True neutral detergent fiber digestibility varied from 50.69% (for A12) to 66.92% (for M5). True organic matter digestibility 48 h varied from 93.91% (for A3) to 94.98% (for control). True dry matter digestibility varied from 230.6 mg (for A12) to 300.9 mg (for M5). Partitioning factor varied from 3.233 mg/ml (for M5A3) to 4.557 mg/ml (for A3). Microbial protein varied from 85.6 mg/g (for M5A3) to 122.7 mg/g (for control). Microbial protein synthesis efficiency varied from 31.89% (for M5A3) to 51.62% (for A3). Truly degradable substrate varied from 50.37% (for A12) to 66.63% (for M5) (Table 5).

Table 5
In vitro dry matter degradability, true NDF degradability and true organic matter degradability parameters after 48 hours of daisy incubation

Trait	Group	C	M5	A3	A6	A12	M5A3	M5A6	M5A12	LSD _{0.05}	F-ANOVA
IVDMD %	Mean	45.02 ^d	59.63 ^a	40.06 ^e	41.5 ^e	42.37 ^e	52.06 ^b	49.7 ^c	48.59 ^c	2.494	58.2 ^{***}
	s.d.	2.781	1.398	1.791	0.837	0.133	1.588	1.246	2.382		
TNDFD %	Mean	57.11 ^c	66.92 ^a	52.66 ^e	53.34 ^d ^e	50.69 ^e	60.55 ^b	58.52 ^{bc}	55.78 ^{cd}	2.786	29.3 ^{***}
	s.d.	3.381	1.583	2.448	0.893	2.530	1.072	0.865	0.358		
TOMD %	Mean	94.98 ^a	94.81 ^{bc}	93.91 ^f	94.36 ^e	93.96 ^f	94.77 ^c	94.86 ^b	94.58 ^d	0.056	456.35 ^{***}
	s.d.	0.017	0.036	0.031	0.030	0.063	0.060	0.028	0.010		
TDMD (mg)	Mean	246.6 ^{de}	300.9 ^a	232.4 ^f	236.5 ^{ef}	230.6 ^f	268.2 ^b	263.3 ^{bc}	254.9 ^{cd}	11.343	36.5 ^{***}
	s.d.	13.104	6.843	10.861	2.710	10.130	4.578	3.962	0.446		
PF (mg/ml)	Mean	4.383 ^a	3.32 ^d	4.557 ^a	3.859 ^b	4.045 ^b	3.233 ^d	3.511 ^{cd}	3.557 ^c	0.21	46.29 ^{***}
	s.d.	0.234	0.107	0.203	0.121	0.178	0.089	0.053	0.029		
MP (mg/g)	Mean	122.7 ^a	101.4 ^b	120.2 ^a	101.6 ^b	105.2 ^b	85.6 ^c	98.3 ^b	97.3 ^b	11.518	9.58 ^{***}
	s.d.	11.579	8.525	10.453	5.411	10.130	5.707	3.962	1.459		
MPSE %	Mean	49.66 ^a	33.66 ^d	51.62 ^a	42.93 ^b	45.5 ^b	31.89 ^d	37.32 ^c	38.15 ^c	2.839	55.78 ^{***}
	s.d.	2.615	2.149	2.226	1.813	2.458	1.840	0.943	0.508		
TDS %	Mean	56.63 ^c	66.63 ^a	52.22 ^e	52.97 ^{de}	50.37 ^e	60.29 ^b	58 ^{bc}	55.35 ^{cd}	2.688	31.82 ^{***}
	s.d.	3.231	1.514	2.432	0.714	2.481	1.110	0.733	0.262		

IVDMD, *in vitro* dry matter degradability after 48 h; TNDFD, true neutral detergent fiber degradability; TOMD, true organic matter degradability after 48 h; TDMD, true dry matter degradability; PF, partitioning factor; MP, microbial protein; MPSE, microbial protein synthesis efficiency; TDS, truly digestible substrate.

*** $p < 0.001$; In rows, mean values marked with the same letter are not statistically different.

Mean values of relative feed value varied from 105.6 (for A12) to 138.1 (for M5). Relative feed quality varied from 111.7 (for A12) to 147.3 (for M5A3). Fatty acids varied from 1.082 (for control) to 1.769 (for M5A12). Non-fibrous carbohydrates varied from 12.83 (for A12) to 26.21 (for M5). Crude protein in NDF varied from 2.895 (for M5) to 3.902 (for A12). Total digestible nutrients varied from 58.65 (for A12) to 69.22 (for M5A3). Dry matter intake varied from 2.342% (for A12) to 2.723% (for M5). Organic matter digestibility after 24 h varied from 38.69% (for control) to 51.45% (for M5). Metabolizable energy varied from 6.744 MJ/kg (for A3) to 9.149 MJ/kg (for M5). Net energy lactation varied from 3.692 MJ/kg (for A3) to 5.39 MJ/kg (for M5). Nitrogen-free NDF varied from 38.46 (for M5) to 51.85 (for A12) (Table 6).

