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The impact of adding hops to alfalfa at different rates on silage quality and methane emissions in vitro

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Abstract: Legumes are typically challenging to ensile due to their low concentration of water-soluble carbohydrates and high capacity for buffering. If legumes and medicinal plants are ensiled together, the silage quality increases, which benefits the digestive system of ruminates. The aim of this study was to research the effects of different alfalfa (*Medicago sativa* L.) and hops (*Humulus lupulus* L.) ratios on silage quality and in vitro rumen fermentation traits. The plant materials were chopped to a particle size of <2 cm; then alfalfa (A) and hops (H) were ensiled in ratios of 100A + 0H, 75A + 25H, 50A + 50H, 25A + 75H and 0A + 100H by weight with 4 replicates. Fresh silage samples were left to ferment for 45 days at 25 ± 2 °C. Subsequently, it was observed that hops added to alfalfa improved the silage's fermentation qualities. At a 75A + 25H ratio, the silage quality increased due to having a lower pH and dry matter ratio as well as increased values for Flieg score, crude protein, relative feed, and condensed tannin concentration. A higher proportion of alfalfa in the silage mixture increased methane (CH_4) and carbon dioxide (CO_2) and decreased lactic acid (LA). Comparing 50A + 50H and 25A + 75H silages to 100A + 0H, the CH_4 production was 26.19% and 27.80% lower, respectively, and the CO_2 production decreased by 25.61% and 28.15%, respectively. Consequently, the 50A + 50H and 25A + 75H silage ratios are advised for feeding ruminants. Furthermore, more research with different forage combinations is required to understand how hops influence rumen fermentation.

Key words: Alfalfa, hops, silage quality, methane

1. Introduction

Livestock activity causes significant greenhouse gas emissions, particularly methane (CH_4) and carbon dioxide (CO_2). Approximately 80% of agricultural CH_4 emissions are caused by animal production, 90% of which is from the intestinal fermentation of ruminant animals, such as cattle and sheep, and 10% is from animal manure (Gerber et al., 2011).

Şahin and Onurbaş Avcıoğlu (2016) reported that CH_4 emissions due to the digestion of ruminants reach 1 million tons per year. Enteric emissions from ruminants can be reduced by increasing the proportion of highly digestible feeds in their daily ration (Vellinga and Hoving, 2011). Gas emissions from the decomposition of animal manure are also an important factor in global warming (Demir and Cevger, 2007). Animal manure produces approximates 9.3 million tons of CH_4 per year, which corresponds to 5% of all CH_4 emissions (Calvet et al., 2007).

Methane and nitrous oxide (N_2O) aid in animal yield and performance. These gasses being released into the atmosphere equals lost energy that does not turn into production (meat, milk, etc.), reducing the yield and quality of animal products. Previous studies have shown that the use of plants containing condensed tannins (CT) in animal nutrition reduces greenhouse gas emissions. On the other hand, CT will divert indigestible nitrogen to feces instead of urine. While nitrogen in the urine is rapidly converted to N_2O and released into the atmosphere, nitrogen in the feces is stored in the soil as organic matter. Johnson and Johnson (1995) and Hristov et al. (1999) revealed that ruminants fed plants lacking bioactive components produce more than 2%–12% CH_4 during anaerobic fermentation in the rumen, corresponding to a 77% gross energy loss in animals.

The intensive use of externally-given antibiotics in modern animal husbandry has raised concerns (Ghimpeţeanu et al., 2022), leading to a greater importance being put on plants with antibiotic-effective content for animal nutrition. Kowalczyk et al. (2013) reported that external antibiotic supplementation in animal nutrition was banned in 2006 by European Union (EU), so plants containing natural antibiotics can be used as an alternative to antibiotics. While plant-based ionophore antibiotics increase feed efficiency, they also reduce excessive

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ammonia (NH_3) and CH_4 production in the rumen (Bergen and Bates, 1983).

Hops (*Humulus lupulus* L.) is a perennial herb from the cannabis family. Hops were first used for their antimicrobial properties, possibly after recognizing that injured animals would rub their wounds on the plant. It has been observed that animals whose birth is approaching are calmer after eating the plant, their birth is easier, and milk yield is increased. In addition, it has been determined that hops have anticonvulsant and hypnotic effects, so animals that eat hops are less restless than other animals. Additionally, hops have attracted attention because they contain ionophore antibiotics, which are important in terms of their potential to reduce NH_3 production (Flythe, 2009; Narvaez et al., 2013; Salfer et al., 2020). Hops are grown for the brewing industry, but only the cones, which make up about 20% of the plant, are used for this purpose. The remaining 80%, consisting of stems and leaves that are generally discarded, can be used as a valuable roughage or silage.

