Original Article

Effects of Hambeles (*Myrtus Communis* L.) leaf on in vitro ruminal fermentation parameters, methane production and protozoa counts

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Abstract

Current ruminant nutrition studies are based on improving rumen fermentation to enhance performance and reduce greenhouse gases. For this purpose, the use of herbal-based feed supplements has become widespread. To determine the effects of adding Hambeles (*Myrtus communis* L.) leaf to some roughage on rumen fermentation parameters by in vitro gas production technique. Corn silage, alfalfa hay and wheat straw were used as roughage. Hambeles leaf was added to roughage at the rate of 0%, 3%, 6%, 9% of the dry matter. According to the data obtained, it was determined that adding 6% and 9% Hambeles leaves to corn silage, 3%, 6%, and 9% to alfalfa hay, and 6% to wheat straw reduced methane levels. The addition of Hambeles leaves at a rate of 9% increased the NH_3-N concentration in corn silage, but in contrast, it decreased it in alfalfa hay and wheat straw. Hambeles leaves added to corn silage increased in vitro organic matter digestibility at all addition rates, while an increase was observed at a 3% addition rate in wheat straw. It was determined that Hambeles leaves added to corn silage at all addition rates, and at a 9% addition rate in wheat straw, reduced protozoa count. The addition of Hambeles leaves at 6% and 9% in corn silage and 3% and 9% in alfalfa hay reduced the total volatile fatty acid concentration. However, the addition of Hambeles leaves at 6% to alfalfa hay increased the total volatile fatty acid concentration. In the light of these data, it is concluded that Hambeles leaves may have the potential to increase ruminal fermentation efficiency in different roughage sources and at different addition rates. In the presented study, it was observed that different addition rates of Hambeles leaves in different roughage sources had positive effects on various parameters in ruminal fermentation. Despite these positive effects, it is thought that more in vitro and in vivo studies are needed to determine the appropriate dosage for adding Hambeles leaves.

Keywords: in vitro, methane, *Myrtus communis* L., rumen fermentation

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Introduction

Many of the current ruminant nutrition studies involve manipulation of rumen fermentation to increase productivity and reduce pollutant emissions (e.g. methane or ammonia) (Gislon *et al*., 2020). Methane gas formed in the rumen is excreted through the rectum. As a result, 12% of the metabolized feed energy is lost (Palangi *et al*., 2022). In addition, methane contributes to global warming due to its greenhouse gas effect (Gu *et al*., 2023). Some applications are made to reduce methane formation in rumen and to improve other fermentation parameters. One of these is herbal products added to the ratio. Since the use of chemical additives causes some safety concerns, there is a rising interest in herbal additives (e.g. plant secondary metabolites). Phenolic compounds in the structure of plants have antioxidant and antimicrobial properties. It is stated that these properties are due to saponins, terpenes, tannins and phenylpropanoids (Abdallah *et al*., 2023).

Hambeles (*Myrtus communis* L.) plant used in this study is an evergreen maquis with medicinal and aromatic properties. It is found along the coastlines of Mediterranean countries and in temperate regions of Austria, Middle East and North America (Vega *et al*., 2025). In Turkey, it grows along the coasts of the Mediterranean and Aegean regions (Tüzün-Kis and İkten, 2022). Hambeles is frequently consumed by small ruminants in the region where it grows. There are many studies showing the antioxidant (Yangui *et al*., 2021) and antimicrobial (Abdulqawi and Quadri, 2021) properties of Hambeles plant. According to the studies, it has been proven that Hambeles plant has various phenolic compounds. Caputo *et al*. (2022) identified 59 compounds in the essential oil of Myrtus communis L. leaves in their study. They reported that the main components were myrtilenyl acetate (29.8%), 1,8-cineole (21.9%), α-pinene (14.7%), and linalool (9.1%). In this study conducted in line with the highlighted properties of Hambeles plant; it was predicted that Hambeles leaf may have positive effects on in vitro rumen fermentation parameters.

Materials and Methods

Feed and plant materials: The corn silage was prepared under laboratory conditions. Alfalfa hay and wheat straw were supplied by Van Yuzuncu Yil University Livestock Application and Research Center. Hambeles (*Myrtus communis* L.) leaves were collected during flowering (July 2020) from the gardens in Osmaniye, Turkey. Corn silage and Hambeles leaves were dried in an oven at 65°C for 48 hours, alfalfa hay and wheat straw were dried at 105°C for 12 hours. All examples were ground to pass through a 1.0 mm sieve and kept in closed jars in a dry, cool place.

