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## Comparison of in vitro ruminal fermentation variables of Pennyroyal (*Mentha pulegium* L.) Herbage with Traditional Culture Forages

### Abstract

The aim of this study is to evaluate the effects of on total gas production, methane production, in vitro metabolic energy (ME) and the individually short chain fatty acids (SCFA) of the in vitro fermentation fluid of pennyroyal (*Mentha pulegium* L.), an aromatic forage, and sainfoin, and conventional forages (lucerne and corn silage). In vitro gas production was carried out in accordance with ruminal fermentation, and the total amount of gas produced at the end of 24 hours of incubation was recorded. The methane volume (ml) in the in vitro total gas methane was measured using an infrared gas analyser the fermentation fluid was analysed for straight short-chain fatty acids (SCFA) (acetic, butyric, and propionic acids) and branched short-chain fatty acids (BSCFA). In the present study, the in vitro total gas production of sainfoin was higher than that of pennyroyal ( $P>0.05$ ). CH<sub>4</sub> (ml/0.2g) production (6.09 ml/0.2g) of lucerne was higher than that of other forages. CH<sub>4</sub> production (4.02 ml/0.2g) of corn silage was lower than that of other forages. ME (MJ/kg) values of sainfoin and lucerne were similar, and corn silage was lower than the others. The acetic acid (AA) concentration of in vitro rumen fermentation fluid for pennyroyal herbage did not differ from that of lucerne herbage and corn silage ( $P>0.05$ ), it was lower than that of sainfoin herbage ( $P<0.05$ ). The propionic acid (PA) concentration of in vitro rumen fermentation fluid for pennyroyal herbage was lower than that of other forages ( $P<0.05$ ). The butyric acid (BA) concentration in the rumen fermentation was higher in lucerne and sainfoin than pennyroyal and corn silage. This study reveals that pennyroyal exhibits a distinct fermentation profile compared to conventional forages, characterized by similar AA levels but lower PA and methane production during in vitro rumen fermentation.

**Keywords:** Aromatic forage, in vitro gas production, methane, pennyroyal, ruminal fermentation



## Yarpuz (*Mentha pulegium* L.) Kuru Otunun Geleneksel Kültür Kaba Yemler ile in vitro Rumen Fermentasyon Değişkenlerinin Karşılaştırılması

### Öz

Bu çalışmanın amacı, aromatik bir yem olan pennyroyal (*Mentha pulegium* L.), korunga ve geleneksel yem bitkilerinin (yonca ve mısır silajı) in vitro fermentasyon sıvısının toplam gaz üretimi, metan üretimi, in vitro metabolik enerji (ME) ve bireysel kısa zincirli yağ asitlerinin etkilerini değerlendirmektir. Ruminal fermentasyona uygun olarak in vitro gaz üretimi gerçekleştirilmiş ve 24 saatlik inkübasyon sonunda üretilen toplam gaz miktarı kaydedilmiştir. Kızılötesi gaz analiz cihazı ile in vitro toplam gaz metanındaki metan hacmi (ml) tespit edildi. Fermentasyon sıvısı, düz kısa zincirli yağ asitleri (SCFA; asetik, bütirik ve propiyonik asitler) ve dallanmış kısa zincirli yağ asitleri (BSCFA)

açısından analiz edildi. Bu çalışmada korunganın in vitro toplam gaz üretimi yarpuzdan daha yüksek bulunmuştur ( $P>0.05$ ). Yoncanın  $CH_4$  (ml/0.2 g KM) üretimi (6.09 ml/0.2 g KM) diğer kaba yemlerden daha yüksekti. Mısır silajının  $CH_4$  üretimi (4.02 ml/0.2 g KM) diğer kaba yemlere göre daha düşüktü. Korunga ve yoncanın ME(MJ/kg) değeri benzer olup mısır silajı diğerlerine göre daha düşüktü. Yarpuz kuru otu için in vitro rumen fermentasyon sıvısının asetik asit (AA) konsantrasyonu, yonca otu ve mısır silajına benzer ( $P>0.05$ ), korunga otundan daha düşük ( $P<0.05$ ) idi. Yarpuz kuru otu için in vitro rumen fermentasyon sıvısının propiyonik asit (PA) konsantrasyonu diğer kaba yemlerden daha düşüktü ( $P<0.05$ ). Rumen fermentasyonunda bütirik asit (BA) konsantrasyonu yonca ve korungada yarpuz ve mısır silajına göre daha yüksekti. Bu çalışma, yarpuz otunun in vitro rumen fermentasyonu sırasında benzer asetik asit düzeyleriyle birlikte düşük PA ve metan üretimi sergileyerek, geleneksel yem bitkilerine kıyasla farklı bir fermentasyon profiline sahip olduğunu ortaya koymaktadır.