Legumes are typically more challenging to ensile due to their low concentration of water-soluble carbohydrates and high capacity for buffering (Phelan et al., 2015). Alfalfa (*Medicago sativa* L.) is a perennial legume rich in secondary metabolites, such as phenolics, flavonoids, and tannins. Wang et al. (2007) stated that when legumes and medicinal plants are ensiled together, the silage quality increases, the digestive systems of ruminates improve, and in vitro rumen CH_4 emissions decrease. Following from that, the objectives of this study were to improve silage quality by varying the amount of hops added to alfalfa and to determine their efficiency in the rumen, how they affect the rumen fermentation profile, and how they affect CO_2 and CH_4 emissions.

2. Material and methods

In this study, alfalfa (*Medicago sativa* L. cv. Kayseri) and hops (*Humulus lupulus* L. cv. Brewers Gold) were used as plant material. The hops were harvested in August, and the discarded stems and leaves were added to the alfalfa silage at different rates. The alfalfa was 3 years old and harvested in the third cutting time and in a flowering period. The plant materials were chopped in a silage machine to a particle size of <2 cm; then, the alfalfa (A) and hops (H) were ensiled in mixtures of 100A + 0H, 75A + 25H, 50A + 50H, 25A + 75H and 0A + 100H by weight with 4 replicates. All silage samples were left to ferment for 45 days at 25 ± 2 °C. Finally, the fresh samples of alfalfa and hops were dried at 65 °C until reaching a constant weight, and were ground to a particle size of <1 mm.

2.1. Organic acid analysis (%)

A 20-g sample was taken from each silage blend, mixed with 100 mL of distilled water for 5 min using an electric blender, and filtered. The pH value of each silage sample was determined using a digital pH meter. Then butyric, lactic, and acetic acid tests were performed using high-performance liquid chromatography (Shimadzu, Kyoto, Japan) (Uden, 2018; Öztürk et al., 2022).

2.2. Dry matter ratio (%), pH, and Flieg score

The Flieg score, a number calculated using pH and dry matter ratio (DM%), is a useful indicator of silage quantity [Flieg score = 220 + (2 × DM% – 15) – 40 × pH)]. Flieg score values of 81–100 indicate very good silage, 61–80 indicate good, 41–60 indicate medium, 21–40 indicate poor, and 0–20 indicate the poorest silage quality.

2.3. Crude protein analysis (%)

Silage samples were dried at 65 °C until they reached a constant weight; they were then ground to a particle size of 0.5–1.0 mL. The nitrogen (N) contents of the silage samples were determined using the Kjeldahl apparatus. The crude protein content (CP) of the silage samples was calculated by multiplying the N concentration by a factor of 6.25 (Simonne et al., 1996).

2.4. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) analysis (%)

ADF and NDF ratios were determined by using near infrared reflectance spectroscopy (NIRS, Foss 6500) with the IC-0904FE software package. The relative feed value (RFV) was estimated according to the following equations adapted from Rohweder et al. (1978).

Digestibility of DM (DDM, %) = 88.9 – (0.779 × ADF)

DM intake (DMI, %) = 120 / NDF

Total digestible nutrients (TDN, %) = (96.35 – (ADF × 1.15))

Relative feed value (RFV) = (DDM × DMI) / 1.29

2.5. Mineral content analysis (%)

The phosphorus (P) contents of the silage samples were determined using a spectrophotometer following the dry burning method given by Kitson and Mellon (1944). The potassium (K), calcium (Ca), and magnesium (Mg) contents were determined using an atomic absorption spectrophotometer using the wet burning method described by Kacar (1972).

2.6. Crude ash analysis (%)

The crude ash ratio was calculated by burning 2 g of the ground samples in an oven at 550 °C for 4 h and was calculated by dividing the burned samples by the remaining part. (Kacar, 1972).

2.7. Condensed tannin (%)

A 6-mL aliquot of tannin solution was added to 0.01 g of ground sample, placed in a tube, and mixed in a vortex. The tubes were tightly capped and kept at 100 °C for 1 h, at which point the samples were allowed to cool. Then, using a spectrophotometer at an absorbance value of 550 nm (Bate-Smith, 1975), the CT values were calculated using the formula [absorbance (550 nm × 156.5 × dilution factor) / dry weight (%)].

