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Assessment of *Pistacia atlantica kurdica* gum essential oil using a rumen simulation technique: effects on nutrient disappearance, volatile fatty acid profile and microbial population

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Abstract The present study evaluated the effects of *Pistacia atlantica Subsp. Kurdica* gum essential oil (PAKEO) on nutrient disappearance, volatile fatty acid (VFA) profile and rumen microbial population using a rumen simulation technique (RUSITEC). Treated vessels were supplied with 0.0 (as control diet), 150 µL/L of PAKEO (EO150) and 300 (EO300) µL/L of PAKEO (two vessels per treatment). Experiments were performed in two runs consisting of a 7-day adaptation followed by an 8-day sampling period per run. The sampling period started on the day after administration of the first treatment. For measurement of nutrient disappearance, samples were collected on the initial (days 8 and 9) and final stages (days 14 and 15) of the period. On day 15, samples for the VFA and microbial population (at 4 h after morning vessels feeding) were collected from the vessel contents. Results showed that the addition of PAKEO at 150 µL/L resulted in an increase ($P<0.05$) in the disappearance of dry matter, organic matter, and crude protein in basal diet. Neutral detergent fiber disappearance increased ($P<0.05$) with the addition of 150 and 300 µL/L of PAKEO. Total VFA concentration increased ($P<0.05$) by 300 µL/L PAKEO supplementation. Addition of PAKEO at 300 µL/L resulted in a lower molar proportion of propionate and a higher acetate: propionate ratio compared with the control ($P<0.05$). However, the molar proportions of acetate, butyrate, valerate, and isovalerate were not affected by PAKEO supplementation. The relative abundance of total bacteria increased (by 10%, $P<0.05$) with the addition of PAKEO. Compared to the control, PAKEO supplementation at 150 µL/L sharply decreased ($P<0.05$) the relative abundance of methanogens. Treatment with PAKEO decreased ($P<0.05$) the relative abundance of major cellulolytic bacteria (i.e., *Ruminococcus albus, R. flavefaciens, and Fibrobacter succinogenes*). Total number of protozoa, Entodinium and Ophryoscolex species also decreased ($P<0.05$) by PAKEO treatment. These findings suggested that PAKEO has the potential as a feed additive to improve rumen microbial fermentation.

Keywords: cellulolytic bacteria, *Pistacia atlantica*, rumen microbes, RUSITEC

Introduction

The exploration of new substances that modulate rumen fermentation to increase the efficiency of feed utilization is an important objective in ruminant

nutrition research. A well-known group of such substances is essential oils (EO), which have been used in ruminants as a 'natural' alternative to growth promotor antibiotics over the last decade (Calsamiglia et al., 2006). Essential oils (EOs) are concentrated extracts of aromatic oily liquids that are obtained using a steam distillation method, and which are usually composed of terpenoids and phenylpropanoids (Patra, 2011). Essential oils (EOs) have shown antimicrobial properties against a wide range of microorganisms such as bacteria, protozoa, and fungi (Patra, 2011). It is believed that EOs exert their antimicrobial activities by disrupting the cell wall structure via transport of electrons, ionic gradients, protein translocation, phosphorylation, and enzyme-dependent reactions (Greathead, 2003). Several individual EOs, or blends of EOs (Castillejos et al., 2008; Patra, 2011; Jahani-Azizabadi et al., 2014), have been studied with *in vitro* rumen fermentative experiments. There is evidence demonstrating positive effects of some EOs on rumen fermentation properties, such as enhancing protein metabolism and neutral detergent fiber (NDF) disappearance, and reducing acetate: propionate ratio and methane production. Additionally, EOs have shown inhibitory effect on ruminal methanogens and protozoa population (Patra and Saxena, 2010; Patra and Yu, 2012).

Pistacia atlantica subsp. Kurdica has traditionally been used as a therapeutic agent against infections (Rasooli and Abyaneh, 2004), and is the major source of an exudate gum that is not widely known around the world (Taran et al., 2010) The genus *Pistacia* belongs to the *Anacardiaceae* family, and consists of 15 known species of *Pistacia*. *Pistacia atlantica* is an evergreen shrub or small tree that is native to parts of Eurasia from the Iranian Plateau to North Africa that has three sub-species: *mutica, kurdica,* and *cabulica (*Sharifi and Hazell, 2011). The gum of this plant is used in medicine, and many studies have shown its considerable antimicrobial activity (Tassou and Nychas, 1995; Taran et al., 2010). In our primary *in vitro* batch culture investigation (Naseri et al., 2018), application of PAKEO demonstrated some promising effects on the inhibition of methane production. Therefore, the aim of the current study was to investigate the effects of PAKEO on rumen microbial anaerobic fermentation and the relative abundance of bacteria, methanogens, and protozoa using a rumen-simulated technique (RUSITEC).

