DOSE-RESPONSE EFFECTS OF SAVORY (*Satureja khuzistanica*) ESSENTIAL OIL AND EXTRACT ON RUMEN FERMENTATION CHARACTERISTICS, MICROBIAL PROTEIN SYNTHESIS AND METHANE PRODUCTION *IN VITRO*

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Abstract

The objective of the present study was to investigate dose-response effects of the essential oil (EO) and dry extract (EX) of *Satureja khuzistanica* (SK) on *in vitro* gas production kinetics, rumen fermentation, ruminal methanogenesis and microbial protein synthesis. So, EO and EX were tested at 0 (as control); 150 (low dose); 300, 450 (intermediate doses) and 600 mg/L (high dose). The gas produced over 24 h of incubation (GP₂₄) decreased linearly with both EO and EX dosages ($P < 0.01$). *In vitro* methane production was reduced by both EO (14–69%, depending on the included dose) and EX (7–58%). Microbial protein (MP) as well as the efficiency of microbial protein synthesis (EMPS) were improved by EO (18.8–49.8% and 20.4–61.5% for MP and EMPS, respectively) and to a lesser extent by EX (8.3–25.7% and 4.6–24.2% for MP and EMPS, respectively). Ammonia concentration was dropped in linear and quadratic manners with EO ($P < 0.05$), and linearly with EX dosages ($P < 0.01$). EO and EX exhibited depressive effects (in linear and quadratic ($P < 0.05$), and linear manners ($P < 0.01$), respectively) on total protozoa count. A mixed linear and quadratic effect was observed from both EO and EX on total VFA concentration ($P < 0.01$). Total VFA concentration increased at 300 mg/L of EX, but decreased at high dose of both EO and EX. The acetate proportion increased with EO intermediate and high dosages, but it decreased at the expense of propionate at low and intermediate doses of EX. In total, these findings confirmed previous research on the great capacity of plant-based feed additives in positively modulating rumen fermentation that their effects may vary depending on the doses used. Specifically, these results suggest that EO and EX have high potentials to improve rumen functions at intermediate doses, which needs to be confirmed by *in vivo* experiments.

Key words: essential oils, feed additives, *Satureja khuzistanica*, gas production, microbial protein

Ruminants play a central role in providing high-quality animal protein for human consumption, accounting for around 70% of total animal protein consumed in human diets (Minson, 1997). However, a relatively low efficiency of the rumen fermentation imposes negative impacts on ruminant performance through partial loss of dietary energy and protein in the forms of methane emission, and faecal and urinary nitrogen excretions (Calabrò, 2015). Among numerous strategies employed for the improvement of ruminal fermentation, including feed processing, physiological interventions (e.g. stimulating saliva secretion, and increasing passage rate and gut motility) (Nagaraja et al., 1997), manipulation of the rumen microbial ecosystem by additives is generally accepted as one of the most effective approaches in improving the rumen functions (Torres et al., 2020; Honan et al., 2021). Plant secondary metabolites (PSMs) or phytochemicals are naturally occurring substances with antimicrobial activity, which have gained a growing interest as a new class of feed additives after severe restriction of antibiotics as animal classic growth promoters in

many countries in the late 20th century. Public concern on the health hazards associated with antibiotic residues in animal products, along with an increasing demand of consumers for organic and healthy products have made PSMs as a safe and green alternative to antibiotics for modulating rumen fermentation over the two past decades (Patra and Saxena, 2010; Torres et al., 2020; Kholif and Olafadehan, 2021). Essential oils as a plant-derived product composed of volatile fraction of PSMs have extensively been studied for their actions on rumen fermentation (Bodas et al., 2012; Stevanović et al., 2018; Nel et al., 2021). However, data on the efficacy of essential oils as rumen modifiers are inconsistent, because their effects may vary depending on different factors, including their chemical composition, the dose ranges used, type of the diet and experimental conditions (Chaves et al., 2008; Kahvand and Malecky, 2018). Plant extracts are another type of phytochemicals composed mainly of non-volatile fraction of PSMs including different classes of phenolic compounds, such as tannins and flavonoids, glycosides and alkaloids (Calabrò, 2015). Many plant extracts have

been found to have the potential to modify favourably rumen fermentation (Broudiscou et al., 2002; Patra and Saxena, 2010; Lemos et al., 2021).

Despite the positive effects that plant extracts or essential oils as feed additives may have on animal productivity, their extraction is still costly and their application, especially essential oils (because of their volatility), in farm animal diets may be faced with some difficulties in field conditions. Therefore, in the case of some aromatic plants with a high PSMs content, direct supplementation of the whole herbal plants into the diet can be cost-effective and more practical for farmers. In this case, investigating both volatile and non-volatile fractions of phytochemicals could help to have a clear image of the whole plant potential for modulating rumen functions (Christaki et al., 2012; Hundal et al., 2019).

Savory (*Satureja*) is a genus of aromatic plants of Lamiaceae family native to North Africa, southern and southeastern Europe, the Middle East and central Asia. *Satureja khuzistanica* (SK) is an aromatic species of *Satureja* genus, growing mainly in Zagros highlands in the southwest of the Iranian plateau especially north of Khuzestan province. This plant with a strong aroma, is used mainly for food flavouring in this region (Abbasi et al., 2017). It has been found that SK has a high essential oil content (up to 4.5% of DM), in which carvacrol is the major component accounting for 89–95% of the essential oil (Hadian et al., 2011; Abbasi et al., 2017). This plant seems to have the potential to modify rumen fermentation; however, data on this plant or its extracted products is missing in the literature.

Therefore, the objective of the present study was to evaluate the effects of using different levels of the essential oil or dry extract of SK on *in vitro* ruminal fermentation, microbial protein synthesis and methane production.

Material and methods

Plant samples

The savory plant samples were collected in early blooming stage from Khuzestan province highlands during May 2020. The samples were mixed to give a representative sample and then air-dried in the shed to a constant weight. One part of the SK sample was used for extraction of essential oil and the other part was reserved to methanol extraction. All of the procedures and experimental protocols associated with the plant samples were conducted in compliance with national guideline and legislation for plant materials research and approved by the National Committee for Ethics in Biomedical Research (Ref No: 1397/1139).