2.8. In vitro gas production

All animal procedures were approved by the Animal Care and Use Committee (decision number 2021-15/03 dated 30.11.2021). To monitor rumen fermentation parameters over time, an in vitro ruminal fermentation trial was carried out. The rumen fluid was obtained from rumen-cannulated 600-kg nonlactating Holstein dairy cows (n = 2). The donor cows were fed daily with a 50/50 mix of corn silage and concentrate. The daily diet was divided into 2 equal meals given at 0800 and 1600. The animals had free access to water and salt blocks. The rumen fluid was withdrawn from the rumen cannula prior to the first feeding each morning. Approximately 1000 mL of rumen contents from the 2 cows were combined in a warm (39°C) thermos flask, immediately transferred to the laboratory, squeezed through cheesecloth, and placed in an Erlenmeyer flask (39 °C). The time between the withdrawal of the rumen content and the laboratory setup did not exceed 35 minutes. The Menke and Steingass (1988) method was used to make a buffer comprising macro and trace elements, a reducing agent, and a resazurin reduction indicator for in vitro rumen fermentation. A 1:2 (v/v) buffer/nutrient solution consisting of 474 mL of purified water, 237 mL of macroelement solution, 0.12 mL of trace element solution, 237 mL of buffer solution, 1.22 mL of resazurin, and 47.5 mL of reducing solution was added under carbon dioxide flush.. Particle-free rumen fluid (15 mL) and buffer medium (25 mL) were mixed in a warm bottle (39 °C) and gassed with CO_2. Glass syringes (Fortuna®, Häberle Labortechnik, Ettlenschieß, Germany) with a calibrated volume of 100 mL were used as incubation vessels. Each syringe contained 0.2 g of dry feed sample and 30 mL of the particle-free rumen fluid (10 mL) and buffer medium (20 mL) mixture. At the 3rd, 6th, 12th, 24th, 48th, 72nd, and 96th hours of incubation, the amount of gas produced in the glass syringes was measured. Three bottles without substrate were used as blanks. Three additional syringes per sample were used to get the CH_4 measurements. After 96 hours of incubation, fermentation was stopped and the rumen fluid samples were centrifuged at 5000 rpm for 15 min. Rumen pH was measured using a calibrated electronic pH meter (Sartorius, Basic PB-20, Göttingen, Germany). The total concentration and profiles of the volatile fatty acids (VFA) in the supernatant were determined using liquid chromatography equipped with a 4 × 250 mm organic acid column (Dionex brand ICS 3000 model device and ICS-VWD model UV detector). After recording the gas production value at 96 h, three additional syringes per sample were used to inject 4 mL of 10 N sodium hydroxide into the plastic hose at the end of the glass syringes. The CH_4 gas (mL) formed was then immediately measured (Fievez et al. 2005).

The values of metabolizable energy (ME), organic matter digestibility (OMD) were determined using the equations from Menke and Steingass (1988), and net energy lactation (NEL) was calculated using the equation from Menke et al. (1979).

$$ME\ (\frac{MJ}{kg}\ DM) = 2.20 + 0.136xGP + 0.057xCP + 0.0028597xCF^2$$

$$OMD\ (\frac{g}{kg}\ DM) = 148.8 + 8.893 \times GP + 0.448 \times CP(mg/g\ DM) + 0.651 \times CA(mg/g\ DM)$$

$$NEL\ (MJ/kg\ DM) = 0{,}101\ x\ GP + 0.051\ x\ CP + 0.112\ x\ CF$$

where GP is net gas production volume at 24 h (mL 200 mg^{-1} DM), and CP, CF, and CA are crude protein, crude fat, and crude ash (g kg^{-1} DM), respectively.

The experiment used a completely randomized design with a 5 × 4 factorial arrangement (5 treatments and 4 duplicates). As an experimental unit, a vacuum-packed bag was used. The experimental data and orthogonal contrast analysis were performed in the Minitab statistical program version 2020 using a general linear model procedure, with the alfalfa proportion in the mixture as a fixed effect and duplicates as a random effect. The least squares mean and standard error of the means were reported per treatment. Duncan's multiple range test was used to separate the means.