Materials and methods

Essential oils

Essential oil of *Pistacia Atlantica Subsp. Kurdica* (PAK) gum was prepared by SaghezSazi Kurdistan (Van®), Kurdistan, Iran. The gum of PAK was collected in the first month of summer in the Kurdistan province in Iran. The gum was hydro distilled for 5 h to obtain a volatile oil in 20% yield based on the fresh weight of the gum. The oils were stored at a low temperature (-20 °C) prior to analysis.

Analysis for the composition of the EO was conducted by gas chromatography coupled to mass spectrometry (GC-MS), as described by Sharifi and Hazell (2011). The main secondary component of PAKEO was α-pinene (934.1 g/kg) (Sharifi and Hazell, 2011) (Table 1).

Table 1. The composition of *Pistacia atlantica subspecies Kurdica* gum essential oil

Compounds	Percent of total
α-Pinene	93.41
β-Terpinene	2.79
α-Fenchene	1.73
Isoterpinolene	1.25
α-Thujene	0.81

Experimental design and treatments

The experiment was carried out using the RUSITEC as described by Czerkawski and Breckenridge (1977) in two experimental runs. The RUSITEC system was equipped with 6 fermenters with a volume of 10,000 mL (Iran, patent Number: 89037). On the first day of each experimental run, 700 mL of ruminal fluid and 300 mL of warmed McDougall's buffer (pH 8.2, as artificial saliva) modified to contain 1.0 g/L of (NH4)2SO4 (McDougall, 1948), was filled into each fermenter. A pair of nylon bags (8 × 12 cm; pore size of 50 µm) was added to each fermenter, one containing the substrate [10 g, dry matter (DM) basis], and the second bag containing solid ruminal digesta (20 g). On the second day, the bags containing rumen solid contents were replaced with a fresh bag containing experimental substrate. Accordingly, every bag containing the experimental diet was incubated for 24 h and was subsequently replaced with a new one. A 60:40 concentrate: forage diet, ground to pass through a 4 mm screen, was used as a basal diet (Table 2). Rumen fluid was obtained 2 h after the morning feeding from 4 ruminally cannulated Sanjabi rams that were fed a diet consisting of alfalfa hay (40%) and a concentrate mixture (60%). To obtain ruminal fluid, ruminal content was strained through four layers of cheese cloth. In addition, pooled solid rumen content was also used as initial inoculation of particles associated bacteria of the fermenters.

Each of the two experimental runs lasted 15 d, with the first 7 d (d 1 to 7) used for microbial adaptation, followed by 8 d of sampling (d 8 to 15). Experimental treatments were: (1) basal diet without additive (as control, CTR); 2) basal diet plus 150 µL/L of PAKEO (EO150); and 3) basal diet plus 300 µL/L of PAKEO (EO300) µL/L of medium culture. At the beginning of d 8, PAKEO was injected once daily into the fermenters. The daily doses of PAKEO were injected directly into the fermenters at the morning feeding. The concentrations of PAKEO were selected based on batch-culture screening in our previous study (not published). During the experiment, artificial saliva was continuously infused into the fermenters at a dilution rate of 2.5%/h. At nylon bag exchange, each fermentation vessel was flushed with O_2-free CO_2 to maintain anaerobic conditions.

Table 2. Ingredients and chemical composition of the diet

	Value
Ingredients (%DM)	
Alfalfa hay	40.0
Barley grain, ground	17.4
Corn grain, ground	16.5
Soybean meal	10.0
Canola meal	3.0
Wheat barn	3.0
Cotton seed, whole	3.6
Fish meal	3.0
Fat powder (MEGAFAT 701)	2.2
Mineral and vitamin mixture[1]	0.48
Sodium bicarbonate	0.37
Salt	0.45
Chemical composition (% DM)	
Organic matter	90.0
Crude protein	16.0
Neutral detergent fiber	30.0
Acid detergent fiber	15.0

[1] Provided per kg of premix: 95000 mg of Ca, 45000 mg of P, 2970 mg of S, 10.72 mg of Na, 16.5 mg of Cl, 2550 mg of Fe, 4144 mg of Mg, 116 mg of Co, 89 mg of I, 60 mg of Se, 1044 mg of Cu, 10000 IU of vitamin E, 350,000 IU of vitamin D3, 1,600,000 IU of vitamin A.