Rumen fluid: The rumen fluid used in the in vitro incubation of feeds was obtained from a slaughterhouse in Ipekyolu, Van, Turkey. The animal material from which rumen fluid is provided consists of Simmental cattle (450±25 kg live weight) belonging to a farm that applies a roughage-based ration. Rumen fluid taken immediately after the animals were slaughtered was quickly brought to the laboratory in a thermos containing 39±1°C water, maintaining anaerobic conditions. Rumen fluid was filtered through a 4-layer cheese bag. The filtered rumen fluid was mixed with artificial saliva prepared on the day of the experiment in a ratio of 1/2 (rumen fluid/artificial saliva) under anaerobic conditions.

Experimental design: In this study, 12 experimental groups were prepared by adding Hambeles (*Myrtus communis* L.) leaf at 4 different doses (0%, 3%, 6%, 9% of the dry matter) to 3 different roughages (corn silage, alfalfa hay and wheat straw). The eperimental groups were designed as follows; corn silage, corn silage + 3% hambeles, corn silage + 6% hambeles, corn silage + 9% hambeles, alfalfa hay, alfalfa hay + 3% hambeles, alfalfa hay + 6% hambeles, alfalfa hay + 9% hambeles, wheat straw, wheat straw + 3% hambeles, wheat straw + 6% hambeles, wheat straw + 9% hambeles. Each experimental group was set up with 4 replicates. A total of 52 (4 blank) gas production syringes (Model: Fortuna, 100 ml:1, 40 mm capillary tube, boro, amber grad, Poulten & Graf GmbH Wertheim, Germany) were used for in vitro incubation of feeds belonging to the experimental groups.

Determination of chemical compositions of experimental feeds and Hambeles (Myrtus communis L.) leaves: Corn silage and Hambeles leaves were dried at room temperature. Then, corn silage, alfalfa hay, wheat straw and Hambeles leaves were ground to pass through a 1 mm sieve. Dry matter, ash, crude protein and crude fat analyses of all feed samples and Hambeles leaves were determined according to the methods reported by AOAC (2006). ADF (acid detergent fiber) and NDF (neutral detergent fiber) analyses were created according to the methods reported by Van Soest *et al*. (1991). Condensed tannin content of experimental feeds and Hambeles leaves was determined by spectrophotometer according to the method reported by Makkar *et al*. (1995). Nutrient compositions of experimental feeds and Hambeles (*Myrtus communis* L.) leaves are shown in Table 1.

In vitro gas production technique: The in vitro gas production technique reported by Menke *et al*. (1979) was used in the experiment. 200±10 mg DM from each experimental feed was weighed in 4 replicates and placed in in vitro gas production syringes. 30 ml of rumen fluid/buffer solution mixture was added to each syringe under 39±1°C and CO_2 gas. Then, the syringes were incubated in a specially made water bath at 39±1°C for 24 hours. The total gas accumulated in the syringe at the end of the incubation was measured and recorded. Net gas production; it was calculated by determining the difference between the total gas accumulated in the syringes of the experimental groups and the total gas accumulated in the blank syringes. Gas production results are given as ml/200 mg DM. The gas accumulated in in vitro syringes was removed with a plastic syringe and transferred to a computer-aided methane detector (Sensors Analysentechnik GmbH & Co. KG, Berlin, Germany). The methane ratio in total gas was recorded as a percentage. Gas production values were determined according to the method reported by Menke and

Staingass (1988). In vitro organic matter digestibility and metabolizable energy values were determined using the calculations reported by Menke *et al*. (1979).

Table 1 Nutrient compositions of experimental feeds and Hambeles (*Myrtus communis* L.) leaf, %DM.

Nutrients	Corn silage	Alfalfa hay	Wheat straw	Myrtus communis leaf
DM	93,21	91.25	92.99	44.38
Ash	9.16	8.71	12.46	4,30
OM	90.17	90.45	86.60	90.31
CP	7.96	15.67	4.65	22.80
EE	2.46	1.55	0.91	7.98
NDF	58.17	49.47	73.62	48.63
ADF	31.09	38.90	41.95	35.06
NFC*	2.23	2.46	0.84	1.63
CT	0.20	0.36	0.18	1.45

DM: dry matter, OM: organic matter, CP: crude protein, EE: ether extract, NDF: neutral detergent fiber, ADF: acid detergent fiber, NFC: non-fiber carbohydrate, CT: condensed tannin, *: determined by calculation.