2.9. Statistical analysis

The data were analyzed using a complete randomized block design and five ratios of alfalfa to hops (100A + 0H, 75A + 25H, 50A + 50H, 25A + 75H, and 0A + 100H) for each block. A one-way analysis of variance was used to determine the linear and quadratic responses to the proportion of alfalfa inclusion on ensiling traits, chemical composition, and in vitro gas production of the silage mixtures. All statistical analyses were performed using Minitab software (V.16, Minitab Inc., State College, PA, USA). The standard error (SEM) was calculated to examine the linear and quadratic effects of the proportion of alfalfa in the silage mixtures.

3. Results

3.1. Fresh alfalfa and hops chemical traits

The fresh alfalfa and hops chemical traits are given in Table 1. Compared with alfalfa, hops has lower values for CP, CT, K, and P, and higher values for DM, ADF, NDF, Ca, and Mg.

3.2. Silage quality of the alfalfa + hops mixtures

As the proportion of alfalfa decreased in the silage mixtures, the silage DM ratio increased whereas the CP and LA content decreased (Table 2). The DM ratio increased quadratically ($p < 0.01$) and LA decreased linearly and quadratically ($p < 0.01$) as the proportion of alfalfa decreased in silage mixtures (Table 2). A positive associative effect on CP was detected as the proportion of alfalfa in the mixture increased ($p < 0.05$). A negative associative effect on Ca was detected as the proportion of alfalfa increased ($p < 0.01$).

Table 1. Quality traits of fresh alfalfa and hops.

Traits	Alfalfa	Hops
Dry matter ratio (DM%)	28.83	30.02
Crude protein content (CP%)	21.21	16.23
Acid detergent fiber (ADF%)	31.21	32.21
Neutral detergent fiber (NDF%)	40.01	41.43
Condensed tannin (CT%)	1.23	1.18
Potassium (K%)	2.54	2.22
Phosphorus (P%)	0.54	0.40
Calcium (Ca%)	1.24	2.01
Magnesium (Mg%)	0.24	0.61

Table 2. Chemical qualities of the alfalfa + hops silages.

Traits	Treatments (alfalfa + hops)					Significance		
	100 + 0	75 + 25	50 + 50	25 + 75	0 + 100	SEM	Linear	Quadratic
pH	4.95	4.58	4.77	4.98	4.94	0.09	0.193	0.489
DM (%)	29.90^{e}	31.86^{d}	34.37^{c}	36.28^{b}	37.80^{a}	0.77	0.187	<0.001
Flieg score	66.92	85.39	82.93	78.23	83.00	3.93	0.476	0.746
CP (%)	22.23^{a}	20.08^{b}	19.26^{b}	18.83^{bc}	17.21^{c}	0.47	0.431	0.002
RFV	148.39	161.72	139.31	144.46	156.51	3.15	0.955	0.528
LA (%)	2.49^{c}	4.80^{a}	3.16^{b}	2.58^{bc}	2.37^{c}	0.25	<0.001	<0.001
AA (%)	0.09	0.15	0.11	0.22	0.15	0.01	0.767	0.587
BA (%)	0.079	0.065	0.064	0.052	0.038	0.00	0.706	0.065
CT (%)	1.08^{d}	1.55^{a}	1.69^{a}	1.38^{b}	1.23^{c}	0.06	0.001	0.003
K (%)	2.66	2.75	2.34	2.15	2.04	0.08	0. 078	0.206
P (%)	0.55	0.54	0.41	0.40	0.36	0.02	0.133	0.003
Ca (%)	1.27^{d}	1.79^{b}	1.57^{c}	2.06^{a}	2.15^{a}	0.08	0.322	<0.001
Mg (%)	0.26^{d}	0.41^{c}	0.51^{b}	0.56^{ab}	0.60^{a}	0.03	0.605	<0.001

Dry matter (DM); Crude protein (CP); Relative feed value (RFV); Lactic acid (LA); Acetic acid (AA); Butyric acid (BA); Condensed tannin (CT); Potassium (K); Phosphorus (P); Calcium (Ca); Magnesium (Mg); Standard error of the mean (SEM). Means in the same line with different letters differ significantly ($p < 0.05$). The different superscript letters in the table indicate the order of statistical significance, with 'a' being the highest and 'e' the lowest."