Essential oil extraction and chemical composition

The EO was extracted by steam distillation using a Clevenger apparatus and stored at 4°C until analysis for its chemical composition. The EO was subsequently analyzed qualitatively and quantitatively by gas chromatog-

raphy-mass spectrometry (GC-MS) and gas chromatography-flame ionization detector (GC-FID), respectively. The GC-MS analysis was performed on a Shimadzu 2010 Plus gas chromatograph (Kyoto, Japan) coupled with a Shimadzu mass selective detector (QP2010), and fitted with a BP-5 capillary column (30 m × 0.22 mm, film thickness = 0.25 µm). Helium was used as the carrier gas with a flow rate of 0.9 mL/min. The oven temperature was held at 60°C for 2 min, and then increased at 5°C/min up to 250°C. The mass scan range was 35–450 m/z with an electron ionization energy of 70 eV. The injector and detector temperatures were set to 250°C and 280°C, respectively.

The GC-FID analysis was accomplished using a Shimadzu GC 2010 Plus (Kyoto, Japan) equipped with a nonpolar BP-5 column (30 m × 0.22 mm, film thickness = 0.25 µm). Helium was used as the carrier gas with a flow rate of 0.9 mL/min. The other operations were the same as described for GC-MS analysis. The EO constituents were identified based on their retention indices compared with those of reference compounds in the literature (Adams, 2007) and the mass spectra in the Wiley Library (Wiley 7.0). Relative percentages of the identified compounds were calculated using the peak area from the GC-FID analysis.

Methanol extraction and determining total phenolic and flavonoid contents

Methanolic extract was prepared according to Skendi et al. (2017). Before extraction, the plant materials were ground to pass through 1 mm sieve. A 5-g (DM) sample was dissolved in 85% methanol at the 1:5 (w/vol) ratio and sonicated in water-bath for 25 min. The extract solution was then centrifuged at 8000×g for 10 min and the remaining particulate matter re-extracted with the same volume of methanol. The supernatants were pooled and subsequently drained in a rotary evaporator and stored at 4°C until analysis for its major components.

Total phenolic compounds of EX were determined using the Folin and Ciocalteu reagents (Singleton and Rossi, 1965). The absorbance of the samples and standards was read using a spectrophotometer (UV-1280 V-VIS, Shimadzu, Japan) at 760 nm against blank. Total phenolic content was calculated as mg gallic acid equivalents/g of dry plant material based on a standard curve of gallic acid (5–500 mg/L). All determinations were carried out in triplicate.

Total flavonoids content of EX was determined in triplicate using the aluminium chloride colorimetric method (Dewanto et al., 2002). Quercetin was used as standard for quantification of total flavonoids in the extract. The absorbance of the reaction mixtures in the samples and standards was measured against blank with a UV-Vis spectrophotometer (UV-1280 UV-Vis, Shimadzu, Japan) at 510 nm. The concentration of total flavonoids in the samples was calculated from the calibration curve of quercetin and expressed as mg quercetin equivalent/g of dried plant material.

HPLC analysis of phenolic acids

Individual phenolic acids in EX including chlorogenic acid, caffeic acid, salvianolic acid and rosmarinic acid were analyzed according to Luis et al. (2007) using a RP-HPLC (Knauer Scientific Instruments, Berlin, Germany) system equipped with a multiple wavelength detector. Before HPLC analysis, the samples were filtered through a 0.45 µm filter. Aliquots of 12 µl were injected into an Apollo C18 reversed phase column (150×4.6 mm i.d.). The column was eluted at a flow rate of 1 ml/min with a mixture of acetonitrile (solvent A) and 2.5% acetic acid in distilled water (solvent B) in a gradient mode as follows: 0 min, 10% A; 15 min, 40% A; 24 min, 70% A; 35 min; 10% A. The detection was set at 280 nm. Identification of individual compounds was based on the comparison of the actual retention time with those of reference authentic standards. Quantification of each compound was made based on its own standard curve.

Treatments and incubations

The present *in vitro* experiment consisted of two sets of short-term and long-term incubations. The first incubations set of 144 h was used to evaluate dose-response effects of EO and EX at 0, 150, 300, 450 and 600 mg/L of incubation culture on ruminal gas production kinetics. The second set of 24-h incubations was devoted to assess treatment effect (the same doses of EO and EX as used for 144 h incubations) on the rumen digestibility and fermentation, microbial protein synthesis and ruminal methanogenesis. Each set of incubations was repeated twice (n = 2 runs) on two separate days.

In vitro experimental diets and gas production

Gas production procedure was conducted according to Menke and Steingass (1988). Three ruminally fistulated mature rams (50 ± 4.5 kg BW) were used to obtain rumen fluid before morning feeding for gas production incubations. The rams received twice daily a maintenance diet composed of (per kg DM): 700 g alfalfa hay and 300 g barley, providing 9.58 MJ metabolizable energy (ME) and 137.4 g crude protein (CP). Rumen fluids were pooled, strained through four layers of cheesecloth into a pre-warmed (38–39°C) insulated flask and immediately transported to the laboratory. A diet for lactating

ewes in early lactation (Table 1) was used as the fermentation substrate. In the first set of incubations, sub-samples of 200 mg (DM basis) of the substrate were incubated in triplicate in 100 ml glass syringes with 30 ml of buffered rumen inoculum under continuous flow of CO₂. The incubation inoculum was prepared by mixing the rumen fluid with the buffer at a ratio of 1:2 (v/v). The buffer was composed of 237 ml macro-minerals solution, 0.12 ml micro-minerals solution, 237 ml bicarbonate buffer solution, 1.22 ml Resazurin solution, 50 ml reducing solution and 474.7 ml distilled water per litre (Makkar et al., 1995). Three syringes containing buffered rumen inoculum without the substrate were used as blanks. The syringes were then incubated in a water-bath at 39°C and the gas produced at 2, 4, 6, 8, 12, 24, 48, 72, 96, 120 and 144 h of incubation was measured. Data on the cumulative gas production were fitted to the model proposed by France et al. (1993) using NLIN procedure of SAS (SAS, 2002) and the kinetic parameters including the asymptotic gas production (A), lag time (L), time of half asymptotic gas production (T_{1/2}) and fractional gas production rate at T_{1/2} (µ) were estimated as described by France et al. (1993).

In the second set of incubations, sub-samples of 500 mg of the substrate were incubated for 24 h (n = 6 for each treatment level) anaerobically with 40 ml buffered rumen inoculum in the glass syringes (Makkar et al., 1995). At the end of incubation and after recording the cumulative gas production, three of the six syringes were used to determine the substrate degradability and fermentation indices. For this group, the syringe contents were transferred to centrifuge tubes and cooled immediately in ice water to stop fermentation. The tubes were then centrifuged at 15000×g for 20 min at 4°C and aliquots of 4 ml from the supernatant were acidified with 1 ml of 25% metaphosphoric acid and kept frozen at –20°C until subsequent analysis for VFA. Additional aliquots of 4 ml of the supernatant were fixed with equal volume of 0.2 N chloridric acid and stored at –20°C for ammonia analysis. Partitioning factor (PF) was calculated as the organic matter truly degraded (mg) to the gas produced (ml) ratio over 24 h of incubation. Microbial biomass (MB) was estimated as difference between fermentation residue at the end of incubation and that after NDS extraction (Blümmel et al., 1997 a).