Determination of rumen fermentation parameters: The contents in the syringes were transferred to 50 ml beakers and pH values were determined quickly with a pH meter. The rumen fluid separated from the solid particles was portioned into 15 ml centrifuge tubes. Samples were centrifuged at 4500 rpm for 15 minutes for ammonia nitrogen and volatile fatty acid analyses. Ammonia nitrogen concentration was determined by the Markham distillation method (Markham, 1942). Volatile fatty acid concentration was determined by high-pressure liquid chromatography according to the method reported by Quiros *et al*. (2009). The number of protozoa in rumen fluid was determined according to the method reported by Boyne *et al*. (1957).

Statistical analysis: Data was analyzed using SPSS statistical software (Ver.22.0 for windows, SPSS Inc., Chicago, IL, USA). The means and their pooled standard errors were presented. Polynomial contrasts were used to test the linear and quadratic effects of Hambeles. Duncan multiple comparison test was used to compare group means. Significance was declared at $P<0.05$.

Result

The effects of different levels of Hambeles (*Myrtus communis* L.) leaf addition to corn silage, alfalfa hay and wheat straw on the nutrient composition are given in Table 2, Table 3 and Table 4, respectively. Hambeles leaf supplementation caused quadratic increase in corn silage ($P<0.001$) and both linear and quadratic increase in alfalfa hay ($P<0.001$, $P<0.001$) and wheat straw ($P<0.001$, $P=0.005$) in terms of dry matter. The supplementation of hambeles leaves to corn silage increased the crude protein ratio quadratically ($P=0.020$), while it decreased linearly ($P=0.006$) in alfalfa hay. The supplementation of hambeles leaf to corn silage and wheat straw decreased the NDF ratio linearly ($P<0.001$, $P<0.001$), while it decreased the ADF ratio both linearly ($P<0.001$, $P<0.001$) and quadratically ($P<0.001$, $P<0.001$) in both experimental feeds.

The effects of different levels of Hambeles (*Myrtus communis* L.) leaf in addition to corn silage on in vitro rumen fermentation parameters are shown in Table 5. There was no statistical difference between the groups in terms of total gas production, pH, metabolizable energy and acetic acid/propionic acid ratio. The ratio of CH_4 in total gas linearly ($P<0.001$) decreased as the Hambeles leaf level in corn silage increased. The ruminal NH_3-N linearly ($P<0.001$) and quadratically ($P<0.001$) increased with increasing levels of Hambeles leaf. It was determined that Hambeles leaf supplemented to corn silage linearly ($P=0.016$) increased in vitro organic matter digestibility. In terms of the number of protozoa in rumen fluid, linear ($P=0.036$) and quadratic ($P=0.036$) decreases were determined in the groups to which Hambeles leaves were supplemented compared to the control. As the level of Hambeles leaves supplemented to corn silage increased, the concentrations of acetic acid ($P<0.001$), propionic acid ($P<0.001$), butyric acid ($P<0.001$, $P=0.009$) and total volatile fatty acids ($P<0.001$) decreased linearly and quadratically.

The effects of different levels of Hambeles (*Myrtus communis* L.) leaf addition to alfalfa hay on in vitro rumen fermentation parameters are shown in Table 6. There was no statistical difference between the groups in terms of gas production, pH, in vitro organic matter digestibility, metabolizable energy, protozoa count and acetic acid/propionic acid ratio. The ratio of CH_4 in total gas linearly ($P=0.012$) and quadratically ($P=0.014$) decreased as the Hambeles leaf level in alfalfa hay increased. The ruminal NH_3-N linearly ($P=0.001$) decreased with increasing levels of Hambeles leaf. The concentration of acetic acid (quadratic $P<0.001$), propionic acid (quadratic $P=0.029$), buturic acid (linear $P=0.003$, quadratic $P<0.001$) and total volatile fatty acids (quadratic $P<0.001$) was highest in alfalfa hay at 6% Hambeles level. At all other levels, these values decreased compared to the control.