Animal handling was performed in accordance with the protocol approved by the Razi University Ethics Committee for Animal Care and Use (AEC# VN931023).

Sample collection and chemical analyses

On the initial (8 to 9 d) and last (14 to 15 d) days of the sampling period, after 24 h incubation, the experimental diet bags were replaced with new bags, and removed from each fermenter, rinsed under cold water until the water was clear, then dried at 55 °C for 72 h. Bag residues were ground through a 1-mm screen in a Wiley mill (standard model 4; Arthur H. Thomas, USA) and subsequently analyzed for organic matter (OM), NDF, and crude protein (CP).

The DM content of the diets and the undigested feed residues in the polyester bags were determined by oven drying at 55 °C for 72 h, ash content was determined by combustion at 550 ˚C overnight, and OM was calculated by difference (AOAC, 1990). Crude protein (CP, ID 920.152) was determined using automatic distiller Kjeldahl apparatus (Kjeltec Auto 1030 Analyser, Tecator, Sweden) and the NDF content as described by Van Soest et al. (1991). Apparent nutrient disappearance was calculated from the difference between the nutrient contents in the nylon bags before and after 24 h of incubation.

On d 14 to 15, fermenter fluid samples were taken directly from the vessels via a syringe equipped with a plastic tube 4 h after the feed bag exchange. Some of the fluid sample was immediately used for protozoa counting and subsamples were used to determine bacterial and methanogens abundance (1.5 mL) and VFA concentration (1.5 mL of culture fluid: 0.5 mL of 25% orthophosphoric acid), and were frozen at –20 °C. The VFA (i.e., acetate, propionate, butyrate, valerate and iso-valerate) were analyzed by gas chromatography (GC), as described previously (Klevenhusen et al., 2014). The GC (Chrompack, Model CP-9002, Chrompack, EA Middelburg, Netherlands) was equipped with a 50 m (0.32 mm ID) silica-fused column (CP-Wax Chrompack Capillary Column, Varian, Palo Alto, CA, USA). The temperatures of the injector and detector were 170 and 190 °C, respectively. Helium gas was used as carrier gas with a flow rate of 1 mL/min. For microbial analysis, additional fluid subsamples were snap frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

DNA extraction and real-time polymerase chain reaction (Real-Time PCR)

Samples of fermenter liquid were thawed and mixed in 1.5 mL tubes containing glass beads. The samples were incubated on ice for 2 min and vortexed. Then, the samples were centrifuged at 2000× *g* for 10 min at 4 °C and total DNA was extracted from approximately 200 µL of supernatants using a standard DNA extraction kit (AccuPrepTM, Bioneer Corporation, Daejeon, Republic of Korea). Amplification and detection were performed in triplicate with an ABI Real-Time PCR System (Applied Biosystems 7300, Waltham, MA, USA). The reaction was assayed in a final volume of 25 µL reaction mixture consisting of 12.5 µL of SYBR Green PCR Master Mix (Maxima® SYBR Green/ROX qPCR Master Mix, K0221, Fermentas), 1 µL of DNA template, 0.5 µL of each primer (containing 10 pmol of primer), and 11 µL of deionized water. The species-specific primers used in the present study are shown in Table 3. The DNA amplification programs were performed as described by Valizadeh et al. (2010). The relative abundances of microbes were determined using the $2^{-\Delta\Delta Ct}$ method.

Table 3. The PCR primer sets for real-time PCR assay

Target species	Forward/ Reverse	Primer sequence	Length (bp)	Primer efficiency (%)	Reference
Total bacteria	F	GTGSTGCAYGGYTGTCGTCA	120	94	Maeda et al. (2003)
	R	ACGTCRTCCMCACCTTCCTC			
Methanogens	F	TTCGGTGGATCDCARAGRGC	140	93	Zhang et al. (2008)
	R	GBARGTCGWAWCCGTAGAATCC			
Fibrobacter succinogenes	F	GTTCGGAATTACTGGGCGTAAA	121	97	Denman and McSweeney (2006)
	R	CGCCTGCCCCTGAACTATC			
Ruminococcus albus	F	CCCTAAAAGCAGTCTTAGTTCG	122	96	Denman and McSweeney (2006)
	R	CCTCCTTGCGGTTAGAACA			
Ruminococcus flavefaciens	F	CGAACGGAGATAATTTGAGTTTACTTAGG	132	94	Denman and McSweeney (2006)
	R	CGGTCTCTGTATGTTATGAGGTATTACC			

Protozoa counts

Samples were prepared for protozoa counting by following the procedure described by Dehority (1984). Protozoa were enumerated microscopically in a Sedgwick–Rafter counting chamber, and twenty squares per sample were counted (Naseri et al., 2022).