**Anahtar Kelimeler:** Aromatik kaba yem, in vitro gaz üretimi, metan, yarpuz, rumen fermentasyonu



## Introduction

Herbage has a fundamental role in the nutrition of ruminant animals and is an important factor in increasing the healthy growth, development, and productivity of animals. Good quality herbage provides the necessary conditions for a healthy process of providing the necessary energy, protein, fiber, and other dietary requirements in animals. The use of natural and herbal alternatives in the animal nutrition industry has become an important research area that supports animal health and productivity (Ersahince and Kara, 2017; Kara et al., 2018; Kara, 2021). *Mentha pulegium*, whose digestibility varies according to the phenological period, easily adapts to the climatic conditions in terms of sustainability. This supports the efficient cultivation of the *Mentha pulegium* in agricultural areas and its widespread use in animal nutrition. Thus, it contributes to the health of the animals and ensures the sustainability of the agricultural processes. (Kara et al., 2022; Pastorelli et al., 2022) *Mentha pulegium*, also known as pennyroyal, is among the *Mentha* species of the *Lamiaceae* family and is a natural vegetation in many regions of the Northern Hemisphere (Ölmez and Makav, 2021).

The fermentation process that takes place in the rumen has a significant impact on the nutritional regimes of ruminant animals. The activity of the rumen microbial population can determine the nutrient utilization efficiency in animals (Ando et al., 2003). Therefore, it is important to investigate the effects of herbage sources on rumen fermentation profiles. In recent years, research on the use of various plants as herbage and the effects of these plants on rumen microbial activity have been examined. This is one of the plants that attracts attention in this context and has potential to be a valuable source that can be used in animal nutrition. The chemical structures of *Mentha pulegium* can be fermented by rumen microorganisms in different ways, causing the formation of different metabolites such as AA, PA, and methane. The aromatic plant, the pennyroyal plant, plays an important role due to its potential health benefits. Thanks to the essential oils found in its leaves, it can increase animal feed consumption and support their health by enhancing palatability. It is also known to have protective effects on animal health with its antibacterial and antiparasitic properties (Boukhebt et al., 2011).

In recent years, studies have increasingly focused on the effectiveness of aromatic plants containing various essential oils in animal nutrition. Their anti-methanogenic effects have been attributed to their terpenoid content, demonstrated through both *in vitro* experiments and in vivo animal trials, highlighting their role as rumen modifiers and immune system stimulants (Benetel et al., 2022; Kara and Pirci, 2023; Pandur et al., 2022; Vakili et al., 2013). This study hypothesizes that a roughage source, naturally rich in terpenes and considered a functional forage, may exhibit different ruminal effects compared to conventional forages. This study aims to examine the impact of *Pennyroyal* on rumen microbial activity and methane production. For this purpose, *in vitro*, rumen fermentation experiments were conducted to compare with pennyroyal's common herbage sources. The obtained

findings shed light on the potential of the use of pennyroyal in animal nutrition and its contribution to the sustainability of the animal production system.

## Materials and Methods

### Forage Material and Feed Analysis

The pennyroyal and sainfoin used in the study were obtained by mowing and drying during the full bloom, and the clover hay was harvested and dried during the beginning of flowering. Herbages were ground in an IKA-A10 laboratory type mill (IKA-Werke, Germany) with a sieve diameter of approximately 1.0 mm to be used in *in vitro* gas production.