Table 6
Relative feed value, relative feed quality, metabolizable energy and net energy lactation of alfalfa silage

Trait	Group	C	M5	A3	A6	A12	M5A3	M5A6	M5A12	LSD _{0.05}	F-ANOVA
RFV	Mean	115.6 ^d	138.1 ^a	106.5 ^e	108.5 ^e	105.6 ^e	131.2 ^b	121.7 ^c	125.1 ^c	3.978	77.82***
	s.d.	1.054	2.659	1.921	1.583	1.098	3.729	5.024	2.170		
RFQ	Mean	128.5 ^{bc}	147.2 ^a	124.6 ^c	116.9 ^d	111.7 ^e	147.3 ^a	132.8 ^b	132.6 ^b	4.72	62.77***
	s.d.	3.331	3.482	1.567	3.096	1.552	3.982	4.901	2.479		
FA	Mean	1.082 ^d	1.72 ^{ab}	1.39 ^c	1.119 ^d	1.443 ^c	1.441 ^c	1.548 ^{bc}	1.769 ^a	0.21	11.98***
	s.d.	0.091	0.126	0.084	0.224	0.183	0.135	0.035	0.177		
NCF	Mean	18.6 ^{bc}	26.21 ^a	15.15 ^d	15.58 ^d	12.83 ^e	21.08 ^b	18.84 ^{bc}	18.09 ^c	2.501	22.92***
	s.d.	0.223	1.644	1.246	1.360	0.979	2.751	2.553	1.517		
NDFCP	Mean	3.353 ^c	2.895 ^e	3.659 ^b	3.722 ^b	3.902 ^a	3.079 ^d	3.298 ^c	3.259 ^c	0.143	48.28***
	s.d.	0.026	0.082	0.037	0.094	0.039	0.104	0.194	0.098		
TDN	Mean	63.43 ^c	66.48 ^b	63.98 ^c	60.35 ^d	58.65 ^e	69.22 ^a	64.74 ^c	64.4 ^c	1.416	46.32***
	s.d.	1.780	0.484	1.016	1.063	0.997	0.755	0.568	0.296		
DMI %	Mean	2.493 ^a	2.723 ^a	2.396 ^a	2.382 ^a	2.342 ^a	2.618 ^a	2.523 ^a	2.533 ^a	0.06	38.3***
	s.d.	0.010	0.052	0.010	0.023	0.007	0.056	0.073	0.043		
OMD %	Mean	38.69 ^f	51.45 ^a	36.63 ^g	40.45 ^e	38.82 ^f	48.76 ^b	45.71 ^c	44.51 ^d	0.834	344.97***
	s.d.	0.972	0.729	0.361	0.456	0.035	0.877	0.013	0.170		
ME (MJ/kg)	Mean	7.106 ^e	9.149 ^a	6.744 ^f	7.339 ^d	7.104 ^e	8.759 ^b	8.229 ^c	8.118 ^c	0.149	291.48***
	s.d.	0.170	0.115	0.045	0.065	0.031	0.184	0.018	0.004		
NE _L (MJ/kg)	Mean	3.946 ^e	5.39 ^a	3.692 ^f	4.114 ^d	3.946 ^e	5.112 ^b	4.74 ^c	4.657 ^c	0.104	297.61***
	s.d.	0.120	0.081	0.029	0.046	0.021	0.128	0.012	0.002		
NDFn	Mean	44.55 ^c	38.46 ^e	48.61 ^b	49.45 ^b	51.85 ^a	40.91 ^d	43.82 ^c	43.29 ^c	1.903	48.28***
	s.d.	0.345	1.090	0.495	1.245	0.522	1.380	2.574	1.300		

RFV, relative feed value; RFQ, relative feed quality; FA, fatty acids; NCF, non-fibrous carbohydrates; NDFCP, crude protein in NDF; TDN, total digestible nutrients; DMI, dry matter intake; OMD, organic matter degradability after 24 h; ME, metabolizable energy; NE_L, net energy lactation; NDFn, nitrogen-free NDF. *** $p < 0.001$; In rows, mean values marked with the same letter are not statistically different.

In addition to between-group comparisons (Tables 2–6), we conducted a non-orthogonal contrast analysis to compare sets of groups (Tables 7–11). The control was significantly different from the mean of A3, A6 and A12 for all traits except the PA, smell, color and structure. The control was significantly different from the mean of M5A3, M5A6 and M5A12 for all traits except the LA, PA, smell, color and structure. M5 and A3 vs. M5A3 was significant for pH, DM, LA, PA and BA. Mean value of M5 and A6 was different from M5A6 for: pH, DM, AA, PA and BA. A comparison between M5 + A12 and M5A12 showed statistical significance for: pH, DM, AA and BA. Comparisons between A3, A6, A12 vs. M5, A3, A6, A12 vs. M5A3, M5A6, M5A12 as well as M5 vs. M5A3, M5A6, M5A12 showed significant differences for most of the observed traits (Table 7).