Statistical analysis

Values of the duplicate treatments in two runs were calculated; thus, overall, four replicates per treatment were obtained by conducting two experimental runs, each lasting for 15 days. Since the first 7 days of each experimental run were considered an adaptation period, mean values of the initial (8 to 9) and last (14 to 15) days of sampling period per treatment were analyzed as a completely randomized design using the MIXED procedure of SAS (Version 9.2, SAS Institute, Cary, NC, USA) with time as repeated measures, according to the following model:

$$Y_{ijk} = \mu + T_j + V_i + R_k + e_{ijk}$$

where Y_{ijk} = the dependent variable, μ = the overall mean, T*j* = fixed effect of PAKEO level, V_i = random effect of the vessels, R_k = random effect of experimental run, and e_{ijk} = residual error. To test the linear influence of PAKEO dosages the orthogonal polynomial contrasts were used. The Tukey–Kramer test was conducted to determine the significant difference amongst the means (P<0.05).

Results

The results of the effects of PAKEO supplementation on nutrient disappearance measured in the RUSITEC are shown in Table 4. Relative to the CTR, supplementation with PAKEO at a dose of 150 µL/L, but not at 300 µL/L, increased (P<0.05) apparent DM, OM, and CP disappearance of the substrate following 24 h incubation. The dietary NDF disappearance increased (P<0.05) at both doses of APKEO.

The effects of PAKEO treatment on VFA concentration at 4h after feeding in the RUSITEC system are shown in Table 5. Total VFA concentration increased (P<0.01) with increasing PAKEO levels, while the molar proportions of valerate, isovalerate, butyrate, acetate, and propionate were not affected by PAKEO supplementation. The ratio of acetate to propionate increased at 300 µL/L PAKEO compared to the CTR.

The relative abundance of total bacteria increased (by 10%, P<0.01) in the fermenters supplemented with PAKEO (Table 6). The PAKEO supplementation at 150 µL/L resulted in a significant decrease (P<0.02) in the abundance of methanogens relative to those of the CTR and 300 µL/L PAKEO. A significant decrease (P<0.01) in ruminal cellulolytic bacterial populations occurred with PAKEO supplementation (Table 6). A linear decrease (P<0.01) in *R. albus* and *R. flavefaciens* populations was found with increasing PAKEO supplementation. In contrast, the relative abundance of *F. succinogenes* decreased with the at 300 µL/L PAKEO level.

The total protozoa population (log_{10}/mL) at 4 h after feeding decreased (P<0.01) with PAKEO supplementation (Table 7); however, no significant differences were observed between 150 and 300 µL/L PAKEO treatments. Additionally, except for a *Diplodinium* species, the population of *Entodinium* and *Ophryoscolex* decreased (P<0.05) with PAKEO supplementation.

Table 4. Effects of *Pistacia atlantica Subsp. Kurdica* gum essential oil on apparent disappearance of DM, OM, CP, and NDF using the rumen simulation technique

Item	Treatments			SEM	*P*-Value			
	CTR[1]	EO150[2]	EO300[3]		Treat[4]	CTR vs. EO	Linear contrast	Quadratic contrast
Apparent disappearance (%)								
Dry matter	54.11[b]	56.51[a]	53.91[b]	0.47	0.02	0.16	0.81	<0.01
Organic matter	53.14[b]	55.49[a]	52.54[b]	0.55	0.02	0.32	0.54	0.01
Crude protein	50.79[b]	53.89[a]	50.59[b]	0.72	<0.01	0.13	0.78	<0.01
Neutral detergent fiber	18.66[c]	26.24[a]	23.04[b]	0.89	<0.01	<0.01	<0.01	<0.01

[a,b:] Within row, means with common superscript (s) are not different (P>0.05).
[1]CTR = control without essential oil, [2] EO150 = *Pistacia atlantica Subsp. Kurdica* gum essential oil at concentrations of 150 µL/L of culture ruminal fluid, [3] EO300 = *Pistacia atlantica Subsp. Kurdica* gum essential oil at concentrations of 300 µL/L of culture ruminal fluid, [4]Treat= treatment effect.