### In Vitro Gas Production Technique

The rumen fluid was obtained from two beef cattle fed a total mixed ration consisting of approximately 80% concentrate and 20% roughage. The concentrate portion included 55% barley, 15% cottonseed meal, 5% wheat bran, 4% sunflower meal and 1% vitamin-mineral premix, while the roughage portion consisted of 10% corn silage and 5% lucerne hay, in addition to 5% wheat straw. Rumen fluid was placed in a glass bottle with a screw cap (Isolab, Germany) at approximately 39±1°C and brought to the laboratory with a lidded thermos container containing water at approximately 39±1°C. Rumen fluid was used in *in vitro* gas production after being filtered through 6 layers of cheesecloth in an anaerobic environment under CO<sub>2</sub> gas.

*In vitro* gas production with 200±10 mg dried pennyroyal in full bloom, sainfoin in full bloom, lucerne and corn silage in 100 ml glass syringes (Model Fortuna, Haberle Labortechnik, Germany) used in accordance with the method of Menke and Steingass (1988). buffer + macromineral + micromineral + reduction + resazurin solutions mixture (20 ml) and rumen fluid (10 ml) mixture were incubated. Incubation was carried out in a thermostatted water bath at 39°C for 24 hours. The glass materials used were used in *in vitro* gas production after being preheated in a thermostatic cabinet (Lovibond, Australia), and the solutions were preheated in a digital magnetic stirrer with a contact thermometer (Wise Stir MSH-D, Witeg, Germany). The solutions+rumen fluid mixture was injected into each fermenter with an automatic dispenser (Isolab, Germany). Syringes were closed using one-way polyethylene clips. In the study, the *in vitro* experiment was performed three times for each group. Three syringes were used blindly (containing no herbage, only buffer and solutions, and rumen fluid) to calculate total gas production.

The ME level of pennyroyal, sainfoin, lucerne and corn silage used in the study was calculated according to the formulas reported by Menke et al. (1979) and Blümmel et al. (1997). The Crude protein (CP) value for pennyroyal was determined by Kara et al. (2015) and the CP values of sainfoin, lucerne and corn silage were calculated according to NRC (2001).

$$\text{ME (MJ /kg DM)} = 2.20 + 0.136 \times \text{GP} + 0.057 \times \text{CP}$$

GP = 24-hour net gas production (ml/200 mg).

CP = Crude protein (g/kg DM)

### In Vitro Methane Production

In the present study, the total amount of gas (ml) produced in each syringe at the end of 24 hours of incubation was determined by reading the syringes. After the total gas production was read, this total gas was taken into a plastic syringe and transferred to the infrared methane measurement device (Sensor, Europe GmbH, Erkrath, Germany) using a three-way tap. Methane measurement of the device was determined as a % value on the computer screen.

### Statistical Analysis

Statistical analysis was performed using the SPSS 22.0 package program. Homogeneity of variances was assessed using Leven's test. One-way analysis of Variance (ANOVA) determined statistical significance between groups. When a significant difference was detected, Tukey's post hoc test was applied to identify pairwise differences among the groups. Differences were considered statistically significant at  $P < 0.05$ .

### Results

*In vitro* total gas production of sainfoin was higher than pennyroyal ( $P > 0.05$ ). Although the methane (%) production of pennyroyal and sainfoin was similar, lucerne was higher than the others ( $P > 0.05$ ). CH<sub>4</sub> (ml/0.2g) production (6.09 ml/0.2g) of lucerne was higher than that of other forages. *In vitro* CH<sub>4</sub> production of pennyroyal was similar to that of corn silage. CH<sub>4</sub> production (4.02 ml/0.2g) of corn silage was lower than that of other forages. ME(MJ/kg) of sainfoin and lucerne were similar, and corn silage was lower than the others (Table 1). The AA concentration of *in vitro* rumen fermentation fluid for pennyroyal herbage was similar to that of lucerne herbage, and co was lower than that of sainfoin herbage ( $P < 0.05$ ). The PA concentration of *in vitro* rumen fermentation fluid for pennyroyal herbage was lower than those of other forages ( $P < 0.05$ ). While the concentrations of iso-BA and iso-VA, which are BSCFA, were high in lucerne herbage, they were low in pennyroyal, sainfoin, and corn silage. The butyric acid concentration in the rumen fermentation was higher in corn silage than others. Butyric acid concentrations of sainfoin and lucerne were similar (Table 2). Concentrations SCFA (valeric acid and acetic acid) in the *in vitro* ruminal fermentation liquid of sainfoin in flower and lucerne were higher than pennyroyal (*Mentha pulegium* L.) and corn silage.

