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Determination of *in vitro* methane production of pomegranate peel and oregano pulp supplementation in ruminant diet

Mehtap Güney[1]*, Çağrı Kale[2]

[1] Van Yuzuncu Yil University, Department of Animal Science, Van, Turkey.
[2] Van Yuzuncu Yil University, Department of Animal Nutrition and Nutritional Diseases, Van, Turkey.

***Corresponding author:**
mguney@yyu.edu.tr

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Eduardo Marostegan de Paula

ABSTRACT - This study was conducted to determine the effects of adding pomegranate peel and oregano pulp at different levels (2, 4, 6, and 8%) on a dry matter basis to sheep rations on methane production and rumen fermentation using the *in vitro* gas production technique. In the study, nine experimental groups were formed by adding different levels (2, 4, 6, and 8%) of pomegranate peel and oregano pulp to the total ration mixture (control ration). Each treatment group was designed with four replications. Rumen fluid was taken from two sheep that were fed mainly roughage. For each feed treatment, four gas production syringes were used. A trial design was created using a total of 40 gas production syringes, and four gas production syringes for blank samples. The prepared syringes were incubated for 24 h. The additive levels of pomegranate peel and oregano pulp did not change total gas, methane (%), and methane (mL) production compared with the control treatment. The use of pomegranate peel and oregano pulp for *in vitro* rations significantly reduced rumen fluid volatile fatty acid (VFA) levels, except for 2% pomegranate peel. *In vitro* organic matter digestibility and metabolizable energy levels of both additives were similar to the control ration. In the study, the addition of pomegranate peel and oregano pulp to total ration mixture at 2, 4, 6, and 8% levels did not change total gas and methane production. Except for the addition of 2% pomegranate peel, the treatments suppressed rumen fluid VFA. Besides, it is thought that the additives used in the study can be used up to 2% in the rations of ruminant animals in regions where they may create waste to reduce feed costs.

Keywords: alternative forage, methane production, rumen parameters

1. Introduction

Severe drought and scarcity of water resources in recent years have increased the costs of conventional feed production for animals in most developing countries (Koyuncu and Nageye, 2020). This situation has increased the interest in alternative feeds for ruminants (Bampidis and Robinson, 2006). In addition to turning to alternative feeds for farm animals, it is seen that low-cost byproducts are used to reduce feed costs and increase profitability (Rogers and Poore, 1994). It has been reported that the use of local feed sources to improve small ruminant production in developing countries is increasing (Ben Salem, 2010). For this purpose, tree leaves and various fruit byproduct residues and aromatic plants are used as modifiers of rumen fermentation in ruminant animals to both increase feed efficiency and reduce methane production in the rumen, as they contain bioactive compounds. It can also be a practical way to improve sustainable animal production (Ben Salem, 2010).

Of these, pomegranate peel is a rich source of phenolic compounds such as flavonoids, anthocyanidins and tannins (Lansky and Newman, 2007). Makkar (2003) stated that the tannin found in pomegranate is stable in the rumen (pH 5.0-7.0) and resistant to rumen microbial degradation, and tannins are secondary metabolites in plants with strong antioxidant activity (Ciampi et al., 2020), which are used as natural feed for farm animals, such as pomegranate peel. It has also been reported that tannins reduce the production of acetic acid in the rumen due to their suppression of cellulolytic microorganisms, thus reducing the carbon dioxide and hydrogen ions needed by methanogenic microorganisms for methane production (Waghorn, 2008). Although pomegranate is known as a tropical and subtropical climate fruit, it can also grow to a limited extent in warm and temperate climate regions. Pomegranate production and consumption in the world and in Türkiye is increasing day by day (Alaçık Develioğlu et al., 2022).

The oil of oregano herb is also as important export source in Turkiye (Bozdemir, 2019). Oregano is cultivated both culturally and naturally in many countries today. After oil is obtained from oregano herbs, the remaining pulp remains as a product processing waste (Abdollahzadeh et al., 2010). It has an antimicrobial effect due to its polyphenols, carvacrol, and thymol active compounds (Mellencamp et al., 2011). Since these products, which may accumulate and become waste, may cause significant environmental pollution, it is thought that it is important to investigate a better alternative for feeding ruminants.