Table 7

Comparisons between particular levels of analyzed groups using the two-sample *t*-test for equal means of silage physical (smell, color and structure) parameters, silo acids, pH, dry matter, ammonia nitrogen concentration of alfalfa silage.

Trait	C vs. A3, A6, A12	C vs. M5A3, M5A6, M5A12	M5, A3 vs. M5A3	M5, A6 vs. M5A6	M5, A12 vs. M5A12	A3, A6, A12 vs. M5	A3, A6, A12 vs. M5A3, M5A6, M5A12	M5 vs. M5A3, M5A6, M5A12
pH	-0.24**	1.19***	0.77***	0.57***	0.43***	1.68***	1.43***	-0.25***
NH ₃ -N (mg/dl)	2.46***	3.29***	-0.18	-0.89	-1.05	3.08***	0.83*	-2.25***
DM %	-5.68***	-9.06***	-3.6***	-2.43**	-3.94***	-0.12	-3.38***	-3.26***
LA %	3.31***	0.17	-4.16***	0.38	-1.33	-2.87***	-3.14***	-0.27
AA %	-3.61***	-4.01***	-0.01	-1.11**	-0.71*	0.42	-0.40	-0.82*
PA %	-0.69	0.19	0.79*	-0.83*	0.10	1.72***	0.88**	-0.84*
BA %	0.99*	3.64***	3.37***	1.56***	1.95***	0.72	2.66***	1.93***
Smell	1.50	0.60	-0.30	0.40	1.20	-2.8*	-0.94	1.80
Color	0.08	-0.08	0.00	0.25	0.12	-0.58	-0.17	0.42
Structure	0.36	0.42	-0.42	0.38	0.38	-0.11	0.06	0.17

DM, dry matter; NH₃-N, ammonia nitrogen concentration; AA, acetic acid; PA, propionic acid; BA, butyric acid; LA, lactic acid. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The control was significantly different from the mean of A3, A6 and A12 for all traits except the crude ash. The control was significantly different from the mean of M5A3, M5A6 and M5A12 for all traits except crude protein and crude ash. M5 and A3 vs. M5A3 was significant for dry matter, crude protein, NDF, ADF and crude fibre. Mean value of M5 and A6 was different from M5A6 for dry matter and crude ash. A comparison between M5 + A12 and M5A12 showed statistical significance for all seven silage chemical compositions. Comparisons between A3, A6, A12 vs. M5, A3, A6, A12 vs. M5A3, M5A6, M5A12 as well as M5 vs. M5A3, M5A6, M5A12 showed significant differences for most of the observed traits (Table 8).

Table 8

Comparisons between particular levels of analyzed groups using the two-sample *t*-test for equal means for silage chemical compositions.

Trait	C vs. A3, A6, A12	C vs. M5A3, M5A6, M5A12	M5, A3 vs. M5A3	M5, A6 vs. M5A6	M5, A12 vs. M5A12	A3, A6, A12 vs. M5	A3, A6, A12 vs. M5A3, M5A6, M5A12	M5 vs. M5A3, M5A6, M5A12
Dry matter %	-2.3***	-3.59***	-1.50*	-1.94**	-2.2***	1.18*	-1.29**	-2.47***
Crude protein %	0.93*	-0.63	-1.40**	-0.77	-2.10***	-0.28	-1.56***	-1.29**
NDF %	-5.83***	2.02*	2.82**	0.15	2.00*	12.38***	7.84***	-4.53***
ADF %	-2.20***	4.76***	4.17***	0.17	2.44***	9.39***	6.95***	-2.44***
Crude fiber %	2.41***	8.64***	4.77***	0.94	4.86***	5.41***	6.23***	0.82
Ether extract %	-0.24**	-0.51***	0.11	-0.13	-0.19*	-0.40***	-0.27***	0.13
Crude ash %	0.65	0.00	-0.93	-1.29*	-1.00*	0.85	-0.65	-1.50**

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The control was significantly different from the mean of A3, A6 and A12 for: TVFA, NH₃-N and methane. The control was significantly different from the mean of M5A3, M5A6 and M5A12 for: TVFA, NH₃-N, net gas and methane. M5 and A3 vs. M5A3 was significant for net gas and methane in ml/200mg. Mean value of M5 and A6 was different from M5A6 for TVFA. A comparison between M5 + A12 and M5A12 showed statistical significance for net gas and methane in ml/200mg. Comparisons between A3, A6, A12 vs. M5, A3, A6, A12 vs. M5A3, M5A6, M5A12 as well as M5 vs. M5A3, M5A6, M5A12 showed significant differences for: TVFA, NH₃-N, net gas and methane in ml/200mg (Table 9).

