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Effects of black seed oil and *Ferula elaeochytris* supplementation on ruminal fermentation as tested *in vitro* with the rumen simulation technique (Rusitec)

F. Klevenhusen[A,D,E], *K. Deckardt*[A,E], *Ö. Sizmaz*[B], *S. Wimmer*[A], *A. Muro-Reyes*[C], *R. Khiaosa-ard*[A], *R. Chizzola*[A] *and Q. Zebeli*[A]

[A]Institute of Animal Nutrition and Functional Plant Compounds, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Veterinaerplatz 1, 1210 Vienna, Austria.
[B]Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Ankara, Turkey.
[C]Faculty of Veterinary Medicine and Zootechnique, Autonomous University of Zacatecas, Zacatecas, Mexico.
[D]Corresponding author. Email: fenja.klevenhusen@vetmeduni.ac.at
[E]F.K. and K.D. contributed equally to this work.

Abstract. Plant bioactive compounds are currently viewed as possible feed additives in terms of methane mitigation and improvement of ruminal fermentation. A range of analyses, including the botanical characterisation, chemical composition and *in vitro* efficiency, have to be conducted before testing the compounds *in vivo*. Therefore, the aims of this study were (1) to identify the main bioactive components of black seed (*Nigella sativa*) oil (BO) and of the root powder of *Ferula elaeochytris* (FE), and (2) to investigate their effects on ruminal fermentation *in vitro*, when supplemented in different dosages to a diet (1 : 1, forage : concentrate), using the rumen simulation technique (Rusitec). Main compounds of BO were thymoquinone and *p*-cymene and α-pinene in FE. Supplementation of the diet with BO and FE did not affect concentration of volatile fatty acids but ammonia concentrations decreased with both supplements ($P < 0.001$). No effects of supplements on protozoal counts were detected but *in vitro* disappearance of DM and organic matter tended to increase with 50 mg/L FE ($P < 0.1$), compared with the control.

Additional keywords: essential oils, methane, volatile fatty acids.

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Introduction

During the last decades a substantial amount of research has been conducted on the role of secondary plant compounds in modulating ruminal fermentation (Calsamiglia *et al*. 2007; Hart *et al*. 2008). Several experiments suggested that the addition of plants or plant extracts rich in bioactive compounds to ruminant diets might have beneficial effects on ruminal fermentation, thus enhancing degradation processes and, at the same time, mitigating ruminal methane (CH_4) formation (reviewed by Calsamiglia *et al*. 2007; Benchaar *et al*. 2008; Hart *et al*. 2008). This research has involved a large number of essential oils, including single active compounds of diverse essential oils. In a meta-analysis, Klevenhusen *et al*. (2012) summarised results of 354 different treatments coming from 20 *in vitro* studies that involved ~70 bioactive compounds tested in the past 7 years. The majority of these studies were conducted with 24-h batch incubation *in vitro* experiments, and fewer studies tested phytochemical compounds as ruminant feed additives with continuous culture systems (e.g. Cardozo *et al*. 2004; Castillejos *et al*. 2006; Soliva *et al*. 2011). The rumen simulation technique (Rusitec) was shown to be a suitable technique for detecting mid-term effects up to 20 days of incubation of bioactive compounds on *in vitro* ruminal CH_4 formation, fermentation variables and nutrient degradability (Soliva *et al*. 2004). Although these studies have provided interesting information about the role of bioactive compounds *in vitro*, many of the existing literature focussed on only a small range of compounds [e.g. thymol, carvacrol, eugenol, extensively reviewed by Hart *et al*. (2008) and Benchaar and Greathead (2011)]. The effects of until now little explored plant extracts and plants, such as black seed (*Nigella sativa*) oil (BO) and the dried root powder of *Ferula elaeochytris* (FE), have not been tested in ruminant feeding so far, although *in vitro* and other animal studies (Sahinler *et al*. 2005; Canogullari *et al*. 2009) have suggested them as promising candidate sources of bioactive compounds because of their antimicrobial and strong antioxidative properties (Burits and Bucar 2000; Ahmad *et al*. 2013). A study with growing goats revealed beneficial effects of *N. sativa* seeds on bodyweight gain and immune status (Habeeb and El-Tarabany 2012).