There are very limited published studies on the effects of dried pomegranate peel and oregano pulp on rumen fermentation. Therefore, the effects of phytocompounds in these feed ingredients on reducing energy and methane losses in the diet of ruminants are of interest.

This study aimed to investigate the effects of adding dried pomegranate peel and oregano pulp to total ration mixture on *in vitro* total gas, methane production, and *in vitro* ruminal fermentation parameters.

2. Material and methods

Research on animals was conduct according to the institutional committee on animal use (case number 2022/02-03). This experiment was performed in Van Province, Turkey (43° latitude 38° longitude, and elevation of 1730 m above sea level).

2.1. *In vitro* diets and animal material

In this study, the basal diet consisting of barley, cottonseed meal, and meadow hay was obtained from commercial sources. Pomegranate peel was purchased from a commercial local vendor in Siirt province of Turkey, and dry oregano pulp was obtained from Antalya province in Turkey.

The experimental diets were formulated to meet the requirements of the National Research Council (NRC, 1985) based on the energy and protein needs for sheep over one year old. The basal ration consisting of barley and cotton meal and meadow hay formed the first treatment (control) of the study. Nine treatments were formulated by adding 2, 4, 6, and 8% pomegranate peel to the total ration mixture (the control ration), and similarly 2, 4, 6, and 8% oregano pulp to the control ration. All feeds were used in the study after being ground in a feed mill with a sieve diameter of 1 mm (Retsch GmbH 5657 Haan, West- Germany). Chemical composition of ingredients and diets is presented in Tables 1, 2, and 3.

Table 1 - Chemical composition of trial feeds (% DM basis)

Item	Barley	Cottonseed meal	Meadow hay	Pomegranate peel	Oregano pulp
DM	91.51	95.35	94.45	93.09	94.66
Ash	2.17	5.44	6.93	2.56	7.58
CP	11.69	21.19	8.93	2.52	7.49
EE	1.25	1.04	1.88	1.14	2.81
NDF	27.47	43.87	45.38	14.07	40.63
ADF	4.78	28.63	26.68	9.26	25.52

DM - dry matter; CP - crude protein; EE - ether extract; NDF - neutral detergent fiber; ADF - acid detergent fiber.

Table 2 - Ingredients and chemical composition of the diets (g/kg DM)

Item	Pomegranate peel level				
	Control	2%	4%	6%	8%
Ingredient (g/kg)					
Barley	294	274	245	225	209.3
Cottonseed meal	1.4	14	31	31	26.4
Meadow hay	595	594	586.4	586.4	586.4
Pomegranate peel	0	20	40	60	80
Oregano pulp	0	0	0	0	0
Vitamin mineral premix[1]	7	7	7	7	7
Dicalcium phosphate	78	79	78.6	78.6	78.9
Limestone	7	7	7	7	7
Salt	5	5	5	5	5
Total	1000	1000	1000	1000	1000
Chemical composition (%)					
Dry matter	94.21	93.97	93.64	93.63	93.40
Ash	15.17	11.67	11.44	11.67	11.02
Crude protein	11.91	14.16	13.51	11.36	10.02
Ether extract	1.08	1.33	1.13	1.35	1.51
Neutral detergent fiber	41.68	41.16	41.63	42.23	41.18
Acid detergent fiber	21.46	22.17	21.72	23.20	23.19
Total digestible nutrients	73.65	72.73	73.31	71.40	71.41

[1] Vitamin A, 200,000 IU; vitamin D3, 100,000 IU; vitamin E, 500 mg; vitamin B12, 40 mg; vitamin B6, 35 mg; niacin, 1000 mg; Mn, 2000 mg; Fe, 2000 mg; Zn, 2500 mg; Cu, 1000 mg; I, 25 mg; Co, 30 mg; aroma, 150 mg; MgO, 1200 mg; $NaHCO_3$, 1000 mg; antioxidant, 1500 mg.