The effects of different levels of Hambeles (*Myrtus communis* L.) leaf in addition to wheat straw on in vitro rumen fermentation parameters are shown in Table 7. There was no statistical difference between the groups in terms of gas production, pH, metabolizable energy propionic acid, total volatile fatty acid and acetic acid/propionic acid ratio. The ratio of CH_4 in total gas linearly ($P=0.003$) and quadratically ($P<0.001$) decreased. The ruminal NH_3-N linearly ($P=0.043$) decreased with increasing levels of Hambeles leaf. It was determined that Hambeles leaf supplemented to wheat straw quadratic ($P=0.073$) increased in vitro organic matter digestibility. The number of protozoa in rumen fluid was highest in wheat straw at 3% Hambeles level. At all other levels, the number of protozoa linearly (P=0.002) decreased compared to the control. The acetic acid concentration quadratically (P=0.003), buturic acid concentration linearly (P=0.004) increased with increasing levels of Hambeles leaf.

Table 2 Nutrient composition of corn silage with different levels of Hambeles (*Myrtus communis* L.) leaf added, DM%.

Nutrients	Hambeles leaf level, %				*P* value		
	0	3	6	9	Treatment	Linear	Quadratic
DM	93.21±0.09[c]	94.40±0.03[a]	93.75±0.14[b]	93.58±0.14[b]	<0.001	0.368	<0.001
Ash	9.16±0.13	8.99±0.29	8.91±0.05	8.84±0.03	0.556	0.182	0.794
CP	7.96±0.37[b]	8.26±0.21[b]	9.30±0.00[a]	7.84±0.42[b]	0.034	0.625	0.020
NDF	58.17±0.30[a]	57.54±0.35[a]	56.12±0.03[b]	54.45±0.24[c]	<0.001	<0.001	0.079
ADF	31.09±0.09[a]	30.55±0.03[b]	29.64±0.12[d]	30.03±0.10[c]	<0.001	<0.001	0.001

[a,b,c]: The difference between means shown with different letters in the same row is significant, P<0.05. DM: dry matter, CP: crude protein, NDF: neutral detergent fiber, ADF: acid detergent fiber.

Table 3 Nutrient composition of alfalfa hay with different levels of Hambeles (*Myrtus communis* L.) leaf added, DM%.

Nutrients	Hambeles leaf level, %				*P* value		
	0	3	6	9	Treatment	Linear	Quadratic
DM	91.25±0.08[c]	92.97±0.08[a]	92.76±0.14[ab]	92.47±0.15[b]	<0.001	<0.001	<0.001
Ash	7.71±0.19[a]	8.65±0.07[a]	8.74±0.02[a]	8.22±0.04[b]	0.024	0.017	0.059
CP	15.67±0.50[a]	14.59±0.20[bc]	15.50±0.14[ab]	13.77±0.17[c]	0.006	0.006	0.295
NDF	49.47±0.86	47.70±1.75	46.99±0.33	47.46±0.21	0.375	0.169	0.293
ADF	38.90±0.10	38.57±1.45	38.56±0.32	39.20±0.59	0.928	0.811	0.558

[a,b,c]: The difference between means shown with different letters in the same row is significant, P<0.05. DM: dry matter, CP: crude protein, NDF: neutral detergent fiber, ADF: acid detergent fiber.

Table 4 Nutrient composition of wheat straw with different levels of Hambeles (*Myrtus communis L.*) leaf added, DM%.

Nutrients	Hambeles leaf level, %				*P* value		
	0	3	6	9	Treatment	Linear	Quadratic
DM	92.99±0.06[b]	93.70±0.15[a]	93.87±0.02[a]	93.95±0.03[a]	<0.001	<0.001	0.005
Ash	12.46±0.36[c]	14.27±0.22[a]	13.32±0.08[b]	12.60±0.03[c]	0.001	0.593	<0.001
CP	4.64±0.51[b]	4.64±0.09[b]	5.59±0.02[a]	4.78±0.03[ab]	0.091	0.280	0.158
NDF	73.62±0.11[a]	70.50±0.06[b]	68.92±0.34[c]	67.98±0.50[c]	<0.001	<0.001	0.008
ADF	41.96±0.20[a]	40.76±0.17[b]	39.25±0.06[c]	39.72±0.13[c]	<0.001	<0.001	<0.001

[a,b,c]: The difference between means shown with different letters in the same row is significant, P<0.05. DM: dry matter, CP: crude protein, NDF: neutral detergent fiber, ADF: acid detergent fiber.

Table 5 Effect of different levels of Hambeles (*Myrtus communis* L.) leaf addition to corn silage on in vitro rumen fermentation parameters.