3.3. In vitro gas production of alfalfa + hops

A rumen simulation system was used to monitor ruminal fermentation patterns, pH values, in vitro gas production at various hours, quantities of total VFA production, VFA profile composition, CH_4 production, and rumen fluid at 96 h (Table 3). The different ratios of alfalfa to hops had no effect on gas production at 3, 12, 48, or 96 h of incubation ($p > 0.05$); however, the 24-h gas production values of all the combined alfalfa + hops silage mixtures were significantly lower (linear, $p = 0.029$; quadratic, $p = 0.043$) than either the fully alfalfa or fully hops silages. The cumulative gas production (linear, $p = 0.004$; quadratic, $p = 0.006$) and 72-h gas production (linear, $p = 0.027$) of the 100A + 0H silage increased more than for any of the others. Both sole silages (100A + 0H and 0A + 100H) had higher ME (linear, $p = 0.034$; quadratic, $p = 0.048$) and OMD (linear, $p = 0.032$; quadratic, $p = 0.043$) values than the other silage mixtures. The NEL values were not affected by the variations ($p > 0.05$). Alfalfa as the sole silage produced the most CH_4, followed by the 100% hops silage. However, increasing the amount of hops in the silage mixture resulted in a linear decrease ($p < 0.05$)

Table 3. Rumen fermentation characteristics of the alfalfa + hops silages.

Traits	Treatments (alfalfa + hops)					Significance		
	100 + 0	75 + 25	50 + 50	25 + 75	0 + 100	SEM	Linear	Quadratic
6 h	14.75^{a}	14.75^{a}	13.00^{b}	12.50^{b}	13.00^{b}	0.27	0.382	0.596
12 h	26.00^{a}	24.25^{b}	24.50^{b}	23.00^{c}	24.50^{b}	0.27	0.320	0.451
24 h	40.00^{a}	38.75^{ab}	36.00^{c}	37.25^{bc}	40.75^{a}	0.53	0.029	0.043
48 h	46.50^{b}	45.25^{c}	43.75^{d}	54.50^{a}	45.50^{bc}	1.02	0.670	0.258
72 h	70.00^{a}	57.50^{c}	60.25^{bc}	58.25^{c}	62.50^{b}	1.30	0.027	0.058
96 h	71.50	70.75	73.00	71.50	70.50	0.38	0.482	0.767
CG	77.00^{a}	57.25^{c}	55.25^{c}	65.00^{b}	66.00^{b}	2.11	0.004	0.006
ME	7.76^{a}	7.58^{ab}	7.20^{c}	7.37^{bc}	7.83^{a}	0.09	0.034	0.048
NEL	5.72^{a}	5.36^{abc}	5.27^{bc}	5.07^{c}	5.47^{ab}	0.09	0.191	0.381
OMD	51.25^{a}	50.28^{ab}	47.80^{c}	48.82^{bc}	51.98^{a}	0.46	0.032	0.043
CO_2	38.44^{a}	34.49^{b}	28.37^{c}	27.75^{c}	33.32^{b}	1.08	0.332	0.621
CH_4	25.61^{a}	20.64^{c}	19.05^{d}	18.40^{d}	22.57^{b}	0.71	0.100	0.109
pH	6.31^{b}	6.00^{c}	6.06^{c}	5.70^{d}	6.57^{a}	0.08	0.010	0.001
C2	57.90^{a}	45.90^{c}	43.45^{cd}	41.25^{d}	51.15^{b}	1.65	0.026	0.094
C3	8.71^{ab}	9.07^{a}	6.64^{c}	6.05^{c}	7.35^{bc}	0.35	0.610	0.837
C4	4.88^{b}	6.19^{a}	3.32^{c}	3.74^{bc}	3.94^{bc}	0.29	0.170	0.012
C2:C3	6.64^{c}	5.06^{e}	6.54^{d}	6.81^{b}	6.95^{a}	0.15	0.064	0.002

Cumulative gas production (CG); Estimated metabolizable energy (ME; MJ kg^{-1}); Net energy lactation (NEL, MJ/kg DM); Organic matter digestibility (OMD; g kg^{-1} DM); Carbon dioxide (CO_2, mL); Methane (CH_4, mL); Molar proportions of acetate (C2, mmol L^{-1}), propionate (C3, mmol L^{-1}), and butyrate (C4, mmol L^{-1}); Ratio of acetate to propionate (C2:C3); Standard error of the mean (SEM). Means in the same line with different letters differ significantly ($p < 0.05$). The different superscript letters in the table indicate the order of statistical significance, with 'a' being the highest and 'd' the lowest."