Table 3 - Ingredients and chemical composition of the diets (g/kg DM)

Item	Oregano pulp level				
	Control	2%	4%	6%	8%
Ingredient (g/kg)					
Barley	294	281	275	271	264
Cottonseed meal	1.4	7	4	4	4
Meadow hay	595	594	584	568	555
Pomegranate peel	0	0	0	0	0
Oregano pulp	0	20	40	60	80
Vitamin mineral premix[1]	7	7	7	7	7
Dicalcium phosphate	78	79	78.5	78	78
Limestone	7	7	7	7	7
Salt	5	5	5	5	5
Total	1000	1000	1000	1000	1000
Chemical composition (%)					
Dry matter	94.21	93.97	94.13	93.81	93.63
Ash	15.17	10.17	10.73	12.96	13.74
Crude protein	11.91	11.77	11.26	10.63	11.42
Ether extract	1.08	1.03	1.22	1.25	1.01
Neutral detergent fiber	41.68	42.73	42.67	41.72	41.68
Acid detergent fiber	21.46	22.69	21.31	22.48	22.29
Total digestible nutrients	73.65	72.06	73.84	72.33	72.57

[1] Vitamin A, 200,000 IU; vitamin D3, 100,000 IU; vitamin E, 500 mg; vitamin B12, 40 mg; vitamin B6, 35 mg; niacin, 1000 mg; Mn, 2000 mg; Fe, 2000 mg; Zn, 2500 mg; Cu, 1000 mg; I, 25 mg; Co, 30 mg; aroma, 150 mg; MgO, 1200 mg; $NaHCO_3$, 1000 mg; antioxidant, 1500 mg.

The animal material used in the *in vitro* incubation of feeds was two Norduz sheep (two years old). The animal material from which rumen fluid was provided consisted of sheep (40±1.0 kg live weight) belonging to a farm implementing a roughage-based feeding program.

2.2. Feed analysis

Dry matter (DM) and ash analysis of experimental diets and rations were determined according to the Association of Official Analytical Chemists (AOAC, 2010; methods 934.01 and 942.05, respectively); crude protein was determined by Kjeldahl steam distiller (AOAC, 2010; method 984.13); and the amount of crude fat level was measured according to ANKOM (2008) using an ANKOM XT15 device (Ankom®, Macedon, USA). Contents of neutral detergent fiber treated with alpha amylase, sodium sülfite, and acid detergent fiber were analyzed as described by Van Soest et al. (1991).

Condensed tannin contents in pomegranate peel and oregano pulp were determined according to Makkar et al. (1995). For this, 0.01 g of ground pomegranate peel and oregano pulp were added into 10 mL glass tubes, and 6 mL of butanol + HCl + regant solution (95 mL butanol + 5 mL HCl + 0.05 g $Fe_2SO_4.7H_2O$) were added, and placed in boiling water for 1 h until color change. The tubes were then cooled rapidly in running tap water, centrifuged at 3000 *g* for 10 min, and read at 550 nm wavelength in a spectrophotometer (Shimadzu, UV-1280, Kyota-Japan). For phenolic compound analysis, 10 mL methanol was added to 0.5 g sample and homogenized at 12000 rpm for 10 s. The samples were shaken in a circular shaker for 2 h. It was then centrifuged at 8000 *g* for 10 min. After the supernatant was removed, the same process was repeated two more times. The resulting supernatants were collected in 25 mL amber bottles and made up with methanol until the final volume was 25 mL. One mililiter of methanolic extract was diluted in 10 mL of methanol. Each dilution (0.2 mL) was mixed with 1 mL of folin-ciocalteu reagent and 0.8 mL of 75 g/L sodium carbonate solution. The mixture was allowed to stand at room temperature for 60 min, and the absorbance was measured at 765 nm on an Agilent, 8453 spectrophotometer (USA). The results were expressed as mg gallic acid equivalent (GAE) per kg pomegranate peel and oregano pulp (Singleton and Rossi, 1965).