Parameters	Hambeles leaf level, %				*P* value		
	0	3	6	9	Treatment	Linear	Quadratic
GP, ml/g DM	51.25±0.48	53.00±0.91	53.75±0.95	53.50±1.04	0.226	0.079	0.274
CH_4, %	16.20±0.04[a]	16.37±0.08[a]	15.68±0.06[b]	15.85±0.10[b]	<0.001	<0.001	0.961
pH	6.87±0.01	6.86±0.01	6.87±0.01	6.86±0.01	0.662	0.706	0.549
NH_3-N, mg/ dl	19.83±0.18[b]	20.74±0.60[b]	20.15±0.43[b]	33.50±1.84[a]	<0.001	<0.001	<0.001
IVOMD, % DM	69.42±0.42[b]	71.62±0.56[a]	71.95±0.84[a]	71.87±0.56[a]	0.037	0.016	0.086
ME, MJ/kg DM	9.39±0.22	9.85±0.12	9.77±0.26	9.89±0.14	0.289	0.126	0.396
Protozoa, $\times10^5$	2.41±0.04[a]	2.08±0.03[b]	2.10±0.03[b]	2.13±0.14[b]	0.034	0.036	0.036
AA, mmol/L	41.39±0.29[ab]	42.11±0.14[a]	31.05±0.29[c]	41.16±0.34[b]	<0.001	<0.001	<0.001
PA, mmol/L	15.50±0.23[a]	15.32±0.39[ab]	11.72±0.39[c]	14.47±0.04[b]	<0.001	<0.001	<0.001
BA, mmol/L	7.50±0.31[a]	6.15±0.20[b]	4.84±0.19[c]	4.85±0.12[c]	<0.001	<0.001	0.009
TVFA, mmol/L	64.39±0.35[a]	63.58±0.58[a]	47.62±0.69[c]	60.48±0.26[b]	<0.001	<0.001	<0.001
AA/PA	2.67±0.05	2.75±0.07	2.66±0.09	2.85±0.02	0.216	0.166	0.450

[a,b,c]: The difference between means shown with different letters in the same row is significant, $P<0.05$. GP: gas production, CH_4: methane, NH_3-N: ammonia nitrogen, IVOMD: in vitro organic matter digestibility, DM: dry matter, ME: metabolizable energy, AA: acetic acid, PA: propionic acid, BA: butyric acid, TVFA: total volatile fatty acids.

Table 6 Effect of different levels of Hambeles (*Myrtus communis* L.) leaf addition to alfalfa hay on in vitro rumen fermentation parameters.

Parameters	Hambeles leaf level, %				*P* value		
	0	3	6	9	Treatment	Linear	Quadratic
GP, ml/g DM	42.00±1.29	42.75±0.48	43.00±0.41	43.00±0.71	0.790	0.389	0.895
CH_4, %	17.35±0.31[a]	16.47±0.17[b]	16.35±0.06[b]	16.55±0.12[b]	0.011	0.012	0.014
pH	6.95±0.02	6.99±0.01	6.97±0.01	6.96±0.00	0.310	0.864	0.143
NH_3-N, mg/ dl	30.52±1.52[a]	28.53±2.02[a]	29.34±1.02[a]	21.78±0.43[b]	0.003	0.001	0.066
IVOMD, % DM	63.81±1.12	64.13±0.42	64.84±0.36	63.77±0.63	0.689	0.853	0.340
ME, MJ/kg DM	8.32±0.33	8.58±0.20	8.87±0.06	8.57±0.23	0.441	0.329	0.245
Protozoa, $\times10^5$	1.73±0.07	1.70±0.02	1.78±0.07	1.84±0.02	0.277	0.097	0.371
AA, mmol/L	35.60±0.53[b]	33.00±0.67[c]	41.46±0.27[a]	32.87±0.13[c]	<0.001	0.904	<0.001
PA, mmol/L	8.56±0.22[b]	7.64±0.37[c]	9.74±0.30[a]	7.52±0.14[c]	<0.001	0.405	0.029
BA, mmol/L	4.08±0.12[b]	3.54±0.12[b]	5.21±0.30[a]	2.48±0.16[c]	<0.001	0.003	<0.001
TVFA, mmol/L	48.24±0.55[b]	44.18±0.82[c]	56.40±0.38[a]	42.86±0.22[c]	<0.001	0.132	<0.001
AA/PA	4.17±0.13	4.35±0.24	4.27±0.14	4.38±0.08	0.805	0.476	0.819

[a,b,c]: The difference between means shown with different letters in the same row is significant, $P<0.05$. GP: gas production, CH_4: methane, NH_3-N: ammonia nitrogen, IVOMD: in vitro organic matter digestibility, DM: dry matter, ME: metabolizable energy, AA: acetic acid, PA: propionic acid, BA: butyric acid, TVFA: total volatile fatty acids.