Only recently, the importance of a detailed characterisation of plants and their bioactive compounds was pointed out and a

five-stage program for the nutritional and safety assessment of phytogenic substances as ruminant feed additives was introduced by Flachowsky and Lebzien (2012). According to this program, the plants and bioactive compounds should be characterised and chemically analysed before being further investigated *in vitro* in a dose–response trial to be finally tested *in vivo*. Therefore, the objective of this study was to analyse and identify the main bioactive compounds of BO and FE in detail and to perform a first screening of their effects on ruminal fermentation parameters and CH_4 production *in vitro* and to additionally analyse the degradation products after 3 h of incubation using a continuous culture approach with the Rusitec system.

Materials and methods

Experimental diets

The diet consisted of a 0.48 : 0.52 ratio of meadow-grass rich hay (second cut, beginning of shooting) to concentrate on a dry matter (DM) basis. Concentrate was composed of barley grain (355 g/kg diet DM), soybean meal (152 g/kg diet DM) and a vitamin-mineral mixture for dairy cows[1] (15 g/kg diet DM). The chemical composition per kg DM of the basal diet was 908 g organic matter, 195 g crude protein, 315 g neutral detergent fibre (NDF) and 230 g acid detergent fibre (ADF). The net energy of lactation content of 7.0 MJ/kg DM was calculated using the values of LfL (2011). Experimental treatments consisted of the diet (pure considered as control; CON) supplemented with either 50 or 500 mg/L of BO or of the root powder of FE. The major components of the test supplements are given in Table 1. Daily BO was added to the diet using a micropipette. *Ferula elaeochytris* root powder was weighed using an analytical balance. All substances were thoroughly mixed with the diet (12.0 g DM) shortly before the morning feeding procedure. Dosages equalled 2.92 and 29.2 mg/g dietary DM and are considered as small and moderate in comparison to other *in vitro* screening studies (e.g. Busquet *et al.* 2005; Castillejos *et al.* 2006). *F. elaeochytris* roots were harvested at the stage of flowering at Hatay district (South of Turkey) at 1000–1200 m above sea level, dried under shelter and subsequently ground for homogenous mixing with feed. Black seed oil was a commercial product obtained in Turkey. Since the products are not standardised, much emphasis was put on the analysis of the volatile compounds (see below; Table 1). As analysed (Suter *et al.*1997; analytical method see below), BO contained 58.7% linoleic acid (18:2 n-6), 24.1% oleic acid (18:1 n-9), 9.4% palmitic acid (16:0), 3.3% stearic acid (18:0), and 3.1% eicosadienoic acid (20:2 n-6) in total fatty acids, which is in good agreement with others (Kilic *et al*. 2011; Piras *et al*. 2013). Total amount of fatty acids in BO was 61.2%.

Table 1. Composition of the volatile fractions present in black seed oil and *Ferula elaeochytris* root powder calculated from the FID signal

For the individual compounds, the same response was assumed as for the internal standard camphor. RI, retention index; Extract, by ethanol extraction; SPME, solid phase microextraction; MD, microdistillation; traces, <0.001

Fraction	RI	Black seed oil		*Ferula elaeochytris*	
		Extract (mg/g)	SPME (mg/mL)	MD (mg/g)	SPME (mg/g)
α-thujene	929	0.527	0.578	–	–
α-pinene	939	0.128	0.141	2.167	2.847
Camphene	954	–	–	Traces	0.011
Sabinene	976	0.048	0.090	–	–
β-pinene	981	0.117	0.191	0.085	0.125
Myrcene	991	–	–	–	0.010
Decane	1000	–	Traces	–	–
p-cymene	1028	1.208	3.447	–	Traces
Limonene	1034	0.129	0.284	Traces	0.037
1,8-cineol	1035	–	–	–	–
γ-terpinene	1062	0.037	0.105	–	–
trans-pinocarveol	1144	–	–	–	0.007
α-campholenal	1132	–	–	0.012	–
cis-verbenol	1147	–	–	0.016	–
trans-verbenol	1151	–	–	0.048	–
Pinocarvone	1168	–	–	–	0.004
Terpinen-4-ol	1181	Traces	0.050	Traces	0.007
Myrtenol	1201	–	–	0.010	0.007
Verbenone	1216	–	–	0.015	0.016
trans-carveol	1223	–	–	–	Traces
Cuminaldehyde	1245	–	0.049	–	–
Carvone	1249	–	0.025	–	–
Thymoquinone	1257	1.713	4.831	–	–
Bornyl acetate	1291	–	0.016	Traces	0.013
Carvacrol	1304	0.069	0.081	–	–
Elemene	1333	–	–	0.011	–
α-longipinene	1359	0.023	0.068	–	–
α-copaene	1382	–	–	–	0.010
Longifolene	1415	0.100	0.265	–	–
β-caryophyllene	1428	–	Traces	–	–
Aristolene	1430	–	–	0.033	0.254
α-guaiene	1434	–	–	–	0.068
α-patchoulene	1439	–	–	–	0.046
Aromadendrene	1448	–	–	–	0.04
Farnesene	1460	–	–	0.056	0.278
ar-curcumene	1487	–	–	–	0.040
β-bisabolene	1512	–	–	–	0.056
Elemicine	1560	–	–	0.021	–
Thymohydroquinone	1564	0.077	–	–	–
Globulol	1572	–	–	0.018	–
Spathulenol	1589	–	–	0.012	–
Ledol	1596	–	–	0.062	–