Table 1. Ingredients and chemical composition of the experimental diet (fermentation substrate)

Ingredients		Chemical composition	
Item	Proportion (g/kg DM)	Item ¹	Content (g/kg DM)
Alfalfa hay	210	OM	920
Corn silage	190	CP	170
Barley grain	190	NDF	295
Corn grain	168	ADF	189
Wheat bran	17	EE	28
Soybean meal	145	ME (Mj/kg)	10.5
Canola meal	60		
Sodium bicarbonate	5		
Salt	5		
Vitamin and mineral premix	5		
Calcium carbonate	5		

¹OM: organic matter, CP: crude protein, NDF: neutral detergent fibre, ADF: acid detergent fibre, EE: ether extract, ME: metabolizable energy.

The other three syringes were used to determine MP content according to Makkar et al. (1982). For this purpose, the syringe contents were transferred to centrifuge tubes and shaken in a magnetic stirrer for 45 s to remove the microbes adhered to feed particles. The tubes were then centrifuged at 400×g for 5 min to remove protozoa and remaining feed particles. Aliquots (5 ml) of the supernatant were centrifuged at 25000×g for 20 min, the supernatant was discarded and the residue was washed with McDougall buffer (McDougall, 1948) and subsequently centrifuged again at 25000×g for 20 min. The supernatant was discarded and the remaining microbial pellet was suspended in 15 ml of 0.25 N NaOH and incubated in boiling water for 10 min. The microbial suspension was then centrifuged at 25000×g for 30 min and 0.5-ml aliquots of the supernatant were used to determine MP by the method of Lowry et al. (1951) using bovine serum albumin as standard. The EMPS was calculated as g bacterial N produced per kg organic matter truly degraded in the rumen. All the experimental protocols and procedures, and care of the fistulated animals were conducted in accordance with relevant guidelines and legislation of Bu-Ali Sina University Animal Care and Use Committee (sub-committee of National Committee for Ethics in Biomedical Research, Ref No: 1397/1139).

Protozoa count

Total protozoa count was made using the separate runs according to the procedure described by Dehority (2003). For this, the whole syringe content was preserved by mixing with equal amount of 50% formalin (18.5% formaldehyde) and the protozoa numbers were quantified microscopically using a counting slide (Hawksley and Sons Limited, Lancing, Sussex, UK).

Methane production measurement

In the second set of incubations of 24 h, the gas produced over 24 h was collected in vacuum gas-tight bottles and a representative gas sample was used for determining methane concentration in the syringe headspace. Methane concentration was determined using a gas chromatograph (HP 5890 Series, Agilent Technologies, USA) equipped with a thermal conductivity detector and a stainless steel packed column (Porapak-Q, 1.8 m × 2 mm i.d., 80/100 mesh size). Helium was used as the carrier gas at a flow rate of 20 ml/min. The oven temperature was held at 60°C and detector was set at 240°C.

Chemical analyses

The AOAC standard methods (AOAC, 2000) were used to determine DM (ID no. 930.15), total ash (ID no. 924.05) and ether extract (EE) (ID no. 920.39). Crude protein was determined using the Kjeldahl method (ID no. 984.13). Neutral detergent fibre (NDFom) and acid detergent fibre (ADFom) were determined according to the amylase-free methods described by Van Soest et al. (1991) and AOAC (ID no. 973.18), respectively, and expressed exclusive of residual ash.

In the incubations of 24 h, after discarding the supernatant, the remaining pellet in the centrifuge tube was oven-dried at 55°C for 48 h and subsequently refluxed with neutral detergent solution (NDS) for 1 h to estimate IVTDMD. The IVTOMD of the samples was determined by subtracting the ash content of the NDF fraction and that of the substrate. The VFAs concentration of the samples was quantified according to Ottenstein and Bartley (1971), using a gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a capillary column (CP-Wax 58 FFAP CB, 50 m×0.53 mm, film thickness = 2 µm). Hydrogen was used as the carrier gas at a constant flow rate of 30 ml/min. Oven temperature was programmed as follows: initial temperature was set at 80°C and held for 1 min; then increased at 30°C/min to 230°C and held at 230°C for 5 min. The injector and detector temperatures were set at 220°C and 240°C, respectively.

Ammonia concentration in the supernatant was determined according to phenol-hypochlorite method as outlined by Broderick and Kang (1980).

Statistical analysis

Data were analyzed by the MIXED procedure of SAS (SAS, 2002) using the completely randomized design (combined analysis). The syringe was the experimental unit and the model included treatment (EO and EX doses) as a fixed effect; and day, treatment × day and replications nested in day as random effects (combined analysis). Orthogonal polynomial contrasts were used to evaluate the EO and EX linear and quadratic effects on the variables in tested doses range. Statistical significance was declared at $P \leq 0.05$ and tendency at $0.05 < P \leq 0.10$. The least square means were compared using Tukey-Kramer test.

Results

Chemical compositions of EO and EX

The EO and EX yields were 2.2% and 7.8%, respectively (W/W DM basis). Chemical compositions of EO and the major components of EX are summarized in Tables 2 and 3, respectively. Based on the results of GC-MS analysis, carvacrol, γ -terpinene, p-cymene and α -terpinene were the major components of EO accounting for 715.7, 88.3, 58.6 and 37.6 g/kg of total essential oil.

In EX, flavonoids were the major constituents of phenolic compounds accounting for around 70% of total phenolics content. In the HPLC analysis, rosmarinic acid was the major constituent of the measured individual phenolic acids (around 27% of total phenolics content) followed by salvianolic acid and chlorogenic acid accounting for around 11 and 10% of total phenolics in SK, respectively.

Gas production kinetics

The asymptotic gas production (A) and the fractional gas production rate (μ) both decreased linearly ($P < 0.01$)

with EO dosages (Table 4). However, lag time (L) and the time of half-asymptotic gas production ($T_{1/2}$) increased linearly ($P<0.05$) with EO increasing doses in the culture. Similar to EO, EX exhibited a linear depressive effect ($P<0.05$) on the 'A' parameter; however, the other parameters of gas production kinetics remained unchanged with EX dosages ($P>0.05$).