Table 7 Effect of different levels of Hambeles (*Myrtus communis* L.) leaf addition to wheat straw on in vitro rumen fermentation parameters.

Parameters	Hambeles leaf level, %				*P* value		
	0	3	6	9	Treatment	Linear	Quadratic
GP, ml/g DM	38.25±0.25	40.25±1.03	38.00±0.41	39.25±1.25	0.269	0.846	0.665
CH_4, %	14.60±0.04[a]	14.50±0.16[a]	12.83±0.14[b]	14.53±0.02[a]	<0.001	0.003	<0.001
pH	6.92±0.01	6.90±0.01	6.92±0.01	6.93±0.01	0.457	0.517	0.237
NH_3-N, mg/ dl	19.45±0.46[a]	19.03±0.60[ab]	19.49±0.15[a]	17.98±0.12[b]	0.059	0.043	0.184
IVOMD, % DM	58.45±0.21[b]	61.40±0.91[a]	59.61±0.48[ab]	59.55±1.10[ab]	0.104	0.665	0.073
ME, MJ/kg DM	7.82±0.16	7.94±0.14	7.29±0.28	7.59±0.28	0.239	0.211	0.702
Protozoa, $\times 10^5$	1.98±0.02[a]	2.00±0.05[a]	1.87±0.07[ab]	1.72±0.06[b]	0.010	0.002	0.127
AA, mmol/L	32.63±0.55[bc]	33.69±0.21[ab]	34.10±0.44[a]	32.29±0.26[c]	0.019	0.733	0.003
PA, mmol/L	9.26±0.49	8.74±0.08	8.87±0.30	8.56±0.32	0.526	0.212	0.757
BA, mmol/L	3.63±0.17[bc]	3.09±0.29[c]	4.48±0.43[ab]	4.77±0.27[a]	0.008	0.004	0.196
TVFA, mmol/L	45.51±0.19	45.52±0.45	47.45±0.94	45.63±0.58	0.114	0.418	0.157
AA/PA	3.56±0.24	3.86±0.02	3.86±0.10	3.79±0.15	0.480	0.333	0.244

[a,b,c]: The difference between means shown with different letters in the same row is significant, $P<0.05$. GP: gas production, CH_4: methane, NH_3-N: ammonia nitrogen, IVOMD: in vitro organic matter digestibility, DM: dry matter, ME: metabolizable energy, AA: acetic acid, PA: propionic acid, BA: butyric acid, TVFA: total volatile fatty acids.

Discussion

In our study, condensed tannin content of Hambeles leaf was determined as 14.53 g/kg DM (1.45 %DM, Table 1). The obtained value was found to be considerably lower than the condensed tannin content (49 mg/kg DM) of *Myrtus communis* L. used in the study conducted by Ammar *et al*. (2005). It was thought that this difference may be due to the fact that the Hambeles used in the studies grew in different climatic and soil conditions. Tannins, which are polyphenolic compounds, can form complex compounds especially with proteins (Barszcz and Skomial, 2011). Tannins show bactericidal and bacteriostatic effects by binding to enzymes and proteins in microorganism cells. They have antibacterial properties. Therefore, they can suppress methanogenic bacteria (Tavendale *et al*., 2005). However, it is also reported that they affect methane production by having an indirect effect on rumen protozoa. They negatively affect cellulolytic microorganisms and reduce acetic acid production. Thus, they limit the production of carbon dioxide and hydrogen ions needed for methane production (Patra and Saxena, 2009). It has been stated that condensed tannins also indirectly reduce cellulose digestion and thus reduce methane production (Goel and Makkar, 2012).