**Table 1.** *In vitro* ruminal fermentation variables of pennyroyal herbage, sainfoin herbage, lucerne herbage and corn silage

Forages	Methane %	Total Gas (ml/0.2 g DM)	CH <sub>4</sub> (ml/0.2 g DM)	ME (MJ/kg DM)
Pennyroyal	14.36 <sup>b</sup>	31.15 <sup>b</sup>	4.47 <sup>bc</sup>	13.98 <sup>b</sup>
Sainfoin	14.50 <sup>b</sup>	37.8 <sup>a</sup>	5.5 <sup>ab</sup>	16.6 <sup>a</sup>
Lucerne	17.20 <sup>a</sup>	35.42 <sup>ab</sup>	6.09 <sup>a</sup>	15.89 <sup>a</sup>
Corn Silage	15.63 <sup>ab</sup>	25.78 <sup>c</sup>	4.02 <sup>c</sup>	11.96 <sup>c</sup>
SD	1.45	4.74	0.92	1.85
SEM	0.39	1.27	0.25	0.50
P value	0.01	<0.01	0.01	<0.01

ME: metabolic energy calculated from *in vitro* total gas production. as MJ/kg. SD: standard deviation.

Methane: *in vitro* methane gas volume (mL) as %. CH<sub>4</sub>: produced for 0.2 g DM at 24 h. SEM: standard error of means. Total Gas: *in vitro* total gas volume (mL) produced for 0.2 g DM at 24 h.

<sup>a-b-c</sup>: different letters in the same column show significant difference as statically.

**Table 2.** The SCFA and BSCFA concentrations in the *in vitro* rumen fermentation fluid of pennyroyal herbage, sainfoin, lucerne and corn silage

Forages	mmol/L rumen fluid						T-SCFA
	BSCFA		OA	SCFA			
	IBA	IVA	VA	BA	PA	AA	
Pennyroyal	0.45 <sup>b</sup>	0.55 <sup>b</sup>	0.50 <sup>b</sup>	4.03 <sup>b</sup>	8.44 <sup>c</sup>	39.58 <sup>b</sup>	54.25 <sup>b</sup>
Sainfoin	0.45 <sup>b</sup>	0.54 <sup>b</sup>	0.61 <sup>a</sup>	4.45 <sup>ab</sup>	11.30 <sup>ab</sup>	45.73 <sup>a</sup>	63.44 <sup>a</sup>
Lucerne	0.54 <sup>a</sup>	0.72 <sup>a</sup>	0.65 <sup>a</sup>	4.51 <sup>ab</sup>	10.24 <sup>b</sup>	44.46 <sup>ab</sup>	61.49 <sup>ab</sup>
Corn silage	0.46 <sup>b</sup>	0.59 <sup>b</sup>	0.53 <sup>b</sup>	4.57 <sup>a</sup>	12.11 <sup>a</sup>	39.77 <sup>b</sup>	58.39 <sup>ab</sup>
SD	0.04	0.08	0.07	0.27	1.51	3.40	4.38
SEM	0.01	0.02	0.02	0.08	0.44	0.98	1.26
P Value	0.00	0.00	0.00	0.03	0.001	0.01	0.02

BSCFA: branched short-chain fatty acid: IBA + IVA. T-SCFA: total short-chain fatty acid: SCFA + VA + BSCFA.

IBA: iso-butyric acid. IVA: iso-valeric acid. VA: valeric acid. BA: butyric acid. PA: propionic acid. AA: acetic acid. SCFA: straight-short-chain fatty acids: BA + PA + AA

<sup>a-b-c</sup>: different letters in the same column show significant difference as statically.