24-h *in vitro* ruminal fermentation, digestibility and methane production

Data on the EO and EX effects on GP_{24} and methane production are shown in Table 5. The GP_{24} decreased linearly with EO increasing doses in the culture ($P<0.01$). Methane production decreased in linear and quadratic manners ($P<0.05$) with EO dosages, as the CH_4 produced at the end of incubation decreased by 69.4% at the high dose of EO. The EO also exhibited a depressive linear effect ($P<0.01$) on the methane production percentage ($CH_4\%$, relative to GP_{24}) and a mixed linear and quadratic effect ($P<0.05$) on methane production per g dry matter degraded (DMD). Approximately the same effect was observed from EX on GP_{24} and methane production. The GP_{24} decreased linearly with EX increasing doses in the culture ($P<0.01$). Both CH_4 production (57.6% reduction at high dose of EX) and $CH_4\%$ decreased linearly and quadratically with EX dosages ($P<0.01$). Methane production per g DMD also declined linearly ($P<0.01$) with EX increasing doses in the culture.

The EO reduced both IVTDMD and IVTOMD (Table 6) in linear and quadratic manners ($P<0.05$). The PF and MB increased quadratically and linearly, respectively, with EO dosages ($P<0.05$). Both MP and EMPS were improved in linear and quadratic manners ($P<0.05$) with increasing doses of EO in the culture with their highest values obtained at 450 mg/L of EO (54.1 and 61.5%

improvement in MP and EMPS, respectively, compared with the control). Ruminal ammonia concentration as well as total protozoa count both decreased in linear and quadratic manners ($P<0.05$) with EO dosages.

There was a mixed linear and quadratic effect ($P<0.05$) from EX on IVTDMD and IVTOMD (Table 7); however, in contrast to EO, EX improved ruminal degradability of DM and OM at low and intermediate doses. The EX also enhanced PF in linear and quadratic manners ($P<0.05$) and tended to increase linearly ($P=0.06$) MB when included in the culture. Similar to EO, EX also improved both MP and EPMS in linear and quadratic manners ($P<0.05$), as the highest values for these variables were obtained at high dose of EX (25.7 and 24.2% improvements for MP and EMPS, respectively, compared with the control). Both ruminal ammonia concentration and total protozoa count decreased linearly ($P<0.01$) with EX increasing doses in the culture.

Data on total VFA concentration and VFA pattern are shown in the Table 8 and Table 9 for EO and EX, respectively. The EO exhibited a depressive mixed linear and quadratic effect ($P<0.01$) on total VFA concentration. There was also a mixed linear and quadratic effect ($P<0.05$) from EO on the acetate, propionate and butyrate molar proportions, as the acetate and butyrate proportions increased at the expense of that of propionate at 450 and 600 mg/L of EO. The proportion of branched chain VFAs (BCVFAs), including isobutyrate and isovalerate decreased linearly and quadratically ($P<0.05$) with EO dosages, but valerate proportion increased in linear and quadratic manners ($P<0.05$) with EO increasing doses in the culture. The acetate to propionate ratio (A:P) was changed in linear and quadratic manners with EO dosages ($P<0.01$), as it increased at high dose of EO.

Table 2. Composition of *Satureja khuzistanica* (SK) essential oil

NO.	Compounds	RI*	Composition (g/kg)	NO.	Compounds	RI*	Composition (g/kg)
1	α -thujene	931	3.7	16	borneol	1165	1.4
2	α -pipene	939	8.0	17	4-terpineol	1177	3.3
3	camphene	953	1.4	18	α -terpineol	1190	1.2
4	β -pinene	980	9.9	19	thymol	1290	7.0
5	myrcene	991	13.4	20	carvacrol	1298	715.7
6	α -phellandrene	1005	4.8	21	thymyl acetate	1355	8.2
7	α -terpinene	1018	37.6	22	α -copaene	1376	1.9
8	p-cymene	1026	58.6	23	trans-caryophyllene	1418	4.0
9	limonene	1024	4.1	24	alloaromadendrene	1439	2.2
10	β -phellandrene	1025	2.6	25	β -bisabolene	1509	5.8
11	trans- β -ocimene	1050	1.1	26	δ -cadinene	1524	2.3
12	γ -terpinene	1062	88.3	27	trans-calamenene	1532	1.4
13	cis-sabinenehydrate	1068	1.2	28	spathulenol	1576	1.3
14	terpinolene	1088	1.1	29	caryophyllene oxide	1581	1.1
15	trans sabinene hydrate	1140	1.3	Total			993.9

*RI: retention indices relative to C8–C20 n-alkanes on the BPX5 column.

Table 3. Chemical composition of *Satureja khuzistanica* (SK) extract (mean $\pm$ SD)

Compounds	Concentration (mg/g DM of plant materials)
Total phenolics	18.52 $\pm$ 0.48
Total flavonoid	12.98 $\pm$ 0.18
Total tannin	5.51 $\pm$ 0.12
HPLC analysis of individual phenolic acids	
rosmarinic acid	5.00 $\pm$ 0.19
salvianolic acid	1.98 $\pm$ 0.03
chlorogenic acid	1.82 $\pm$ 0.03
caffeic acid	0.45 $\pm$ 0.01

Table 4. Dose-response effects of *Satureja khuzistanica* (SK) essential oil (EO) and extract (EX) on gas production kinetics

Parameters ¹	EO dose (mg/L)					SEM	P-Value ²		
	0	150	300	450	600		Tr	L	Q
A	109.4 a	98.8 ab	97.6 ab	93.3 ab	80.1 b	5.31	0.035	0.006	0.584
μ	0.033 abc	0.040 a	0.034 ab	0.017 c	0.021 bc	0.004	0.017	0.006	0.168
L	0.003	0.005	0.007	0.007	0.014	0.004	0.204	0.048	0.454
T _{1/2}	11.7	10.2	12.8	28.0	24.3	6.10	0.123	0.035	0.641
Parameters	SK EX dose (mg/L)					SEM	P-Value		
	0	150	300	450	600		Tr	L	Q
A	102.9	97.5	93.4	93.0	88.9	4.96	0.224	0.043	0.653
μ	0.041	0.052	0.054	0.034	0.032	0.011	0.339	0.224	0.194
L	0.008	0.002	0.004	0.007	0.004	0.004	0.637	0.667	0.596
T _{1/2}	10.6	8.47	7.92	13.7	11.9	3.25	0.473	0.342	0.460

¹A: asymptotic gas production (ml/ 200 mg fermentation substrate); μ : fractional gas production rate (/h); L: lag time (h); T_{1/2}: the half time of asymptotic gas production (h); SEM: standard error of the means.

²Tr: treatment, L: linear, Q: quadratic, ^{a,b}: different superscript letters indicate differences (P<0.05) between the treatments.