2.3. Implementation of *in vitro* gas and methane production technique

The effect of treatments on *in vitro* ruminal 24-h gas production was conducted based on the method of Menke and Steingass (1988). The rumen fluid used in incubation was taken from two male Norduz sheep (two years old), brought to the slaughterhouse from a private farm that implemented a roughage-based feeding program. The rumen fluid taken was quickly brought to the laboratory with the help of a thermobax containing 39 °C water. Rumen fluid was previously prepared with artificial saliva (macromineral solution = Na_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$; micromineral solution = $CaCl_2.2H_2O$, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $FeCl_3.6H_2O$; buffer solution = $NaHCO_3$, $(NH_4)HCO_3$; resazurin solution and reduction solution = NaOH, $Na_2S.7H_2O$) in a water bath at 39±1 °C and under carbon dioxide gas (rumen fluid/artificial saliva, 1/2). Experimental diets (200 mg) were incubated in 100-mL *in vitro* gas production syringes with 30 mL rumen fluid + artificial saliva mixture in a custom-made water bath at 39±1 °C for 24 h.

For the measurement of methane gas, the gas formed in the glass syringes was taken with a three-way plastic syringe system. The obtained gas was injected into a computer aided methane gas measuring device (Sensors Europe Analysentechnik GmbH, Erkrath, Germany), and the methane gas value (%) was read on the computer.

After incubation, pH measurement was made immediately with a digital pH meter (Thermo, Orion Star A11, USA; 0.01) from the content transferred from the *in vitro* gas production syringe to a 250-mL bottle. Then, ammonia nitrogen analysis in rumen fluids filtered through four layers of cheesecloth was performed according to the Kjeldahl method. For this, rumen fluids were centrifuged at 4500 rpm for 5 min. Rumen fluid (5 mL) was poured into glass tubes connected to Kjeldahl distiller (Tecnal® TE 036/1). Water and sodium hydroxide were then added, and the samples were distilled into a boric acid solution (30 g/L). Methyl red was added to the boric acid solution and used as an indicator.

The solution obtained from distillation (about 100 mL) was titrated with sulfuric acid (H_2SO_4; N/70) (Markham, 1942).

Immediately after incubation, rumen fluid samples were stored at −18 C° for volatile fatty acids (VFA) by adding 2 mL of 1/1 HCl (absolute) to a 10 mL tube (Şenyüz and Karsli, 2021). After approximately one month, the samples were removed from the deep freezer and thawed in the refrigerator overnight. The supernatant was centrifuged at 4,500 rpm for 15 min and transferred to vials using a 0.45 µm syringe filter. The column (TR 5 ms Q) placed in the autosampler compartment of a high-pressure liquid chromatography (HPLC) (Agilent, 1100) was then read at a flow rate of 1.7 mL per minute, injection volume of 1 µL, and injection temperature of 230 °C (de Quirós et al., 2009).

The metabolizable energy (ME) content and organic matter digestibility (OMD) of feed treatments were calculated with the following formula using 24-h gas production and chemical composition parameters (Menke and Steingass, 1988).

$$\text{ME (MJ/kg DM)} = 2.20 + 0.136\ \text{GP} + 0.057\ \text{CP} + 0.002859\ \text{CF} \quad (1)$$

$$\text{OMD (\%)} = 14.88 + 0.889\ \text{GP} + 0.45\ \text{CP} + 0.0651\ \text{Ash} \quad (2)$$

In these equations, GP = 24-hour net gas production (mL/200 mg), CP = crude protein ratio (%), CF = crude fat ratio (%), and ash = crude ash ratio (%).

2.4. Statistical analysis

Statistical analysis was determined according to a completely randomized design as a linear model, and differences of P<0.05 were considered statistically significant. Means were separated with Duncan Multiple comparison test. All analyses were run on SAS software (Statistical Analysis System, version 9.4).

$$Y_{ij} = \mu + a_i + b_j + e_{ij} \quad (3)$$

in which Y_{ij} is the j-th observation of the i-th feed ingredient, μ is the overall average, a_i is the feed ingredient effect of the i-th feed ingredient, and e_{ij} is the random error.

3. Results

In the study, the total phenolic content of pomegranate peel was determined as 3695.03 mg gallic acid/kg, and the average condensed tannin content was determined as 7.14 g/kg, while in oregano pulp, they were determined as 2197.71 mg gallic acid/kg and 5.78 g/kg, respectively.