Discussion

To the best of our knowledge, this is the first study to evaluate the influence of PAKEO on nutrient disappearance, VFA profile, and rumen microbial population using the RUSITEC system. The results revealed that PAKEO has antimicrobial potential. As shown in Table 1, the main active compound of PAKEO is α-pinene (up to 93%). Alpha-pinene is a monoterpene compound with antimicrobial activity against the Gram (+) and Gram (−) bacteria, protozoa, and fungi (Sharifi and Hazell, 2011). The mechanism of action by monoterpenes is similar to other phenolic compounds, that is, the disturbance of the proton motive force, electron flow, active transport, and coagulation of cell contents (Burt, 2004).

The PAKEO increased the DM, OM, and CP disappearances at 150 µL/L, and the NDF disappearance at both doses (Table 4). These results suggested that PAKEO increased the overall fermentation of the diet, an observation that is inconsistent with the antimicrobial activity of this EO (Naseri et al., 2022). The increase in nutrient disappearance with the decrease in relative abundance of some ruminal bacteria demonstrated that PAKEO supplementation may cause an increase in the

population of some kinds of bacteria with higher fermentation activity although their population was not investigated in current study. In addition, the increase in relative abundance of total bacteria and *Fibrobacter succinogenes* could be the reason for the increase in the digestibility of nutrients, including NDF. *Fibrobacter succinogenes* is the main members of cellulolytic bacteria and have high rate constant of cellulose degradation (Weimer, 1993; Stevenson et al., 2007). In agreement with our findings, Kim et al. (2019) showed that supplementation with a mixture of EO (1 and 2 g/kg of substrate) containing cinnamaldehyde, thymol, and eugenol (monoterpene compounds) increased (P<0.05) DM and NDF degradability in an *in vitro* system after 24 h incubation. In agreement with the present study, Klevenhusen et al. (2014) observed that *Ferula elaeochytris* (contained 2.17 mg α-pinene/g DM) at a dosage of 50 mg/L increased (P<0.05) nutrient disappearance compared with control using a RUSITEC system that fed a 48:52 forage: concentrate diet. In contrast, Sizmaz (2016) noted that the addition of Laurel oil (containing α-pinene, Limonene, 1,8-Cineol, α-Terpinolene, *p*-Cymene and Menthol) at 50 and 100 mg/L did not affect DM, OM and CP disappearance in a RUSITEC system fed a 48:52 forage: concentrate diet. The differences among these findings could be attributed to the differences in the type, dose, or chemical composition of EO and the basal diet.

Table 5. Effect of *Pistacia atlantica Subsp. Kurdica* gum essential oil on VFA concentration at 4 h post-feeding measured using the rumen simulation technique

	Treatments				*P*-Value			
Item	CTR[1]	EO150[2]	EO300[3]	SEM	Treat[4]	CTR vs. EO	Linear contrast	Quadratic contrast
Total VFA[5] (mmol/L)	90.63[b]	93.37[b]	104.67[a]	2.46	<0.01	0.18	0.31	<0.01
VFA (mol/100 mol)								
Valerate	7.35	7.28	7.07	0.19	0.87	0.89	0.91	0.62
Isovalerate	5.08	4.14	5.20	0.25	0.18	0.07	0.13	0.24
Butyrate	16.11	17.08	17.50	0.32	0.15	0.69	0.19	0.13
Propionate	20.86	21.17	18.33	0.57	0.06	0.12	0.77	0.02
Acetate	50.60	50.33	51.90	0.71	0.69	0.59	0.89	0.41
Acetate: propionate	2.43[b]	2.37[b]	2.83[a]	0.08	<0.01	0.01	0.51	<0.01

[a,b,c,] Within row, means with common superscript (s) are not different (P>0.05).
[1]CTR = control without essential oil, [2]EO150 = *Pistacia atlantica Subsp. Kurdica* gum essential oil at concentrations of 150 µL/L of culture ruminal fluid, [3] EO300 = *Pistacia atlantica Subsp. Kurdica* gum essential oil at concentrations of 300 µL/L of culture ruminal fluid, [4]Treat= treatment effect, [5]VFA = volatile fatty acids.