Table 9

Comparisons between particular levels of analyzed groups using the two-sample *t*-test for equal means for pH, total volatile fatty acids, ammonia nitrogen concentration, methane and gas production during *in vitro* experiment.

Trait	C vs. A3, A6, A12	C vs. M5A3, M5A6, M5A12	M5, A3 vs. M5A3	M5, A6 vs. M5A6	M5, A12 vs. M5A12	A3, A6, A12 vs. M5	A3, A6, A12 vs. M5A3, M5A6, M5A12	M5 vs. M5A3, M5A6, M5A12
pH	-0.02	-0.01	-0.01	0.01	-0.01	0.02	0.01	-0.02
TVFA (mmol/L)	9.11***	6.19***	-0.11	2.72**	1.87	-8.83***	-2.92***	5.91***
NH ₃ -N (mg/dl)	63.60***	44.60***	7.70	-17.30	16.50	-42.60***	-19.00**	23.60*
Net gas (ml/200mg)	-0.05	-8.99***	-5.41***	0.44	0.96*	-15.22***	-8.94***	6.27***
CH ₄ (ml/200mg)	0.20*	-1.44***	-0.91***	0.01	0.26**	-2.85***	-1.64***	1.21***
CH ₄ (%)	0.83***	0.88***	0.24	-0.25	0.28	-0.07	0.05	0.13

TVFA_s: total volatile fatty acids; NH₃-N: ammonia nitrogen; CH₄: methane. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The control was significantly larger from the average of A3, A6 and A12 for: IVDMD, TNDFD, TOMD, TDMD, PF, MP, MPSE and TD. The control was significantly different from the average of M5A3, M5A6 and M5A12 for: IVDMD, TOMD, TDMD, PF, MP and MPSE. M5 and A3 vs. M5A3 was significant for IVDMD, TOMD, PF, MP and MPSE. Mean value of M5 and A6 was different smaller from M5A6 for TOMD. A comparison between M5 + A12 and M5A12 showed statistical significance for IVDMD, TNDFD, TOMD, TDMD and TD. Comparisons between A3, A6, A12 vs. M5, A3, A6, A12 vs. M5A3, M5A6, M5A12 as well as M5 vs. M5A3, M5A6, M5A12 showed significant differences for most of the observed traits (Table 10).

Table 10

Comparisons between particular levels of analyzed groups using the two-sample *t*-test for equal means for dry matter degradability, true NDF degradability and true organic matter degradability after 48 hours of daisy incubation, and in vitro degradability parameters from gas production after 24 hours.

Trait	C vs. A3, A6, A12	C vs. M5A3, M5A6, M5A12	M5, A3 vs. M5A3	M5, A6 vs. M5A6	M5, A12 vs. M5A12	A3, A6, A12 vs. M5	A3, A6, A12 vs. M5A3, M5A6, M5A12	M5 vs. M5A3, M5A6, M5A12
IVDMD %	3.71***	-5.09***	-2.20*	0.90	2.40*	-18.31***	-8.80***	9.51***
TNDFD %	4.90***	-1.20	-0.80	1.60	3.00*	-14.70***	-6.05***	8.60***
TOMD %	0.90***	0.24***	-0.42***	-0.28***	-0.20***	-0.73***	-0.66***	0.07**
TDMD (mg)	13.50**	-15.50**	-1.50	5.40	10.80*	-67.70***	-29.00***	38.70***
PF (mg/ml)	0.23*	0.95***	0.71***	0.08	0.13	0.83***	0.72***	-0.11
MP (mg)	13.70**	29.00***	25.20***	3.20	6.00	7.60	15.30***	7.70
MPSE %	3.00*	13.90***	10.70***	1.00	1.40	13.00***	10.90***	-2.10
TD %	4.80***	-1.20	-0.90	1.80	3.10**	-14.80***	-6.03***	8.80***

IVDMD, *in vitro* dry matter degradability after 48 h; TNDFD, true neutral detergent fiber degradability; TOMD, true organic matter degradability after 48 h; TDMD, true dry matter degradability; PF, partitioning factor; MP, microbial protein; MPSE, microbial protein synthesis efficiency; TDS, truly degradable substrate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The control was significantly different from the average of A3, A6 and A12 for: RFV, RFQ, FA, NCF, NDFCP, TDN, DMI and NDFn. The control was significantly different from the average of M5A3, M5A6 and M5A12 for: RFV, RFQ, FA, NDFCP, TDN, DMI, ME, NE_L and NDFn. M5 and A3 vs. M5A3 was significant for: RFV, RFQ, NDFCP, TDN, DMI, ME, NE_L and NDFn. Mean value of M5 and A6 was different smaller from M5A6 for TDN. A comparison between M5 + A12 and M5A12 showed statistical significance for: FA, NDFCP, TDN and NDFn. Comparisons between A3, A6, A12 vs. M5, A3, A6, A12 vs. M5A3, M5A6, M5A12 as well as M5 vs. M5A3, M5A6, M5A12 showed significant differences for most of the observed traits (Table 11).