Table 5. Dose-response effects of *Satureja khuzistanica* (SK) essential oil (EO) and extract (EX) on *in vitro* 24-h gas and methane production

Variable ¹	EO dose (mg/L)					SEM	P-Value ²		
	0	150	300	450	600		Tr	L	Q
GP ₂₄	143.8 a	130.0 b	126.2 bc	122.5 bc	116.7 c	2.48	0.002	<0.001	0.069
CH ₄ (ml/24 h)	12.1 a	10.4 b	6.6 c	4.6 d	3.7 d	0.40	<0.001	<0.001	0.010
CH ₄ (%)	8.32 a	7.72 a	5.16 b	3.96 bc	3.26 c	0.277	<0.001	<0.001	0.194
CH ₄ (ml/g DMD)	28.5 a	24.9 a	16.2 b	12.3 c	10.8 c	0.788	<0.001	<0.001	0.013
Variable	SK EX dose (mg/L)					SEM	P-Value		
	0	150	300	450	600		Tr	L	Q
GP ₂₄	141.3 a	136.7 ab	135.0 abc	130.8 bc	127.8 c	1.93	0.012	0.002	0.878
CH ₄ (ml/24h)	12.5 a	11.6 ab	10.4 b	8.5 c	5.3 d	0.40	<0.001	<0.001	0.008
CH ₄ %	8.9 a	8.4 ab	7.8 abc	6.9 c	4.2 d	0.32	<0.001	<0.001	0.006
CH ₄ (ml/g DMD)	29.6 a	26.2 ab	24.1 bc	20.3 c	14.6 d	1.10	0.001	<0.001	0.119

¹GP₂₄: gas produced after 24 h of incubation (/500 mg DM), CH₄ (%): the methane production percentage (relative to total gas volume), CH₄ (ml/g DMD): the methane production per g dry matter degraded, SEM: standard error of the means.

²Tr: treatment, L: linear, Q: quadratic, ^{a,b}: different superscript letters indicate differences (P<0.05) between the treatments.

Similar to EO, a mixed linear and quadratic depressive effect (P<0.01) was observed from EX on total VFA concentration; however, total VFA was higher at 300 mg/L of EX compared with the control (P<0.05). There was also a mixed linear and quadratic effect (P<0.01) from EX on the acetate and propionate proportions; however, their variations were in opposite directions. The acetate proportion decreased at the expense of propionate at doses up to 300 mg/L of EX, but a reverse

trend was observed at higher doses. Consistently, the acetate to propionate ratio decreased in the dose range of 0–300 mg/L of EX, but increased at higher doses. Both butyrate and valerate proportions were changed quadratically (P<0.01) with EX dosages, as they increased at moderate dose of EX. There was a quadratic (P<0.01) and a mixed linear and quadratic effect (P<0.01) from EX on isobutyrate and isovalerate proportions, respectively.

Table 6. Dose-response effects of *Satureja khuzistanica* (SK) essential oil (EO) on *in vitro* 24-h ruminal digestibility and fermentation

Variable ¹	EO dose (mg/L)					SEM	P-Value ²		
	0	150	300	450	600		Tr	L	Q
IVTDMD	0.845 a	0.832 a	0.815 ab	0.779 b	0.714 c	0.011	0.002	<0.001	0.016
IVTOMD	0.848 a	0.863 a	0.820 ab	0.787 b	0.725 c	0.012	0.002	<0.001	0.019
PF	2.94 b	3.21 ab	3.23 a	3.19 ab	3.07 ab	0.06	0.041	0.185	0.008
MB	64.9	75.0	86.1	99.5	84.2	8.40	0.080	0.028	0.095
MP	62.5 c	74.2 c	83.4 b	93.6 a	86.2 ab	2.11	<0.001	<0.001	0.003
EMPS	25.6 d	30.9 c	35.4 b	41.4 a	41.3 a	0.97	<0.001	<0.001	0.023
NH ₃	12.3 a	9.17 b	8.53 b	7.97 bc	6.85 c	0.36	<0.001	<0.001	0.014
Total Protozoa	2.53 ab	2.60 a	1.98 abc	1.67 c	0.93 d	0.157	0.002	<0.001	0.035

¹IVTDMD: *in vitro* true dry matter degradability, IVTOMD: *in vitro* true organic matter degradability, PF: partitioning factor (mg OM degraded/mL gas produced), MB: microbial biomass (mg), MP: microbial protein (mg), EMPS: efficiency of microbial protein synthesis (g bacterial N/Kg organic matter truly degraded); NH₃: ammonia (mM); total protozoa: ruminal protozoa count ($\times 10^5$ /ml); SEM: standard error of the means.

²Tr: treatment, L: linear, Q: quadratic, ^{a,b} different superscript letters indicate differences (P<0.05) between the treatments.

Table 7. Dose-response effects of *Satureja khuzistanica* (SK) extract (EX) on *in vitro* 24-h ruminal digestibility and fermentation

Variable ¹	EX dose (mg/L)					SEM	P-Value ²		
	0	150	300	450	600		Tr	L	Q
IVTDMD	0.844 bc	0.874 ab	0.883 a	0.871 ab	0.810 c	0.009	0.006	0.025	0.001
IVTOMD	0.847 bc	0.876 ab	0.884 a	0.873 ab	0.816 c	0.009	0.006	0.032	0.001
PF	2.99 b	3.20 ab	3.27 a	3.33 a	3.17 ab	0.05	0.015	0.012	0.005
MB	65.7	67.2	68.4	68.7	72.0	2.50	0.299	0.065	0.691
MP	65.3 d	70.7 c	81.0 ab	82.1 a	78.2 b	0.83	<0.001	<0.001	<0.001
EMPS	26.87 a	28.11 a	31.9 b	32.76 b	33.36 b	0.83	<0.001	<0.001	0.018
NH ₃	11.6 a	9.98 ab	9.68 b	9.55 b	8.43 b	0.44	0.014	0.002	0.378
Total Protozoa	1.74 a	2.30 a	1.08 b	1.07 b	0.97 b	0.128	<0.002	<0.001	0.748

¹IVTDMD: *in vitro* true dry matter degradability, IVTOMD: *in vitro* true organic matter degradability, PF: partitioning factor (mg OM degraded/mL gas produced), MB: microbial biomass (mg), MP: microbial protein (mg), EMPS: efficiency of microbial protein synthesis (g bacterial N/Kg organic matter truly degraded); NH₃: ammonia (mM); total protozoa: ruminal protozoa count ($\times 10^5$ /ml); SEM: standard error of the means.

²Tr: treatment, L: linear, Q: quadratic, ^{a,b} different superscript letters indicate differences (P<0.05) between the treatments.