The anti-methanogenic classification of a feed or additive can be made according to the in vitro methane percentage. The anti-methanogenic potential of feeds based on methane ratio of the gas released through fermentation was classified as low (>%11 and ≤%14), medium (%>6 and <%11) and high (>%0 and <%6) by Lopez *et al*. (2010). In this study, the methane content of Hambeles leaf in the total gas produced in vitro was determined as 11.4% on average. According to the classification of Lopez *et al*. (2010), it was determined that the antimethanogenic effect of Hambeles leaf used in this study was low.

In the study, the difference between the groups in terms of total gas production of Hambeles leaf added to corn silage, alfalfa hay and wheat straw at different doses was not statistically significant (Tables 5–7). However, with the increase in the level of Hambeles leaves, numerical increases in gas production were observed. This status indicates that Hambeles leaves improved the ruminal fermentation. In addition, increased in vitro organic matter digestibility with Hambeles leaf levels also explains the increase in gas production. It has been reported that volatile compounds in the leaves may be the main reason for the increase in gas production (Elghandour *et al*., 2015). It has been noted that there are multiple mechanisms by which essential oils affect gas production; however, their primary mechanism is their antimicrobial properties (Macheboeuf *et al*., 2008). Furthermore, it has been reported that some components in the essential oils of plants, such as monoterpenoids with hydrocarbon and alcohol structures, have low antimicrobial potential and may act as a carbon source for certain rumen microorganisms (Broudiscou *et al*., 2000). In the study conducted by Bettaieb *et al*. (2016); Myrtus communis essential oil was added to ryegrass and concentrate based diet at different doses (0, 5, 10, 20, 40, 80, 120 µL/50 ml). It was recorded that Myrtus communis essential oil added to the ration decreased the total amount of gas at doses ranging from 10 µL to 120 µL. It was reported that this decrease may be due to the antimicrobial properties of phenolic compounds.

In all experimental feeds to which Hambeles leaves were added, a decrease in methane content was determined depending on different supplementation ratios (Tables 5–7). In a study designed based on the tannin in its structure, willow tree (*Salix alba*) leaves were added to wheat straw, alfalfa hay and corn silage at different doses. In this study, which has a similar starting point with our study, it was reported that willow tree leaf decreased the CH_4 ratio in the total gas produced in vitro depending on the dose increase. The reason for this decreases was explained as the tannin in willow tree leaves may have reduced methane gas production by suppressing methanogenic bacteria in the rumen (Oruç and Avcı, 2018). It has been noted that tannin fed to ruminants can reduce methane emission directly through suppression of methanogenic archaea and indirectly through reduced fiber digestion in the rumen (Patra and Saxena, 2011). It has been reported that increasing tannin concentrations (condensed or hydrolyzable) reduce methane production in vitro and in vivo, but reliable and distinguishable effects of tannins can only be seen at levels >20 g/kg DM (Jayanegara *et al*., 2012). Although the available tannin content of the Hambeles leaf used in our study was lower than the mentioned amount, it was thought that it may have reduced methane production by suppressing methanogenic bacteria.

To evaluate the stability and balance of the rumen environment in ruminant animals, rumen pH is measured at values ranging from 5.0 to 7.5 (Kholif, 2024). The administration of Hambeles leaves at different levels did not affect pH in all experimental feeds and pH values were between 6.86 and 6.99 (Tables 5–7). The pH value was within the reference ranges required for optimal microflora growth and nutrient digestion. In a study conducted in vivo and in vitro, the effects of *Myrtus communis l*eaves added at 0.2, 0.4 and 0.6% to the ration consisting of 70/30% concentrate / roughage on rumen fermentation in vitro were investigated. In the study, 0.4% *Myrtus communis l*eaf was determined as the suitable dose. It was recorded that *Myrtus communis l*eaves added to the ration at this dose did not cause statistical difference between the groups in terms of rumen pH value in Arabian sheep (Salehpour *et al*., 2018). Many authors have reported an increase (Ben Salem *et al*., 2000), decrease (Bhatta *et al*., 2007) or no change (Jolazadeh *et al*., 2015) in rumen pH depending on the amount of tannins in the ration.

In the present study, it was determined that ruminal NH_3-N concentration increased linearly and quadratically with the increase in the dose of Hambeles leaf supplemented to corn silage, on the contrary, there were linear decreases in alfalfa hay and wheat straw groups (Tables 5–7). The increase in ruminal ammonia in the corn silage groups was associated with an increase in vitro organic matter digestibility. In the study conducted by Correddu *et al*. (2019); it was recorded that ruminal NH_3-N concentration decreased in the groups given Hambeles fruit (50 and 100 g/day/animal) compared to the control. This reduction has been attributed to the ability of polyphenols to affect the growth of rumen proteolytic bacteria, either directly by reducing the activity of protease enzymes or indirectly through their ability to bind proteins. It is reported that tannins, which are found in the structure of many plants, decrease the rate of protein degradation in the rumen and increase the by-pass protein rate when used at suitable doses (Carulla *et al*., 2005).