It was determined that pomegranate peel and oregano pulp added to the diet did not change methane values (%, mL) at all rates compared with the control. Similarly, it was observed that total gas production did not change when pomegranate peel and oregano pulp were added to the basic ration at different rates (Table 4).

The addition of pomegranate peel and oregano pulp levels to the diets did not change OMD and ME levels compared with the control treatment, and were not found to be different (Table 4).

In the study, adding 2, 4, 6, and 8% levels of pomegranate peel and oregano pulp to experimental diets did not change *in vitro* rumen fluid pH and ammonia nitrogen levels compared with the control treatment (Table 5). With the addition of both feed ingredients, *in vitro* rumen fluid pH values ranged from 6.59 to 6.68 and ammonia nitrogen levels ranged from 37.46 to 55.39 mg/dL.

As a result of the addition of different levels of pomegranate peel to the basal ration, acetic (AA) and propionic (PA) acid levels showed a significant decrease, except for the 2% level of pomegranate peel. Butyric acid levels decreased compared with the control treatment at all addition levels (Table 5; P<0.0001). When oregano pulp used in *in vitro* diets was examined in terms of VFA, acetic acid, propionic acid, and butyric acid levels were significantly decreased when all levels were compared with the control treatment (Table 5; P<0.001).

Table 4 - Effect of pomegranate peel and oregano pulp on *in vitro* total gas and methane production

Item	N	Pomegranate peel level					P-value
		Control	2%	4%	6%	8%	
		x̄±Sx̄	x̄±Sx̄	x̄±Sx̄	x̄±Sx̄	x̄±Sx̄	
Total gas (mL/g DM)	4	315.00±1.79	314.79±1.89	313.69±3.30	313.66±1.73	312.89±2.72	0.725
Methane (%)	4	22.98±0.39	23.15±0.20	22.93±0.25	22.83±0.21	22.50±0.46	0.693
Methane (mL)	4	14.83±0.60	16.03±0.38	14.94±0.63	15.76±0.50	14.94±0.48	0.390
OMD (%)	4	81.34±1.58	83.57±1.68	79.71±2.93	82.09±1.54	79.22±2.42	0.598
ME (Mj/kg DM)	4	11.99±0.24	12.43±0.26	11.85±0.45	12.24±0.24	11.82±0.37	0.628
		Oregano pulp level					
		Control	2%	4%	6%	8%	
Total gas (mL/g DM)	4	315.00±1.79	314.80±7.82	315.34±1.65	314.26±3.14	313.66±2.98	0.113
Methane (%)	4	22.98±0.39	23.20±1.10	22.35±0.42	23.00±0.20	25.00±1.27	0.237
Methane (mL)	4	14.83±0.60	11.84±1.85	14.46±0.35	14.04±0.78	14.58±0.16	0.222
OMD (%)	4	81.34±1.58	66.18±6.92	78.21±1.47	74.74±2.79	73.14±2.65	0.094
ME (Mj/kg DM)	4	11.99±0.24	9.81±1.06	11.65±0.22	11.11±0.43	10.84±0.41	0.115

OMD - organic matter digestibility; ME - metabolizable energy; x̄±Sx̄ - mean±standard error of the mean.
a,b - Different letters in the same row represent significant difference.

Table 5 - Effect of addition of pomegranate peel and oregano pulp to diets on rumen fermentation parameters