Table 8. Dose-response effects of *Satureja khuzistanica* (SK) essential oil (EO) on *in vitro* ruminal volatile fatty acids (VFA) profiles

Variable ¹	EO dose (mg/L)					SEM	P-Value ²		
	0	150	300	450	600		Tr	L	Q
Total VFA (mM)	106.7 ab	108.5 a	102.9 bc	101.0 c	94.0 d	1.12	<0.001	<0.001	0.008
VFA (mol/100 mol)									
Acetate	65.8 c	64.6 c	66.6 bc	67.3 b	70.7 a	0.54	<0.001	0.005	0.002
Propionate	24.4 a	24.2 a	22.5 a	22.1 b	17.2 c	0.49	<0.001	<0.001	0.003
Iso-butyrate	2.45 a	2.48 a	2.52 a	2.10 c	2.25 b	0.021	<0.001	<0.001	0.021
Butyrate	5.32 c	5.83 c	5.80 c	6.85 b	7.94 a	0.186	<0.001	<0.001	0.010
Iso-valerate	1.36 ab	1.59 a	1.25 b	0.52 c	0.64 c	0.066	<0.001	<0.001	0.028
Valerate	0.70 b	1.27 a	1.29 a	1.14 ab	1.26 a	0.120	0.031	0.021	0.030
A:P	2.70 b	2.67 b	2.96 b	3.04 b	4.12 a	0.086	<0.001	<0.001	<0.001

¹A:P: acetate to propionate ratio, SEM: standard error of the means.

²Tr: treatment, L: linear, Q: quadratic, ^{a,b} different superscript letters indicate differences (P<0.05) between the treatments.

Table 9. Dose-response effects of *Satureja khuzistanica* (SK) dry extract (EX) on *in vitro* ruminal volatile fatty acids (VFA) profiles

Variable ¹	EX dose (mg/L)					SEM	P-Value ²		
	0	150	300	450	600		Tr	L	Q
Total VFA (mM)	104.3 b	109.6 ab	115.9a	106.6b	87.6c	1.72	<0.001	<0.001	<0.001
VFA (mol/100 mol)									
Acetate	66.8 b	64.7 bc	62.6 c	65.1b c	73.1 a	0.58	<0.001	0.001	<0.001
Propionate	23.7 b	25.2 b	26.93 a	23.6 b	18.6 c	0.367	<0.001	<0.001	<0.001
Iso-butyrate	2.21 a	2.14 ab	2.05 ab	2.04 b	2.34 a	0.037	0.006	0.172	0.001
Butyrate	5.36 b	5.68 b	6.29 ab	7.31 a	5.08 b	0.295	0.008	0.186	0.004
Iso-valerate	0.93 a	0.973 a	0.89 a	0.68 a	0.13 b	0.088	0.003	<0.001	0.005
Valerate	0.97 ab	1.30 a	1.20 a	1.31 a	0.78 b	0.094	0.015	0.155	0.004
A:P	2.82 b	2.57 b	2.33 c	2.76 b	3.93 a	0.071	<0.001	<0.001	<0.001

¹A:P: acetate to propionate ratio, SEM: standard error of the means.

²Tr: treatment, L: linear, Q: quadratic, ^{a,b}: different superscript letters indicate differences (P<0.05) between the treatments.

Discussion

Chemical compositions of EO and EX

The EO yield was in the range reported in the literature (0.6–3%) (Sefidkon and Ahmadi, 2000; Hashemi et al., 2012). Chemical composition of EO with carvacrol as the major compound was also consistent with those reported previously (Hadian et al., 2011; Abbasi et al., 2017).

In the current study, carvacrol was the major compound of EO, which is characterized by a potent antimicrobial activity (Dorman and Deans, 2000; Burt, 2004). Carvacrol is a monoterpene phenol that has extensively been studied *in vitro* for its potential to modify rumen fermentation (Benchaar et al., 2007; Calsamiglia et al., 2007).

Phenols, including mainly flavonoids, phenolic acids, tocopherols and steroids, have been reported as the main constituents of the methanolic or ethanolic extracts from *Satureja* genus (Hadian et al., 2011; Mahboubi and Kazempour, 2016). Flavonoids and tannins are also among the phytochemicals vastly used in modulating the rumen fermentation (Kamra et al., 2006; Tekippe et al., 2012; Oskoueian et al., 2013).

Gas production kinetics

In the current study, a depressive effect of both EO and EX on the asymptotic gas production (as an index of the fermentation extent) could most likely be due to their antimicrobial effects on certain populations of the rumen microorganisms. As stated earlier, phytochemicals are well known for their antimicrobial property (Dorman and Deans, 2000; Wallace, 2004). The SK essential oil and extract have also been found to possess antimicrobial activity (Mahboubi and Kazempour, 2016). However, it should be noticed that the impact of SK additives on rumen fermentation in the current study varied depending on the type and dose used in the culture. Consistent with previous research, the reduction in gas production was more pronounced at higher doses of both EO and EX, reflecting their dose-depending effects on the rumen

microbial activity (Jouany and Morgavi, 2007). In addition, the extent of the EO depressive effect on the 'A' parameter was different from that of EX (around 26% vs. 14% decrease in 'A' parameter at high dose of EO and EX, respectively). This different impact is due mainly to different types and composition of their bioactive compounds. As stated previously, carvacrol and flavonoids were the major constituents of EO and EX, respectively, that possess different antimicrobial activities (Bodas et al., 2012).

It should also be noted that a decreased 'A' parameter with SK additives might also be a result of the shift in ruminal fermentation from gas production pathway to that of MB production (Blümmel et al., 1997 b). This was confirmed by an increased PF with SK additives inclusion in the culture in the 24-h incubations. Lag time, as a period of feed particle colonization by the rumen microbes, is crucial for subsequent digestion of feed particles especially those of roughages (Dehority, 2003). A long lag time and $T_{1/2}$ with EO dosage especially with high doses of EO was coherent with a low asymptotic gas production and gas production rate in these cultures, implying that the rumen microbial activity has been slowed down at high dose EO.