SD: Standard Deviation of Means. SEM: Standard Error of Means

Phenological Stage	CP	CF	Ash	ADFom	NDFom	HC	NFC
%, KM							
Vegetative	10.38 <sup>a</sup>	4.91 <sup>b</sup>	10.96 <sup>a</sup>	45.36 <sup>a</sup>	51.64 <sup>a</sup>	5.61 <sup>b</sup>	22.08 <sup>b</sup>
Full flowering	9.89 <sup>b</sup>	5.83 <sup>b</sup>	11.41 <sup>a</sup>	37.89 <sup>b</sup>	42.80 <sup>b</sup>	5.61 <sup>b</sup>	30.06 <sup>a</sup>
Seed bulking	11.04 <sup>a</sup>	9.86 <sup>a</sup>	8.69 <sup>b</sup>	46.13 <sup>a</sup>	55.35 <sup>a</sup>	10.06 <sup>a</sup>	15.03 <sup>c</sup>
Total	10.44	6.87	10.35	43.13	49.93	7.09	22.39
SD	0.34	2.34	1.27	4.23	5.91	2.23	6.86
SEM	0.21	0.78	0.42	1.41	1.97	0.74	2.28
L	0.138	<0.001	<0.001	0.622	0.090	<0.001	0.014
Q	0.048	0.013	<0.001	0.001	0.001	<0.001	0.001

**Table 3.** Chemical composition of pennyroyal herbage at different phenological stages

SD: Standard Deviation, SEM: Standard Error of the Mean, L: Linear, Q: Quadratic, CP: Crude Protein, CF: Crude Fat, ADFom: Ash-free Acid Detergent Fiber, NDFom: Ash-free Neutral Detergent Fiber, HC: Hemicellulose, NFC: Non-Fiber Carbohydrates.

<sup>a-b-c</sup>: different letters in the same column show significant difference as statically.

## Discussion

It has been reported that drought-resistant *Mentha* species, which are widely found in the world, can be used as an alternative to medium-quality forage plants. However, their nutritive value may be moderate (Franz Vienna et al., 2007). The pennyroyal plant, which grows naturally in pastures, contains aromatic volatile compounds as well as macronutrients like other traditional forage plants.

The use of feeds that reduce methane production is important in reducing the undesirable effects of global warming. (Terrett and Dupree, 2019; Onel et al., 2022). In this study, the methane production of pennyroyal was observed to be similar to that of corn silage. *In vitro* total gas production of pennyroyal was at reference values (Kara et al., 2024). Concentrations of T-SCFA and SCFA such as AA, PA, and BA in the *in vitro* ruminal fermentation fluid of the pennyroyal herbage are an indicator of the *in vitro* fermentation capacity, and the fact that they did not change with the progress in the phenological stage in this forage plant is consistent with the results of ME and NEL. (Kara et al., 2018). The butyric acid concentration in the rumen fermentation was higher in lucerne and sainfoin than in pennyroyal and corn silage. On the other hand, the high butyric acid production of corn silage should be evaluated carefully, as it may be related to lactate breakdown and pH changes during the fermentation process. The fact that pennyroyal is similar to corn silage (4.02 ml/0.2 g), which has lower methane production than other forages, shows that it can be an environmentally friendly alternative feed source. Especially considering the goals of reducing methane emissions from livestock in the fight against global warming, plants such as pennies can offer an important opportunity. The moderate ME value of pennyroyal indicates that this plant can be used as a supplementary or complementary feed source in feed rations.

The concentrations of SCFA and BSCFA obtained after *in vitro* rumen fermentation of pennyroyal, sainfoin, lucerne, and corn silage support this finding. Pennyroyal has lower values than other feeds regarding PA and total SCFA (T-SCFA) production, indicating that its rumen fermentation capacity is limited. Sainfoin and lucerne show higher levels of AA and total SCFA production, indicating that these feeds are profitable regarding fiber fermentation and energy production. Corn silage has been shown to be an important source of rumen epithelial development, prominent in the butyrate type. Additionally, lucerne reflected its protein fermentation potential with higher branched SCFA (BSCFA) production compared to other forages.

## Conclusion

As a result, sainfoin and lucerne show higher fermentation activity, while pennyroyal has lower fermentation capacity, its methane reduction potential makes it a promising alternative forage. In this study where pennyroyal was compared, the low *in vitro* methane production and high ME levels indicate that it can be used as an alternative to roughage in animal feeding, but further *in vitro* and field studies are needed.



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This work is original.

**2. Author Contributions:**

**Concept:** GP, KK; **Conceptualization:** GP, KK, SY; **Literature Search:** GP; **Data Collection:** GP, KK, SY; **Data Processing:** GP, KK; **Analysis:** GP, KK; **Writing – original draft:** GP, SY, KY; **Writing – review & editing:** GP, SY, KK, KY.

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The authors declare no competing interests.

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