Item	N	Pomegranate peel level					P-value
		Control	2%	4%	6%	8%	
		x̄±Sx̄	x̄±Sx̄	x̄±Sx̄	x̄±Sx̄	x̄±Sx̄	
pH	4	6.63±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.59±0.01	0.242
Ammonia (mg/dL)	4	38.60±3.56	45.02±2.33	37.46±3.99	45.36±8.33	41.20±5.94	0.757
Acetic acid (mmol/L)	4	50.97±1.80a	46.39±0.89a	35.79±5.47b	22.39±0.53c	24.45±0.52c	<0.0001
Propionic acid (mmol/L)	4	14.15±0.34a	13.83±0.41a	7.92±2.57b	4.02±0.77c	3.89±0.63c	<0.0001
Butyric acid (mmol/L)	4	13.60±1.87a	8.97±0.42b	4.95±0.66c	2.72±0.14c	3.83±0.22c	<0.0001
		Oregano pulp level					
		Control	2%	4%	6%	8%	
pH	4	6.63±0.01	6.66±0.03	6.61±0.01	6.64±0.01	6.68±0.02	0.125
Ammonia (mg/dL)	4	38.60±3.56	45.25±4.52	51.72±0.84	41.52±1.65	55.39±8.48	0.112
Acetic acid (mmol/L)	4	50.97±1.80a	28.27±1.03b	27.35±0.91b	27.85±0.42b	27.71±0.59b	<0.0001
Propionic acid (mmol/L)	4	14.15±0.34a	5.80±0.63c	5.56±0.76c	4.80±0.40c	8.07±0.98b	<0.0001
Butyric acid (mmol/L)	4	13.60±1.87a	4.46±0.49b	4.80±0.21b	5.34±0.31b	5.40±0.70b	<0.0001

x̄±Sx̄ - mean±standard error of the mean.
a,b,c - Different letters in the same row represent significant difference.

4. Discussion

Gases such as CO_2 and CH_4, which are the products of microbial fermentation in the rumen, are an important cause of energy loss in the rumen environment. It is stated that the main economic losses in ruminant animals are energy losses from methane (Kebreab et al., 2006; Kim, 2012). In this study, the effects of using pomegranate peel, oregano pulp, and their different ratios in sheep rations on *in vitro* gas, methane, and rumen fermentation parameters were investigated.

Adding pomegranate peel and oregano pulp to diets did not alter *in vitro* gas production (mL/g DM), methane (%), and methane (mL) compared with control (Table 4). There is limited information about the effects of oregano pulp on *in vitro* gas and methane production in the rumen, but there are studies on pomegranate fruit byproducts. It is stated that the gas volume of 200 mg of dried pomegranate peel in 24 h of incubation is 47.42 mL/200 mg DM (Mirzaei-Aghsaghali et al., 2011).

It has been reported that *in vitro* gas production decreased with the inclusion of pomegranate peel in 24-h *in vitro* incubations with rumen inoculum from sheep (Natalello et al., 2020). Patra and Saxena (2011) reported that the tannins in pomegranate are considered as modulating agents for ruminal fermentation and methane reduction. Baladi et al. (2014) reported that the effect of tannin extract obtained from pomegranate pulp on *in vitro* gas production volume of soybean meal at different incubation times varied depending on the doses. However, our results showed that adding pomegranate peel and oregano pulp to *in vitro* incubations at a level of up to 8% did not reduce total gas and methane production compared with the average of the control treatment. Moreover, the presence of plant phenolic compounds showed no effect in reducing gas and methane production. These effects may be due to the fact that the tannins in feed ingredients cannot suppress methanogenic bacteria or form a tannin-fiber complex. Rubanza et al. (2003) stated that the differences in gas and methane production are related to the difference between the fiber content and the structure of the fiber in the feed. In addition, it has been stated that differences in tannin content, which may negatively affect the activity of rumen microorganisms, may also be effective (Goel and Makkar, 2012).

Addition of different levels of pomegranate peel and oregano pulp to experimental diets did not alter OMD and ME levels compared with control (Table 4). It also did not change total gas and methane production and *in vitro* organic matter digestibility. Eliyahu et al. (2015) stated that the soluble components of fresh byproducts, including proteins, ether extract, and phenols, are considered fully digestible *in vitro*, whereas their true *in vivo* digestibility is only partial. Based on this statement, a higher OMD and ME of pomegranate peel compared with oregano pulp may be due to its solubility. In a study, dietary inclusion of tannin rich pomegranate peel extract had no effect on OMD in dairy cows (Abarghuei et al., 2013). Gül et al. (2017) determined that addition of 8.6% oregano to maize silage increased *in vitro* OMD compared to 4.3% additive. Abbas et al. (2019), in a study conducted with dairy cows, stated that 2% pomegranate peels increase the digestibility coefficients, nutritional values, and milk yield and can be used without any harm to the productivity and health of the animals. In an *in vivo* study, despite the positive effects of polyphenolic compounds on the health of ruminants, Broderick et al. (1991) reported that they can reduce proteolytic activity in the rumen and thus endanger protein digestion. Therefore, it has been stated that it would be an appropriate decision to measure the optimum dose level when pomegranate byproducts are added to the rations, and that the addition of pomegranate peel extract in the range of 1 to 4% in the total ration can promote animal health and milk production (Jami et al., 2012).