24-h *in vitro* ruminal fermentation, digestibility and methane production

The GP₂₄ variation with both EO and EX was the same as that observed for asymptotic gas production. As argued previously, the decrease in gas production (both 'A' parameter and GP₂₄), especially at intermediate doses of EO and EX, does not appear to be a result of their inhibitory effects on the rumen microorganisms, but rather is a result of modification in the rumen fermentation pathways. Indeed, a decreased GP₂₄, especially at low and intermediate doses (up to 300 mg/L for EO and 450 mg/L for EX), was inconsistent with IVTDMD and IVTOMD variations in these dose ranges that either remained unaffected by EO or even increased by EX. These results were in accord with those of Khatlab et al. (2020) who reported that thyme oil has a negative effect

on gas production without adversely affecting ruminal digestibility. As mentioned above, these results could be due to shift in the rumen fermentation pathways, resulting in a higher partitioning of digested OM to the microbial biomass synthesis pathway than to that resulting in gas production. This is supported by an increased PF as well as MP with low and intermediate doses of both EO and EX in the current study.

One of the most interesting findings of the current research was that both EO and EX exhibited great potential to mitigate ruminal methane production without adversely affecting rumen functions at low and intermediate doses. Part of methanogenesis inhibition by SK additives seems to be mediated indirectly through restricting the availability of its precursors such as CO₂ and H₂ (Patra and Yu, 2012). This is supported by a decreased total gas production (GP₂₄ composed mainly of CO₂) in all the cultures supplemented with both EO and EX. Furthermore, a decreased methane production might also be linked to a partial suppression of protozoal populations that are generally considered as natural habitat for some methanogenic populations and contribute to H₂ supply to methanogens (Finlay et al., 1994; Moss et al., 2000). However, reducing methane percentage (relative to total gas production) by EO and EX implies the fact that methanogens might also be inhibited directly by these additives that needs to be further explored. In this connection, numerous studies have demonstrated that terpenoids and most phenolic compounds, like flavonoids and tannins, have a direct inhibitory impact on rumen methanogens (Kamra et al., 2006; Patra and Saxena, 2010; Yadeghari et al., 2015). Among a vast array of phytochemicals tested *in vitro*, including essential oils, plant extracts or their individual bioactive compounds, many of them have reduced ruminal methane production (El-Zaiat and Abdalla, 2019; Joch et al., 2019; Yu et al., 2020). However, it is important to notice that in many cases, methane inhibition by phytochemicals has been accompanied with a concomitant decrease in ruminal DM and OM digestibility (Oskoueian et al., 2013; Joch et al., 2019; Garcia et al., 2020). Hence, the phytochemicals with the potential to reduce ruminal methane production without negatively affecting rumen fermentation are generally of great importance. Because ruminal methane emission accounts for 2–12% loss of dietary energy, any reduction in methane production could improve the efficacy of feed energy utilization by the host animal (Benchaar and Greathead, 2011; Stevanović et al., 2018).

The results of IVTDMD and IVTOMD revealed that EO has a depressive effect on the rumen microbial activity and in consequence on ruminal digestibility at 450 and 600 mg/L levels. Earlier studies have shown that essential oils have a typically dose-dependent effect on rumen fermentation with a specific selective effect at low and moderate, and a general inhibitory effect at high doses that generally varies depending on the type of the essential oil (Hart et al., 2008; Yadeghari et al., 2015; Yu et al., 2020). In this connection, Benchaar et al.

(2007) found that carvacrol reduces ruminal digestibility at doses higher than 400 mg/L *in vitro*. Despite increasing MB and MP with EO, partial suppression of protozoal populations (which actively participate in digestion of particulate feeds) seems to be the principal cause of decline in IVTDMD and IVTOMD at 450 mg/L of EO. In contrast to EO, EX improved ruminal degradability of DM and OM at doses up to 450 mg/L. There is very few data in the literature reporting a positive impact from phytochemicals on ruminal digestibility (Benchaar et al., 2006; Kim et al., 2019; Zhou et al., 2020); therefore, the mechanisms by which phytochemicals may improve ruminal feed degradability are poorly understood. In this context, there are evidences demonstrating that certain bioactive compounds of phytochemicals may interact simultaneously with bacterial enzymes and fermentation substrate, resulting in the improvement in ruminal feed degradability (Calsamiglia et al., 2007; Liu et al., 2013). Specifically, flavonoids have been found to promote ruminal digestibility through being partially degraded by certain rumen microorganisms (Lowry and Kennedy, 1996; Broudiscou et al., 2002). In this regard, it has been speculated that some intermediate metabolites from flavonoids degradation in the rumen, including mainly phenolic acids, are used by rumen microorganisms for the biosynthesis of certain aromatic amino acids (Stack et al., 1983; Stack and Cotta, 1986). This might have stimulated the growth of rumen microorganisms, consequently improving ruminal feed degradability.

The other important result in the current study was that both EO and EX increased PF as well as MB and MP. Indeed, PF is an index of digested OM partitioning between the gas production pathway and that yielding MB. Hence, the greater PF, the higher proportion of digested OM would be expected to be incorporated into microbial biomass (Blümmel et al., 1997 a), signifying an improvement in the efficiency of feed energy utilization (Ungerfeld, 2015). However, it is important to notice that a high PF is only beneficial to be accompanied with a proportional increase in MB or in MP. In the current study, the MB variation with SK additives was not completely coherent with that of MP, as PF had a higher correlation with MP than MB. This might be partially due to a gravimetric error resulting from an incomplete extraction of MB especially that of the microorganisms solidly adhered to feed particles. The other cause of a moderate difference between MB and MP is likely related to protozoa removal from the culture in the Lowry method, as the protozoa biomass has not been taken into account in calculating MP. Therefore, a different impact of SK additives on the rumen predominant bacteria and protozoa may explain the moderate difference observed in the variations of MB and MP. Hence, regarding different effects that phytochemicals may have on rumen bacteria and protozoa (Hart et al., 2008; Patra and Yu, 2012), and the fact the protozoal protein has a minor contribution to the microbial protein reaching small intestine (Sylvester et al., 2005; Belanche et al., 2012), it can be conclud-

ed that MP may be a better and precise indicator of the predicted microbial protein that can be supplied to the host animal. There was a close linkage between MP and EMPS variations, demonstrating that one great part of MP improvement in both EO and EX supplemented cultures has been due to an increased efficiency of microbial protein synthesis likely as a result of partial removal of rumen protozoa. Predation of rumen bacteria by protozoa has been found to contribute to around 20–50% of bacterial turnover in the rumen (Russell and Hespell, 1981; Williams and Coleman, 1992). Consequently, in addition to reducing bacterial turnover, partial defaunation seems also to have a role in balanced supply of nutrients, especially amino acids to rumen bacteria, promoting their growth (El-Zaiat and Abdalla, 2019). This is supported by a decreased proportion of BCVFAs along with a decline in protozoa count (known to contribute to ruminal deamination activity) when including EO and EX in the culture. In the present study, MP was enhanced by around 19–50% (depending on the included dose) and 8–26% by EX and EO, respectively, that could benefit the host animal by supplying an additional high quality microbial protein.