Table 11

Comparisons between particular levels of analyzed groups using the two-sample *t*-test for equal means for relative feed value, relative feed quality, metabolizable energy and net energy lactation of alfalfa silage.

Trait	C vs. A3, A6, A12	C vs. M5A3, M5A6, M5A12	M5, A3 vs. M5A3	M5, A6 vs. M5A6	M5, A12 vs. M5A12	A3, A6, A12 vs. M5	A3, A6, A12 vs. M5A3, M5A6, M5A12	M5 vs. M5A3, M5A6, M5A12
RFV	8.70***	-10.40***	-8.90***	1.60	-3.20	-31.20***	-19.10***	12.10***
RFQ	10.80***	-9.10***	-11.40***	-0.80	-3.20	-29.50***	-19.90***	9.60***
FA	-0.24**	-0.51***	0.11	-0.13	-0.19*	-0.40***	-0.27***	0.13
NCF	4.08***	-0.73	-0.40	2.10	1.40	-11.68***	-4.81***	6.87***
NDFCP	-0.41***	0.14*	0.20**	0.01	0.14*	0.87***	0.55***	-0.32***
TDN	2.43***	-2.69***	-3.99***	-1.33*	-1.83**	-5.49***	-5.13***	0.36
DMI %	0.12***	-0.07*	-0.06*	0.03	0.00	-0.35***	-0.18***	0.17***
ME (MJ/kg DM)	0.04	-1.26***	-0.81***	0.02	0.01	-2.09***	-1.31***	0.78***
NE _L (MJ/kg DM)	0.03	-0.89***	-0.57***	0.01	0.01	-1.47***	-0.92***	0.55***
NDFn	-5.42***	1.88*	2.62**	0.14	1.86*	11.51***	7.29***	-4.22***

RFV, relative feed value; RFQ, relative feed quality; FA, fatty acids; NCF, non-fibrous carbohydrates; NDFCP, crude protein in NDF; TDN, total digestible nutrients; DMI, dry matter intake; ME, metabolizable energy; NE_L, net energy lactation; NDFn, nitrogen-free NDF. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 12

Mahalanobis distances between groups estimated on the basis of all traits.

Group	C	M5	A3	M5A3	A6	M5A6	A12	M5A12
C	0							
M5	33.46	0						
A3	36.95	32.91	0					
M5A3	58.30	33.60	31.34	0				
A6	51.77	37.28	18.14	18.37	0			
M5A6	51.79	30.73	23.98	8.86	12.34	0		
A12	62.20	46.41	27.87	19.95	10.97	17.08	0	
M5A12	66.44	44.53	38.08	13.87	22.08	16.47	17.69	0

The greatest variation in all the traits, based on the measured Mahalanobis distances, was found for the control and M5A12 (the Mahalanobis distance between them amounted to 66.44). The highest similarity was found between M5A3 and M5A6 (8.86). The Mahalanobis distances for all pairs of groups are listed in Table 12.

Multivariate comparisons

Figure 1 shows the variability of quantitative traits for the groups in terms of the first two canonical variables. In the graph, the coordinates of the points for particular groups are the values for the first and second canonical variables, respectively. The first two canonical variables accounted for 96.81% of the total multivariate variability among individual groups. Significant positive linear relationships with the first canonical variable were observed for butyric acid (0.73), TVFAs (0.78), and ruminal NH₃ (0.80). The first

canonical variable was negatively correlated with silage dry matter (−0.89) and fresh silage dry matter (−0.87). The second canonical variable was significantly positively correlated with smell (0.80), color (0.81), net gas (0.97), and methane (0.98); degradability parameters such as RFV (0.99), NCF (0.91), TDN (0.73), DMI (0.96), RFQ (0.88), OMD (0.97), ME (0.97), and NE_L (0.97); and daisy incubator traits such as IVDMD (0.98), TNDFD (0.91), OMD (0.71), TDMD (0.96), and TDS (0.92). The second canonical variable was significantly negatively correlated with the chemical composition of silage: NDF (−0.93), ADF (−0.98), crude fiber (−0.78), silage quality parameter pH (−0.93), propionic acid (−0.80), two degradability parameters: NDFn (−0.93) and NDFCP (−0.93), and two daisy incubation traits: PF (−0.87) and MPSE (−0.89).