The pH, ammonia, and volatile fatty acid concentrations have a significant effect on the rumen microflora. Therefore, it is necessary to know how the addition of pomegranate peel and oregano pulp to the diets affects *in vitro* ruminal fermentation. In this study, the ruminal pH levels and ammonia concentration of pomegranate peel and oregano pulp were similar to the control, and all pH values were obtained within acceptable limits for rumen fermentation (Vargas et al., 2023). In addition, it was observed that ammonia nitrogen did not change in all treatments. This can be explained by the fact that the deamination step, which occurs as a result of the breakdown of proteins into ammonia, can be inhibited by the phenolic compounds found in pomegranate peel and oregano pulp (Table 5).

Volatile fatty acids are the end products of microbial fermentation in the rumen and represent the main energy source for ruminant animals. In this study, it was observed that adding pomegranate peel to the experimental diets significantly reduced acetic and propionic acid concentrations compared with the control treatment, except for the 2% pomegranate peel treatment (Table 5). Increasing levels of oregano pulp decreased the concentrations of acetic and propionic acids. However, it was seen that propionic acid increased in 8% oregano pulp level; although this increase was lower than the control treatment, we observed that the increase at this level can improve fermentation (Table 5).

Butyric acid concentrations decreased as pomegranate peel and oregano pulp levels increased and were found to be significantly lower than the control treatment (Table 5). Salem et al. (2012) explained that the stimulatory effects of plant secondary metabolites on rumen fermentation have favorable effects on rumen function. In this study, the addition of pomegranate peel and oregano pulp to the control diet did not reduce *in vitro* gas and methane production. However, rumen fermentation was negatively affected by the treatments, and accordingly, acetic and propionic acid concentrations were reduced. It is thought that this is due to the suppression of rumen fermentation as a result of the addition of tannin-rich feed ingredients to the treatment diets. The results from studies using some byproducts (Natalello et al., 2020; Tan et al., 2011; Odhaib et al., 2018) support our conclusions. It has been reported that the ruminal total VFA concentrations is lower than the control ration as the pomegranate peel levels increase (Abbas et al., 2019).

In the Rusitec system, adding 2.8 g tannic acid equivalents kg^{-1} DM to high-grain diets also resulted in decreased VFA concentrations (Refat et al., 2015). Contrary to these results, however, Shakeri et al. (2017) stated that the addition of different levels of pomegranate peel extract increases the production of VFA, and Abarghuei et al. (2013) reported that adding 400, 800, and 1200 mL of pomegranate peel extract per cow per day in the diets does not affect VFA levels.

5. Conclusions

The waste product remaining after obtaining oregano oil and pomegranate peel does not change *in vitro* gas and methane production, organic matter digestibility in the rumen. However, it significantly reduces the volatile fatty acid levels of rumen fluid, except for the 2% pomegranate peel treatment. It is thought that adding pomegranate peel to ruminant rations up to 2% in areas where waste products may occur may be beneficial in reducing feed costs, and both feed ingredients need to be supported by *in vivo* studies at the doses used.

Author contributions

Conceptualization: Güney, M. **Data curation:** Güney, M. and Kale, Ç. **Formal analysis:** Güney, M. **Funding acquisition:** Güney, M. and Kale, Ç. **Investigation:** Güney, M. and Kale, Ç. **Methodology:** Güney, M. and Kale, Ç. **Project administration:** Güney, M. **Resources:** Güney, M. **Software:** Güney, M. and Kale, Ç. **Supervision:** Güney, M. **Validation:** Güney, M. and Kale, Ç. **Visualization:** Güney, M. **Writing – original draft:** Güney, M. **Writing – review & editing:** Güney, M. and Kale, Ç.

Conflict of interest

The authors declare no conflict of interest.

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