Table 6. Effect of *Pistacia atlantica Subsp. Kurdica* gum essential oil on bacteria population at 4 h post-feeding using the rumen simulation technique

	Treatments				*P*-Value			
Item	CTR[1]	EO150[2]	EO300[3]	SEM	Treat[4]	CTR vs. EO	Linear contrast	Quadratic contrast
Total bacteria	1.00[b]	1.10[a]	1.10[a]	0.016	<0.01	<0.01	<0.01	0.31
Methanogens	1.00[a]	0.78[b]	0.95[a]	0.035	0.02	0.04	0.49	<0.01
Ruminococcus albus	1.00[a]	0.65[b]	0.41[c]	0.093	<0.01	<0.01	<0.01	<0.01
Ruminococcus flavefaciens	1.00[a]	0.62[b]	0.45[c]	0.098	<0.01	<0.01	<0.01	0.79
Fibrobacter succinogenes	1.00[a]	1.02[a]	0.79[b]	0.035	0.01	0.14	0.01	0.07

[a,b,c,] Within row, means with common superscript (s) are not different (P>0.05).
[1] CTR = control without essential oil, [2] EO150 = *Pistacia atlantica Subsp. Kurdica* gum essential oil at concentrations of 150 µL/L of culture ruminal fluid, [3] EO300 = *Pistacia atlantica Subsp. Kurdica* gum essential oil at concentrations of 300 µL/L of culture ruminal fluid, [4]Treat= treatment effect.

Table 7. Effect of *Pistacia atlantica Subsp. Kurdica* gum essential oil on protozoa population (log_{10}/mL) at 4h post-feeding using the rumen simulation technique

Item	Treatments				*P*-Value			
	CTR[1]	EO150[2]	EO300[3]	SEM	Treat[4]	CTR vs. EO	Linear contrast	Quadratic contrast
Total protozoa	4.57[a]	4.40[b]	4.35[b]	0.04	<0.01	<0.001	<0.01	0.05
Diplodinium	3.40	3.30	3.20	0.05	0.29	0.18	0.13	1.00
Entodinium	4.49[a]	4.34[b]	4.28[b]	0.03	0.01	<0.01	<0.01	0.21
Ophryoscolex	3.41[a]	3.20[b]	3.18[b]	0.05	0.07	0.03	0.04	0.21

[a,b,c,] Within row, means with common superscript (s) are not different (P>0.05).
[1]CTR = control without essential oil, [2]EO150 = *Pistacia atlantica Subsp. Kurdica* gum essential oil at concentrations of 150 µL/L of culture ruminal fluid, [3]EO300 = *Pistacia atlantica Subsp. Kurdica* gum essential oil at concentrations of 300 µL/L of culture ruminal fluid, [4]Treat= treatment effect.

The increase in total VFA concentration with the addition of PAKEO was consistent with the associated increment in nutrient disappearance, suggesting an increase in overall rumen microbial fermentation. Additionally, the increase in total VFA concentration in response to PAKEO supplementation may have a positive effect on ruminant production efficiency. The increase in total VFA concentration is attributed to increase in relative abundance in total bacteria population. This assumption is based on the reality that VFA are the main energy substrates for ruminants (Chaves et al., 2008; Morgavi et al., 2010), and any increase in ruminal VFA formation would subsequently increase the energy supply to the host animal (Klevenhusen et al., 2012). According to the published literature (Benchaar and Greathead, 2011; Klevenhusen

et al., 2014), the effects of EO on VFA production are highly variable, and depend on types and doses of EO (Patra, 2012). In this study the VFA profile was not influenced by PAKEO supplementation. Similar VFA patterns were also observed when *Laurel oil* (containing α-pinene, Limonene, 1,8-Cineol, α-Terpinolene, *p*-Cymene, and Menthol) was added to semi-continuous culture systems (Sizmaz, 2016). The effects of PAKEO on VFA profile in our study are inconsistent with those reported by Klevenhusen et al. (2014), who observed that the addition (50 and 500 mg/L) of black seed and *Ferula elaeochytris* oils (containing 0.13 and 2.17 mg/g DM of α-pinene, respectively) did not affect the VFA profile compared with the control using RUSITEC. Additionally, Joch et al. (2016) noted that molar proportions of acetate, propionate, and butyrate were not affected by the addition of α-pinene (1000 µL/L). In contrast, Jacques et al. (2015) showed that *Ocimum gratissimum* EO (at doses of 100, 200 and 400 mg/L) linearly increased the molar proportion of acetate, whereas the molar proportions of propionate, butyrate, branch-chained VFA, and valerate responded quadratically. These results suggested that EOs can affect individual VFAs by different mechanisms. This is reflected in the total VFA concentrations and VFA profiles.

Diet supplementation with PAKEO altered the population of some ruminal bacteria and protozoa species. The PAKEO contains terpenoid compounds, especially α-pinene (bicyclic monoterpene compound, >93%). Alpha-pinene affects microbial activity and survival by multiple mechanisms, which include metabolic disruption, inhibition of microbial efflux, and decreased membrane integrity (Jasna, 2015). Therefore, the antimicrobial properties of α-pinene confirm the antimicrobial potency of PAKEO shown in this study.