in CH_4 production. The CO_2 production was unaffected by the treatments ($p > 0.05$). Hops as the sole silage had a significantly higher rumen pH than the other silages (linear, $p = 0.010$; quadratic, $p = 0.001$). Up to 75% of the energy consumed by ruminants is derived from acetate, propionate, and butyrate, which together make up 95% of the total volatile fatty acid (VFA) produced in the rumen (Bergman 1990). The highest concentration of rumen acetate was found in the 100A + 0H silage, followed by the 0A + 100H silage ($p < 0.05$). However, increasing the amount of hops in the mixture linearly ($p < 0.05$) decreased the rumen acetate concentration. The rumen propionate concentration was not affected by ratio variation ($p > 0.05$). The highest concentration of butyrate (quadratic, $p = 0.012$) was found in the 75A + 25H silage mixture. The highest ratio of acetate to propionate (quadratic, $p = 0.002$) was found in both sole silages, while the lowest was found in the 75A + 25H silage mixture.

3.4. Relationship between silage and in vitro gas traits of the alfalfa + hops silage ratios

According to Figure 1, the sole alfalfa silage produced the most CH_4 and CO_2, while the 75A + 25H and 50A + 50H mixtures produced the least. Additionally, the highest CT and LA values were observed in these same two mixtures. These findings show that mixed silages in certain ratios emit less CH_4 and CO_2 than sole silages, implying that this may be a more environmentally friendly application in animal feeding.

3.5. Correlation between silage chemical composition, in vitro rumen fermentation parameters, and CH_4 production

The OMD, CH_4, 24h, CG and C2, and CH_4 were found to be negatively correlated with silage CT concentration ($p < 0.05$ and $p < 0.01$). Cumulative gas production ($r = 0.741$), 24-h gas production values ($r = 0.727$), OMD ($r = 0.729$), rumen acetate ($r = 0.994$), propionate concentration ($r =$

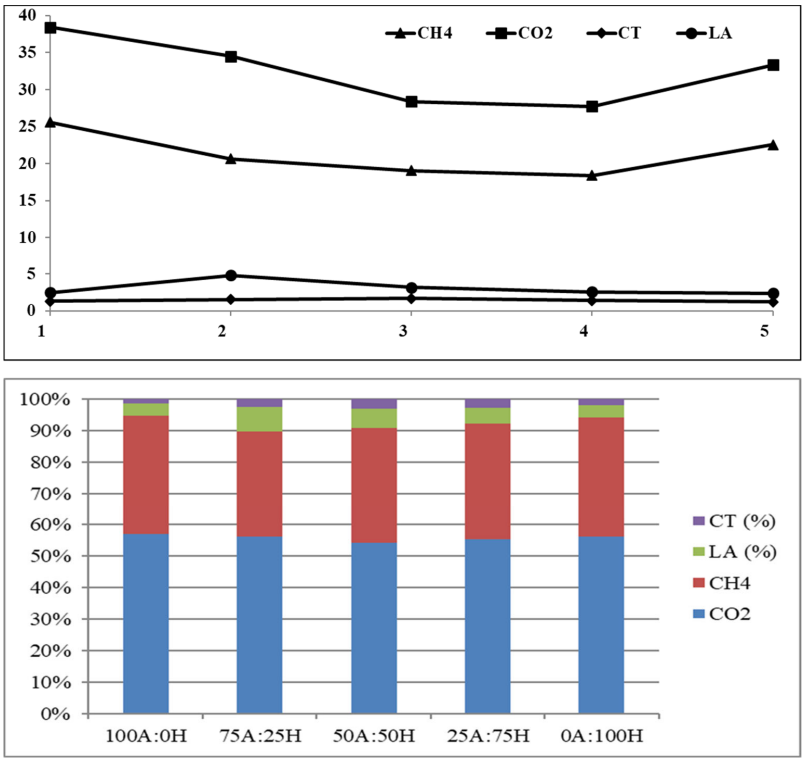

Figure 1. The relationship between alfalfa and hops silage mixture ratio and rumen fermentation gas traits. Alfalfa (A); Hops (H); Methane (CH_4, mL); Carbon dioxide (CO_2, mL), Condensed tannin (CT, %); Lactic acid (LA, %).