Discussion

Alfalfa silage chemical composition

According to a previous study, the primary factor responsible for silage DM losses was the growth of unfavourable microbes, such as yeast and mold, during the ensiling process; however, the addition of molasses may reduce such losses (Jian et al., 2017). The effectiveness of alfalfa nutrient preservation by the ensiling process was demonstrated in the current investigation because the DM content remained stable or even slightly increased with the added amount of M. The protein content of the raw ingredient for ensiling is conserved at low pH levels, which restricts the proteolytic activity of bacteria (Van Man et al., 2002). In the current study, alfalfa silage supplemented with M5 and a mixture of acacia leaves and molasses (M5A3, M5A6) showed stable CP content, demonstrating the effectiveness of pH decline and protein content preservation. However, in the acacia leaves supplemented groups (A3, A6, and A12), the CP content was also stable, which could be due to the high CP content of the acacia leaves themselves. However, M5A12 showed a slight increase in CP content of the silage. During the ensilage process, a lower pH promotes more acid hydrolysis of plant cells (Ni et al., 2017) resulting in greater NDF reduction. In M5 and M5A3, NDF content was lower than in the control, which is similar to previous studies (Luo et al., 2021). Tannins have been shown to increase the concentration of NDF by binding to fibers (Chen et al., 2021). Acacia leaves are rich sources of condensed tannins. In this study, acacia leaf supplementation in alfalfa silage resulted in a higher NDF content than that of the control.

Alfalfa silage fermentation characteristics

Silage pH is a key factor for good quality fermentation; a lower pH inhibits the proteolytic activity and growth of undesirable microbes (Muck et al., 1994). Previous studies indicated that a pH value of 4.5 or lower indicates good fermentation quality (Hashemzadeh-Cigari et al., 2014). In the present study, M5 and M5A3 effectively reduced the pH of alfalfa after ensiling. Meanwhile, the pH value increased by increasing the level of acacia, such as A6 and A12. However, acacia with molasses addition also reduced the silage pH. The lactic acid bacteria (LAB) fermentation pattern indicated that LA was predominantly formed during homo-fermentation, whereas acetic acid (AA) and lactic acid (LA) were produced during hetero-fermentation (Woolford 1975; Guo et al., 2018). In the present study, the amount of added M5 transformed the alfalfa silage into hetero-fermentation and increased the AA content, which resulted in pH reduction. Thus, the results showed a reduction in BA and PA contents. In the acacia-supplemented groups, AA content was higher, while BA content was lower than that in the control. However, the combination of acacia and molasses significantly increased the AA content but reduced the butyric acid (BA) content in the silage. The LA content did not change in any of the silage groups. The production of propionic acid (PA), which is mostly carried out by *Propionibacterium*, *Clostridium propionicum* and *Selenomonas ruminantium* (Hashemzadeh-Cigari et al., 2014), is inhibited in environments with a pH less than 4.5 (Bjornsdottir et al., 2006). BA is mainly produced by *Clostridium butyrate* and repressed under lower pH conditions. BA is an indicator of poor preservation and is produced during amino acid fermentation, leading to nutritional loss (Szymanowska-Powalowska et al., 2014).

The hetero-fermentation pattern in the silage may be due to the high protein contents of alfalfa (26.2% in DM) and acacia leaves (30.3% in DM) and with added amount of M5 had not effectively turned the silage into homo-fermentation. Thus, LA content of the silage was not affected by M5. In legume silages, the major reason for high levels of soluble nitrogen and NH₃-N is the strong proteolysis caused by *Clostridia* (Kung Jr et al., 2018). The protein-binding properties of tannins have been associated with a reduction in NPN and NH₃-N concentrations during silage fermentation (Cavallarin et al., 2007). The amount of NH₃-N produced in silage is directly associated with proteolytic activity during the ensiling process and inversely correlated with the voluntary intake of silage (Jeyanathan et al., 2014). In this study, the addition of M5, A, and a mix of M5 and A to alfalfa silage significantly reduced BA and NH₃-N. This could be due to lower proteolytic activity (CP consumption) and better preservation of alfalfa silage. The physical characteristics (smell, color, and structure) of the silage were improved by the addition of M5, but the addition had more negative

effects on these characteristics than the control. However, there was no significant difference between the physical characteristics of all treatments and the control.