A significant increase in the rumen total bacteria population with the addition of PAKEO can be attributed to the stimulatory effect of PAKEO on microbial growth and the increase in nutrient availability for rumen microbes, which is in accordance with the increase in nutrient disappearance (Table 4). Our findings confirmed the results of Sizmaz (2016), who used laurel oil (containing α-pinene, limonene, 1, 8-cineol, and α-terpinolene) as a rumen modifier. Patra and Yu (2012) suggested that EOs at low doses can increase ruminal bacteria species enrichment, which is in line with the results of our study.

Essential oils can have important effects on rumen methanogens, either by affecting methanogens directly, or indirectly by affecting rumen protozoa (Morgavi et al., 2012). Newbold et al. (1995) demonstrated that rumen methanogenesis, and thereby the ruminal methanogens, is directly correlated with the population of protozoa. Several studies have illustrated that the absence of protozoa in the rumen ecosystem has a significant effect on the methanogen population, and thereby the level of methane production (Cieslak et al., 2009; Morgavi et al., 2012). Thus, a noticeable reduction in methanogens with PAKEO could be related to the direct effect of PAKEO on methanogens (Table 6) or indirectly on the population of protozoa (Table 7). Patra and Saxena (2010) reported that the addition of juniper berry oil [containing myrcene (25%), citronellol (18%), and α-pinene (14%)] decreased the population of methanogenic archaeal, which is likely due to changes in protozoal populations. Moreover, in accordance with Goel et al. (2009), reduction in methanogens could be attributed to decrease in the bacteria populations, such as *Ruminococci* that produce remarkable amounts of hydrogen. Therefore, in the current study, the reduction in *R. flavefaciens* and *R. albus* populations with PAKEO might have inhibited the methanogens. Microbial interactions in rumen ecology are usually complex and unpredictable. Studies on interactions of methanogens (hydrogen consumers) and other microbial groups (hydrogen producers) have been very effective (Goel et al., 2009), even though such studies are lacking for the rumen ecosystem.

In this study, the reduction in the populations of methanogens and protozoa with PAKEO supplementation illustrated that this EO has an inhibitory effect on methanogenesis, although methane production was not directly measured in the current study. In addition, the capability of PAKEO to reduce the abundance of *R. albus*, a hydrogen-producing bacterium, points to a potential in lowering the production of hydrogen, the electron donator of methanogenesis.

The PAKEO showed adverse effects on three of the rumen cellulolytic bacteria. The populations of *F. succinogenes*, *R. flavefaciens*, and *R. albus* were notably decreased by PAKEO. In general, Gram-positive bacteria are more sensitive to EOs than Gram-negative ones, due to the lack of a protective outer membrane of the cell wall (Patra and Yu, 2012). Accordingly, the present study shows a decrease in the population of *F. succinogenes*, a Gram-negative species, significantly smaller than those of *R. flavefaciens* or *R. albus*, both of which are Gram-positive bacteria.

The negative effect on the population of *R. flavefaciens* and *R. albus*, might be due to the increased partial pressure of hydrogen accumulated in the system, because of the reduction in methanogens. It has been reported that this hydrogen pressure affects the metabolism of the two important fiber-degrading species, *R. flavefaciens* and *R. albus*, inhibiting NADH oxidation in both species and diverting hydrogen to form other products such as succinate and ethanol (Goel et al., 2009).

In this study, the response of *F. succinogenes* differed from those of *R. flavefaciens* and *R. albus* when different doses of PAKEO were applied to the system. Therefore, the *F. succinogenes* population decreased at the high level of PAKEO (300 µL/L), while PAKEO at a low level did not affect this fibrolytic bacterium. According to Lee et al. (2020) the *F. succinogenes* and *R. flavefaciens* populations decreased with supplementation of EO from the *Artemisia montana* plant (containing monoterpene