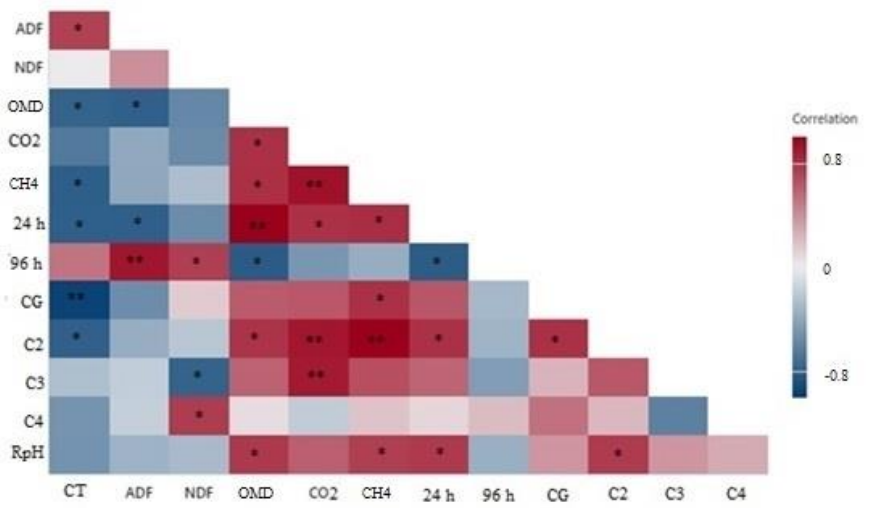

Figure 2. The correlation between silage chemical composition, in vitro rumen fermentation parameters, and CH_4 production. Acid detergent fiber (ADF, %); Neutral detergent fiber (NDF, %); Condensed tannin (CT, %); Rumen pH (RpH); Cumulative gas production (CG); Organic matter digestibility (OMD; g kg^{-1} DM); Molar proportions of acetate (C2), propionate (C3), methane (CH_4, mL), and carbon dioxide (CO_2, mL); Ratio of acetate to propionate (C2:C3).

0.595), and rumen pH (0.739) all had a strong positive correlation ($p < 0.05$ and $p < 0.01$) with in vitro CH_4 yield. Furthermore, it was observed that the acetate to propionate ratio and CH_4 production are dependent on the substrate and ruminal pH ($p < 0.05$ and $p < 0.01$) (Figure 2). In an in vivo study with fistulated ewes, it was suggested that the CT directly inhibit some ruminal gram-positive specialized fibrolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Butyrivrio proteoclasticus*), indicating that tannins may inhibit cellulolytic, proteolytic, or methanogen bacteria. In general, there are significant correlations between enteric CH_4 production and the ratio of rumen acetate to propionate (Russell 1998; Tavendale et al. 2005).

4. Discussion

4.1. Fresh alfalfa + hops chemical traits

The fresh alfalfa CP ratio was 21.21% in current study. Wang et al. (2022) reported the CP of fresh alfalfa to be 22.74%. In the current study, the crude protein increased to 22.23% with an increase in the alfalfa ratio in the silage mixture. In this study, the CT of alfalfa was higher than that found by Wang et al. (2022), who stated that the level of CT in alfalfa was 0.88 g kg^{-1}.

In this study, the CP content of fresh hops prior to ensiling was 16.23%. Lavrencic et al. (2013) founded the CP ratio in hops to be 18.4%. This variation in results for the same trait between studies can be attributed to differences in growing conditions, cultivars, and ecology.

4.2. Ensiling and chemical quality of alfalfa, hops, and their mixture

By adding 25% hops to alfalfa silage, the quality of the silage increases due to having a lower pH and DM ratio as well as higher Flieg score, crude protein, relative feed, and condensed tannin concentration values (Table 2). Filya (2001) reported that the pH of quality silage should be 3.7–4.8. In this sense, the silage fermentation was good when the percentage of hops was 25% and 50%, with the pH ranging from 4.58 to 4.77. In this study, the DM of the silage varied between 29.90%–37.80%. Kılıç (1986) indicated that quality silage should contain 30%–35% DM as a high DM content decreases palatability and with a low DM content, most of the carbohydrate may be leached (Çakmak et al., 2013). In this regard, the silage was well preserved at hops percentages of 25% and 50%, with DM content ranging from 31.86% to 34.37%. Öztürk et al. (2020) reported that the DM content of maize, soybean, and hops silage mixtures were between 27.33%–31.67%.

RFV, indicating forage quality, is >151 for top quality , 125–151 for first quality , 103–124 for second quality , 87–102 for third quality, 75–86 for fourth quality, and <75 for fifth quality (Rohweder et al., 1978). The RFV of this study ranged from 139.31 to 161.72 and showed silage between the second and top forage quality standards.