In vitro total gas and CH₄ production

Tannins are known to limit ruminal CH₄ production either by decreasing the activity of CH₄-producing rumen microbes or by forming stable complexes with carbohydrates such as cellulose or hemicellulose to reduce their digestion (Byeng Ryel Min 2019). In the present study, there was a decline in the percentage of CH₄ production with the addition of acacia leaves alone or mixed with molasses, indicating that CT in acacia leaves inhibited ruminal carbohydrate fermentation. Meanwhile, the results showed that the VFAs proportion was decreased in acacia supplemented alone or mixed with molasses, which was similar to the results of another study. Rumen gases are produced by the breakdown of carbohydrates into volatile fatty acids (VFAs). Tannin (CT and HT) treatments in alfalfa silage were found to reduce total VFAs concentrations, which partially supported this finding (Chen et al., 2021). In the present study, A6, or a mix of acacia and molasses groups, increased the total gas production. However, A3-supplemented silage groups reduced the gas production from the silage was reduced in the silage. M5 supplementation reduced the CH₄ percentage and increased the total gas production from the silage, but did not affect the ruminal NH₃-N and total VFAs compared to the control. The increase in gas production could be due to an increase in WSC content in alfalfa silage. Acetogenic bacteria are also present in the rumen (10^7 – 10^8 cells/g). It can grow heterotrophically by utilizing sugars, or autotrophically by utilizing H₂ and CO₂ (Jeyanathan et al., 2014). *In vitro* studies have also revealed that acetogenesis may be a viable alternative to methanogenesis for ruminal H₂ elimination (Morvan et al., 1996). In the current study, all treatments reduced CH₄ production (%) but increased AA production, which could be due to the acetogenesis pathway that supports our current findings as well.

In vitro ruminal fermentation and nutrient degradability

In vitro ruminal pH, all the treatments illustrated that pH varied between 6.95 and 6.98 which were close to the normal range of pH (5.5–7). In this study, all treatment groups with acacia leaves had decreased ruminal NH₃-N concentrations, which indicates that protein degradation was reduced during rumen fermentation. Acacia leaves were the source of CT (12.5%) in this study. Similarly, supplementation with tannins (Getachew et al., 2008) in *in vitro* and *in vivo* studies (Beauchemin et al., 2007) also confirmed a reduction in ruminal NH₃-N concentration. Tannins can bind to proteins and reduce ruminal protein degradation. Furthermore, the present study showed a reduction in MP and MPSE in all treatment groups compared to the control, which was opposite to previous findings. The extent to which tannins affect fermentation depends on the substrate used, dose, interaction with other compounds and type of tannins (Makkar 2003). Thus, we used acacia leaves as a substrate for CT and analyzed it only; there might be other phytochemicals that divert the current findings from previous studies.

The amount of total VFAs was reduced by CT-containing acacia leaf treatments compared to the control group; therefore, the results were similar to those of previous studies (Dschaak et al., 2011; Byeng Ryel Min 2019). In this study, the decrease in total VFAs resulted in lower *in vitro* dry matter degradability (IVDMD) and true NDF degradability (TNDFD) in acacia leaf-treated silages. However, when acacia leaves were mixed with molasses (M5A3), IVDMD and TNDFD improved but TOMD was reduced. Molasses supplementation, in addition to supplying sufficient substrates for the sharp decline in the pH value, boosts microbial protein synthesis, and improves nutritional quality (Chen et al., 2020). In this study, the amount of M5 added decreased the MPSE while improving the ME, NE_L, RFV, and RFQ of the silage compared to the control. In the acacia leaf-treated groups, IVDMD, TDMD, TNDFD, OMD, RFQ, and RFV were lower than those in the control. Meanwhile, acacia with molasses mix also improved the IVDMD, TDMD, TNDFD, RFV, RFQ, ME, and NE_L but reduced the TOMD compared to the control. In general, the combination of acacia leaves and molasses, that is, M5 supplementation to A3, A6, and A12 (M5A3, M5A6, and M5A12, respectively) resulted in greater variation compared to the use of A3, A6, and A16 in terms of all traits taken together (Fig. 1, Table 12).

Conclusions

The results illustrated that the addition of agroforestry (acacia) leaves with sugar beet molasses in alfalfa silage is more potent in improving silage fermentation quality, reducing ruminal CH₄ production, and NH₃-N concentration in alfalfa silage. These findings support our hypothesis that a combination of acacia and molasses is more efficient. However, the addition of a low level of acacia leaves (A 3%) in combination with M 5% (M5A3) was highly effective in preserving the nutrient content of the silage. Furthermore, M5A3 reduced pH, proteolysis, ruminal CH₄ production, and NH₃-N concentration without any negative impact on the nutrient

degradability of alfalfa silage. Meanwhile, improved nutrient degradability (IVDMD and TNDFD) of the silage was observed. It was concluded that adding a lower level of acacia leaves (3% DM) and molasses (5% DM) could improve alfalfa silage fermentation quality, and reduce the environmental impact of alfalfa silage without any adverse effects on *in vitro* ruminal fermentation. Nonetheless, it is recommended to undertake additional *in vitro* and *in vivo* studies delving into silvopasture aspects, exploring phytochemicals beyond CT, and determining the optimal acacia (*Robinia pseudoacacia* L.) dosage in alfalfa silage.