In vitro organic matter digestibility of corn silage increased linearly with increasing dose of Hambeles, while the difference between the groups was not significant in alfalfa hay (Tables 5, 6). In vitro organic matter digestibility of wheat straw showed a quadratic increase with the increase in Hambeles dose, but the significance was higher than 0.05 (Table 7). Plant phytochemicals have been reported to stimulate fibrolytic microbial activities in the rumen (Morgavi *et al*., 2000), which increases rumen fermentation and substrate degradability (Kholif *et al*., 2021). It has been reported that rumen microbiota can utilize plant phytochemicals (e.g. phenolics and essential oils) as energy sources for their growth and activities (Kholif *et al*., 2021). Önel *et al*. (2021) noted that Myrtus communis essential oil added to alfalfa hay decreased in vitro organic matter digestibility compared to the control. It has been reported that appropriate doses of essential oils can be beneficial for fibrolytic bacterial activity that enhances the degradation and fermentation of substrates (Kholif *et al*., 2017), whereas high concentrations of essential oils potentially inhibit the growth of cellulolytic bacteria and reduce the degradability of feedstuffs (Agarwal *et al*., 2009; Kumar *et al*., 2022). In the study conducted by Kılınç (2021), it was reported that the addition of different doses of *Myrtus communis l*eaf to wheat straw did not create a statistical difference between the groups in terms of in vitro organic matter digestibility. In the present study, it is thought that the results in terms of in vitro digestibility may be due to the different digestibility rates of the forages used in the experiment or the phytochemicals in the structure of the supplemented Hambeles leaf.

The count of ruminal protozoa in corn silage and wheat straw groups decreased with the increase in Hambeles leaf dose, while the difference between alfalfa hay groups was insignificant (Tables 5–7). It has been reported that there may be differences in the anti-methanogenic and antiprotozoal effects of plant species and extracts due to structural and dose differences (Kamra *et al*., 2006). It is thought that the phenolic compounds in the Hambeles leaf caused the results in terms of the number of ruminal protozoa as a result of fermentation of different roughages used in our study. Salehpour *et al*. (2018) reported that *Myrtus communis l*eaves added at a dose of 0.4% to the rations of Arabian sheep fed with 70/30% concentrate/coarse feed did not create a statistical difference between the groups in terms of total protozoa count. Önel *et al*. (2021) reported that Myrtus communis essential oil added to alfalfa hay increased rumen protozoa count compared to the control group.

Hambeles leaf supplemented to corn silage caused a general decrease in all volatile fatty acid concentrations (Table 5). Hambeles leaf supplemented to alfalfa hay decreased the concentrations of all volatile fatty acids at 3% and 9% and increased them to 6% (Table 6). In the study, Hambeles leaf increased in vitro organic matter digestibility in three forage sources. In parallel with this increase, volatile fatty acids concentrations are also expected to increase. However, there was a general decrease in volatile fatty acids concentrations. It has been reported that the reason for the increase in digestibility against the decrease in volatile fatty acids concentration may be related to the production of microbial extracellular enzymes that increase dry matter and fiber digestibility (Priest, 1977), and that hydrolysis products may not be fermented into volatile fatty acids. Önel *et al*. (2021) reported that Myrtus communis essential oil added to alfalfa hay decreased all VFA values compared to the control.

According to the data obtained from this study, Hambeles leaf supplemented to the roughages used in the experiment caused statistical differences in many rumen parameters. The main prediction of the study was that the tannin in the structure of Hambeles leaf supplemented to roughages suppresses methane formation in the rumen without adversely affecting other rumen parameters. It was generally observed that Hambeles leaf suppressed methane and increased vitro organic matter digestibility in three different forages. Overall, the findings of the present study underline the potential of Hambeles leaf to improve ruminal fermentation efficiency. Additional research should focus on studying the in vivo effect of Hambeles leaf administration to further elucidate its impact on methane production and dietary digestibility.

***Conflict of interest*:** The authors declare that there is no conflict of interest.

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