compounds) *in vitro*. Additionally, Lin et al. (2012) showed that the supplementation of a diet containing ground maize (50%) and *Leymus chinensis* hay (50%) with a combination of different EO reduced the populations of *F. succinogenes* and *R. flavefaciens*. In another study by Patra and Yu (2012), the population of main cellulolytic bacteria (e.g., *F. succinogenes* and *R. flavefaciens*) decreased with the addition of clove oil, eucalyptus oil, garlic oil, origanum oil, and peppermint oil to a mixed substrate containing alfalfa and concentrate (1:1 ratio). These studies showed that the use of EOs has adverse effects on *F. succinogens* and *R. flavefaciens* populations, which supports the results obtained with PAKEO supplementation in the current study. In contrast, several studies have shown that appropriate doses of different EO increase the populations of fibrolytic bacteria (Calsamiglia et al., 2007; Benchaar et al., 2008; Patra and Yu, 2012). Nonetheless, the populations of specific fibrolytic bacteria are increased with certain EOs, suggesting that different bacterial species have different levels of sensitivity to various EO. For instance, in the soybean meal substrate, addition of *San wormwood* EO resulted in the highest increases in the *R. albus* and *S. bovis* populations, while *Ganghwa* and *Injin wormwood* EOs enhanced the population of *R. flavefaciens* (Lee et al., 2020). Therefore, different active compounds of EO may impart varying effects on certain fibrolytic rumen bacteria. In general, even though our study indicated the impotence of PAKEO (at 150 µL/L) for improving the population of *Fibrobacter succinogenes*, further investigation is necessary to understand why PAKEO has beneficial and detrimental effects.

The protozoa count decreased with the addition of PAKEO in the medium (Table 7). There are conflicting reports on the effects on rumen protozoa. Klevenhusen et al. (2014) observed that the numbers of ciliate protozoa, both *Holotrichs* and *Entodiniomorphs*, were unaffected due to dietary supplementation of black seed oil and *Ferula elaeochytris* (containing α-pinene as the main active compound) in RUSITEC. Similarly, Sizmaz (2016) reported that supplementation of laurel oil with 6.6% α-pinene content did not affect protozoa counts, including *Holotrichs* and *Entodiniomorphs*, in a semi-continuous culture system. In contrast, Patra and Yu (2012) showed that different doses of EOs containing monoterpene compounds *in vitro* decreased the abundance of archaea and protozoa linearly with increasing EO doses. Additionally, Fraser et al. (2007) reported that the addition of EO from cinnamon leaf reduced protozoa numbers by as much as 2 logs in a RUSITEC system.

In the current study, the antiprotozoal activity of PAKEO seems to be mainly related to the presence of monoterpenes principally α-pinene. The antimicrobial mechanism of α-pinene was studied using electron microscopy and the incorporation of isotope-labeled precursor. After treatment with α-pinene, the microbial morphology and ultrastructure showed obvious changes: their cell walls and cytoplasmic membrane ruptured; intracellular components were released, and the cell residue fused to form irregular masses. In addition, the synthesis of DNA, RNA, and polysaccharides in the cell wall and ergosterol of the cytoplasmic membrane was inhibited (Salehi et al., 2019). It has been indicated that these changes are related to the antimicrobial mechanism of α-pinene (Salehi et al., 2019). As mentioned by Russell (2002), there is an inverse relationship between the number of protozoa and rumen bacteria due to bacterial predation by protozoa. Therefore, a decrease in protozoa number as a consequence of supplementation of PAKEO could lead to an enhancement in the population of ruminal bacteria (Table 6) owing to a decrease in predatory activity. Additionally, ruminal protozoa play a substantial role in methane production (Moss et al., 2000), since a direct relation has been recognized between the number of ruminal protozoa and methanogenesis (Klieve and Hegarty, 1999). Rumen protozoa are estimated to provide a habitat for up to 20% of ruminal methanogens, and methanogens living on and within protozoa are thought to be responsible for an estimated 37% of methane emissions from ruminant animals (Klieve and Hegarty, 1999). Therefore, the results of this study support the existence of a synergism between protozoa and methanogens.

Conclusions

The potential of *Pistacia atlantica Subsp. Kurdica* gum essential oil (PAKEO) as a ruminal modifier was evaluated using a RUSITEC system. The PAKEO exhibited antimicrobial activity through an increase in nutrient digestibility, especially fiber digestion and total VFA concentration at lower doses. The PAKEO significantly decreased the abundance of methanogens and protozoa. Although PAKEO supplementation decreased relative populations of some fibrolytic bacteria, it exerted no negative effects on fiber degradation. The increase in relative abundance of total bacteria and *Fibrobacter succinogenes* could be the reason for the increase in the nutrients digestibility. Therefore, given the results of this study, PAKEO may be effective for application in ruminant nutrition to improve the efficiency of feed utilization. Even so, more research is needed to evaluate the effects of PAKEO on *in vitro* and *in vivo* rumen microbial fermentation.

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Institutional Review Board Statement

All procedures with the animals were performed in accordance with the protocol approved by the Razi University Ethics Committee for Animal Care and Use (AEC# VN931023).

Conflicts of Interest

The authors confirm that there are no recognized conflicts of interest associated with this publication.

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