Declarations

Author contributions

Conceptualization, methodology and validation: Mahmood Ul Hassan. Investigation, data collection and analysis: Mahmood Ul Hassan and Mehmet Gül. Writing—review and editing: Mahmood Ul Hassan. Supervision and project administration: Mehmet Gül. All authors have read and agreed to the published version of the manuscript.

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Ethical approval

The animal study protocol was approved by the Local Ethical Committee of Ataturk University (protocol code E-43216829-000-2200198267) for the collection of rumen fluid from the two experimental cannulated cows.

Conflicts of Interest

The authors declare no conflict of interest.

Data availability

Not Applicable

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Figures

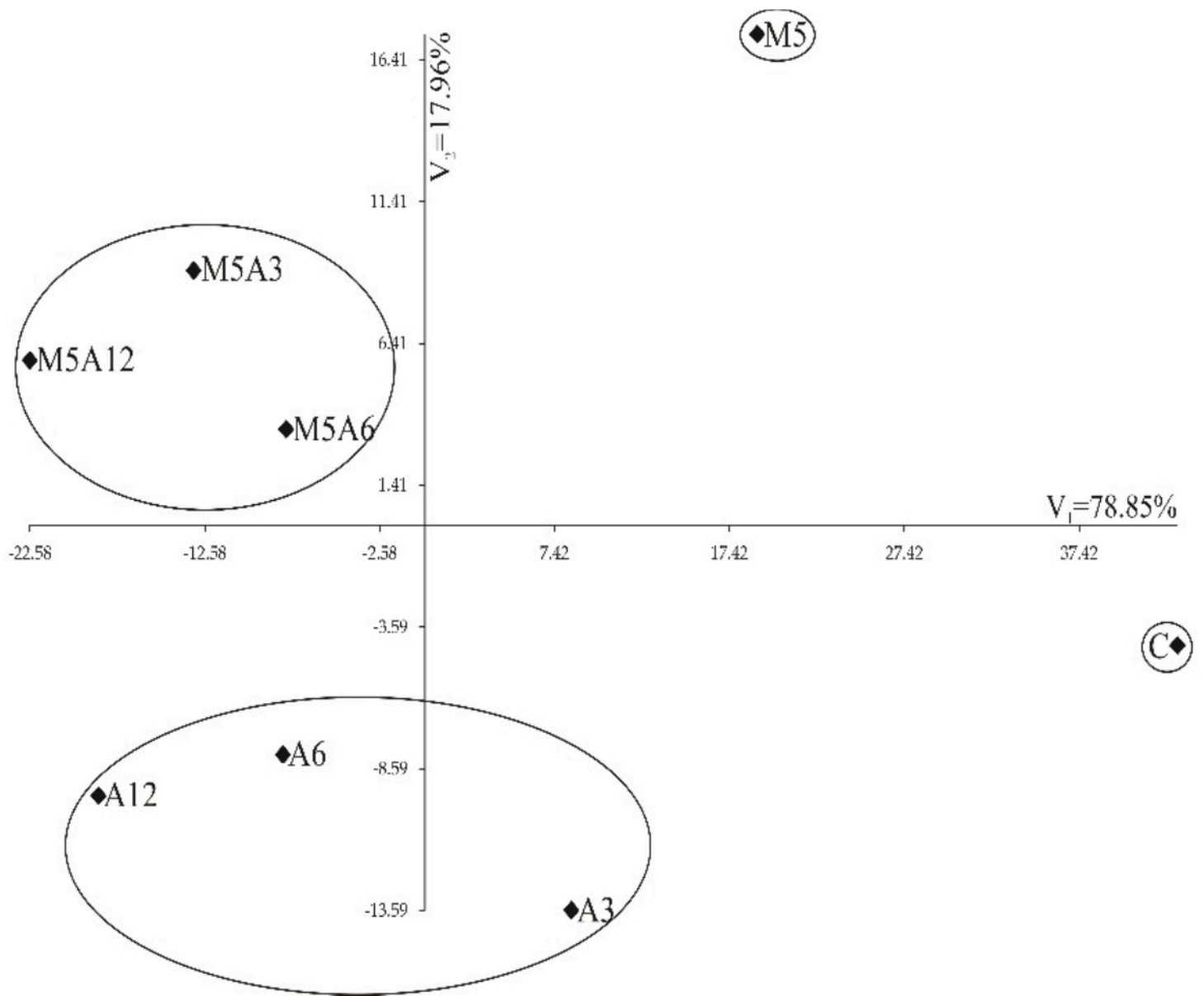


Figure 1

Distribution of groups in the space of the first canonical variable (V_1) and the second canonical variable (V_2).