The CT level dropped from 1.23% DM in fresh alfalfa to 1.08% DM in the corresponding silage. Ensiling reduced the amount of extractable CT in the alfalfa, possibly by increasing CT binding to fiber and protein (Scharenberg et al., 2007). Furthermore, Kondo et al. (2004) found that microbial activity during silage fermentation can degrade tannins to low-molecular-weight polyphenols. According to Kumar and Singh (1984), high tannin levels in feeds have a negative impact on protein digestion, microbial activity, and enzyme activity in ruminants. In this regard, tannins up to 2%–3% are beneficial to rumen health (Barry, 1987), and the CT contents of all silages in this study were less than 2%. Furthermore, silages with low levels of CT are valuable for both feeding and reducing carbon emissions (Öztürk et al., 2022).

LA is a desirable organic acid in silage, whereas acetic acid and butyric acid are undesirable (Woolford, 1984). The LA content of silage should be greater than 2.0% (Kılıç, 2006). The highest LA in this study was found in the 75A + 25H (4.80%), followed by the 50A + 50H (3.16%), and was the lowest in the sole silages. The K, P, Ca, and Mg silage contents were between 2.04%–2.75%, 0.36%–0.55%, 1.25%–2.15%, and 0.26%–0.60%, respectively. The mineral nutrients of all silages were found to be adequate for livestock requirements (Kidambi et al., 1993).

4.3. In vitro gas production of the alfalfa + hops silage mixtures

According to the results of this study, the concentration of ruminal acetate increased with the amount of alfalfa in the mixed silage, probably due to the higher protein content (Table 1), creating a more favorable environment in the rumen for cellulolytic bacteria (Wang et al. 2022). On the other hand, increasing the level of hops in the silage mixture decreased rumen acetate concentration but increased the acetate to propionate ratio. This finding was consistent with that of Wang et al. (2010), who claimed that adding hops to diets did improve in vitro ruminal fermentation. In that study, the increase in the level of hops in the diet affected rumen fermentation in terms of a linear increase in gas production, DDM, and total VFA production. Schmidt and Nelson (2006), in contrast to this study, found that adding hop ß-acids (1.25 to 3.75 ppm) to forage and grain substrates decreased the acetate to propionate ratio, gas production, VFA production, and DDM. Schmidt and Nelson (2006) used sole ß-acids and sole forage and grain as substrates in their work, whereas we used hops silage as a substrate, which may be the cause of the discrepancy. Furthermore, we realized that, in this study, ruminal pH was a factor in the decrease of acetate concentration at high hops levels in the silage mixture. This research revealed that low ruminal pH decreased acetate concentration, which in turn reduced CH_4 production (Russell 1998).

It is unknown how supplementing with whole hops reduced ruminal CH_4 production. However, the fact that whole hops reduced methane production from all substrates used and that other fermentation products' reactions to the addition of hops were diet-dependent raises the possibility that whole hops contain substances that specifically inhibit ruminal methanogenesis (Narvaez et al. 2011). In vitro ruminal cellulose digestion can be affected by even minor changes in ruminal pH (Mouriño et al. 2001). In vitro OMD values revealed that a 25A + 75H silage mixture reduced cellulose digestibility when the final pH of the rumen was 5.7, which was lower than the sole silages. The higher OMD of alfalfa and hops as sole silages compared to an alfalfa + hops silage mixture is most likely due to lower ADF concentrations in the sole forages (Table 1 and 2). Our results are in line with earlier research (Niderkorn et al. 2011), which found that silages made from alfalfa and hops had higher OMD values in vitro.

4.4. Relationship between silage and in vitro gas traits of alfalfa + hops silage mixtures

By increasing the proportion of alfalfa in the silage mixture, the amount of CH_4 and CO_2 increased while the amount of LA decreased. The lowest amount of CH_4 and CO_2 were observed in the 50A + 50H and 25A + 75H silage mixtures in this study. It was also observed that variation in mixture did not have an effect on the CT content (Figure 1).

5. Conclusion

The CH_4 yield and rumen fermentation patterns provide information on how ruminal microbes degrade substrates. The mixture of 50A + 50H and 25A + 75H improved silage quality and had the greatest impact on reducing OMD, ruminal pH, acetate, and CH_4 yield compared to sole silages. The mechanism of action is unclear as to how hop silage affects rumen fermentation and methanogenesis. More research with different forage combinations is needed to understand how hops positively affect rumen fermentation.

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Conflict of Interest

The authors declare no conflict of interest.

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