The rumen ammonia concentration is generally an outcome of the balance between its release from protein degradation and its uptake by the rumen bacteria in *in vitro* cultures (Nolan and Dobos, 2005; Zhou et al., 2020). In the current study, the decrease in ammonia concentration with SK additives was not coherent with IVTDMD and IVTOMD variations, but was negatively correlated to MP, suggesting that an improved microbial growth has likely played a major role in reducing ammonia concentration. However, a more pronounced decrease in ammonia concentration at high dose of EO and EX seems to be mainly a result of a decreased protein degradation as well as inhibition of deamination activity. In this context, previous research has shown that a large number of phytochemicals including essential oils and plant extracts have some potential to reduce ruminal ammonia concentration (Wallace, 2004; Joch et al., 2019). The decreased ammonia concentration by phytochemicals in most of this research has mainly been attributed to a partial inhibition of deamination activity of HAP bacteria (Castillejos et al., 2007; Hart et al., 2008). Moreover, partial removal of protozoa by SK additives might also have contribution in reducing ammonia concentration especially regarding that protozoa are known as the ammonia net exporter in the rumen (Cotta and Russell, 1997; Busquet et al., 2005; El-Zaiat et al., 2019).

Partial suppression of protozoal populations can be considered as another positive effect of EO and EX on the rumen microbial ecosystem at their intermediate and high doses. Previous studies screening a large number of phytochemicals have identified many PSMs with some antiprotozoal activity (Bodas et al., 2012; Cieslak et al., 2013; Khiaosa-Ard and Zebeli, 2013). Essential oils have shown a varied impact on protozoal numbers depending on their dose and chemical composition (Patra and Yu,

2012). However, in most cases, they have shown a depressive effect on the rumen protozoa count (Patra and Yu, 2012; El-Zaiat and Abdalla, 2019; Zhou et al., 2019). Plant extracts, especially those of high tannins and flavonoids content, also have shown potent antiprotozoal activity (Patra et al., 2006; Wang et al., 2013). Despite contribution of protozoa to ruminal feed degradability and supplying digested nutrients to rumen bacteria, partial defaunation by phytochemicals has mostly resulted in a net improvement in rumen functions through reducing methane emission and deamination activity as well as increasing the efficiency of microbial protein synthesis (Williams and Coleman, 1992; Jouany and Morgavi, 2007; Cieslak et al., 2013). In the current study, part of the improvement in MP and VFA pattern (including a decreased acetate to propionate ratio and BCVFAs proportion) as well as decline in methane production with EO and EX could be linked to partial inhibition of rumen protozoa.

Despite relatively different impacts of EO and EX on the rumen fermentation, both reduced total VFA concentration at their high dose. The decrease in total VFA was consistent with a decreased degradability of DM and OM indicating that both EO and EX have a general inhibitory effect on the rumen microbial ecosystem at their high dose. A decreased total VFA at intermediate and high doses of EO, as mentioned previously, could likely be a result of the modification in the rumen fermentation favouring the microbial protein synthesis as reflected by a higher PF and MP. This is also probably the case with EX, as the improvement in degradability of DM and OM at intermediate and high doses resulted in a greater increase in MP than in total VFA concentration.

The VFA profiles were also altered by the SK additives indicating a different susceptibility of the rumen microorganisms to these additives. The EO and EX had certain similarities in their impacts on the VFA molar proportions as both enhanced acetate proportion (at their high dose), reduced branched chain fatty acids and increased butyrate proportion. Enhancement of propionate proportion by the medium and to a lesser extent by low dose of EX might be in part a result of a higher availability of H_2 redirected from methane production pathway (because of partial inhibition of the rumen methanogenesis), consequently favouring propionate production (Patra and Yu, 2013; Ungerfeld, 2015). These results are in line with previous research indicating that the inhibition of methanogenesis or partial suppression of protozoa had a positive impact on the propionate proportion and propionate to acetate ratio (Patra et al., 2006; El-Zaiat and Abdalla, 2019; Torres et al., 2020). However, the susceptibility of propionate producing bacteria to higher doses of SK additives seems to have counteracted the advantage of excess reducing equivalents for propionate production. The increased proportion of butyrate with EX and especially with high dose of EO was consistent with earlier studies reporting an increased proportion of

butyrate when using flavonoids (Oskoueian et al., 2013) and carvacrol at doses higher than 300 mg/L in the culture (Benchaar et al., 2007; Calsamiglia et al., 2007; Olihoek et al., 2019). An increased proportion of butyrate as well as valerate with EO and to a lesser extent with EX in the current study might be associated to a restricted production of methane, because their production pathways are considered as alternative sink of H₂ to methanogenesis (Ungerfeld, 2015). Moreover, other research has revealed that some rumen fermentation modifiers (e.g. ionophores, essential oils and plant extracts) causing H₂ accumulation in the rumen may increase butyrate molar proportion through inhibition of butyrate utilizing bacteria (Van Nevel and Demeyer, 1996; Busquet et al., 2005; Tekippe et al., 2012).

A decrease in BCFVAs molar proportions with EO as well as EX was consistent with the improvement in MP and a decreased ammonia concentration, implying the possibility of higher incorporation of BCFVAs into microbial biomass when using the SK additives in the culture. Previous research has indicated that some essential oils and extracts have the potential to reduce BCFVAs through depressing protein degradation as well as inhibiting the deamination activity in the rumen (Cardozo et al., 2006; Castillejos et al., 2006). Therefore, a marked decrease in BCFVAs especially at the high dose of both EO and EX is most probably due to a result of depressive effect of these additives on protein as well as amino acids degradation in the culture. These results revealed that EX positively modified the rumen VFA pattern, which could improve feed energy utilization at doses up to 300 mg/L.

Conclusions

The results from this study revealed that both EO and EX have the potential to profoundly modify rumen functions in a dose-response manner. Both EO and EX improved rumen fermentation at intermediate doses through reducing methane production and enhancement of MP and EMPS; however, they exhibited a general negative impact on the rumen microbial activity at their high dose. Moreover, EX enhanced ruminal feed degradability and total VFA concentration, and altered positively the VFA pattern towards greater propionate proportion at 300 mg/L. These findings provide direct evidence that both EO and EX have great capacity as new feed additives for positively modulating rumen fermentation that need to be confirmed by *in vivo* experiments.

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Ethics approval and consent to participate

All the experimental protocols and procedures, and care of the fistulated animals were approved by the Bu-Ali Sina University Animal Care and Use Committee (Ref No: 397/1139).

Conflict of interest

The authors declare that there is no conflict of interest associated with this manuscript.

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