Experimental procedure

With a rumen stimulation technique (Rusitec), three experimental runs were conducted, with each comprising 10 fermenters, except in the second run only five fermenters were used. Each run lasted for 10 days and included all dietary treatments in a randomised design, Run 2 and 3 even in duplicate. Every fermenter, considered as single experimental unit, had a capacity of 800 mL. Artificial saliva (McDougall 1948) was continuously

[1]Contained (g/kg): Ca, 187; P, 40; Mg, 40; Na, 110; Zn, 6.6; Mn, 4.5; I, 0.083; Se, 0.04; Co, 0.1; Cu, 1; vitamin A, 800 000 (IU); vitamin D_3, 75 000 (IU), vitamin E, 2.

infused at a rate of 326 mL/day (±19.2) using a 12-channel peristaltic pump (Model ISM932, ISMATEC, IDEX Health & Science GmbH, Wertheim, Germany). Regular vertical movements of the feed containers (~7 cm and 8 cycles/min) were ensured by an electric motor. On the first day of each experimental run every fermenter was inoculated with ruminal fluid and solid digesta obtained randomly from two out of three rumen-cannulated dairy cows (all non-lactating Brown Swiss) kept at the Clinic for Ruminants at the University of Veterinary Medicine, Vienna. Cows were kept according to the Austrian guidelines for animal welfare.

As suggested by Soliva and Hess (2007), the ruminal fluid of the two cows was mixed and filtered through four layers of medical gauze (~1-mm pore size). Afterwards 600 mL of ruminal fluid and 100 mL of artificial saliva were filled into each fermenter. A pair of nylon bags (120 × 65 mm, 150-µm pore size, Linker Industrie-Technik, Kassel, Germany) was added to every fermenter, one containing the experimental diet, the second bag containing solid ruminal digesta from the donor cows. On the second day, bags with the digesta were replaced with a fresh bag containing the respective experimental diet. Experimental diets were ground to pass a 2-mm sieve before feeding. Accordingly every bag containing the experimental diet was incubated for 48 h, and was afterwards replaced with a new one. Every day after exchanging the feed bags the system was flushed with N_2 gas for 3 min in order to maintain the anaerobic milieu. The incubation temperature was kept constant at 39.5°C.

Sample collection and laboratory analyses

Daily fermenter fluid samples were taken directly from the fermenters via a three-way valve using a syringe equipped with a plastic tube 1 h before the feeding procedure and analysed for pH, redox potential, and NH_3 concentration using the respective electrodes (Inlab Expert Pro-ISM pH, Inlab Expert 501 Redox and ammonia electrode type 15 230 3000, Mettler-Toledo GmbH, Schwerzenbach, Switzerland) connected to a pH-meter (Seven Multi TM, Mettler-Toledo GmbH, Schwerzenbach, Switzerland). Numbers of ciliate protozoa, classified into entodiniomorph and holotrich, were counted by light microscopy using Bürker counting chambers (0.1 mm depths; Blau Brand, Wertheim, Germany). Before counting, microbial samples were fixed with Hayem solution (mg/mL; $HgCl_2$, 2.5; Na_2SO_4, 25; NaCl, 5.0). In addition, part of the fermenter fluid samples was frozen at −20°C to determine the concentration of volatile fatty acids (VFA). The VFA (i.e. acetate, propionate, *iso*-butyrate, *n*-butyrate, *iso*-valerate and *n*-valerate) were analysed by gas chromatography (GC). For this, fermenter fluid samples were centrifuged at 20 000*g* for 20 min at 20°C. The supernatant (0.8 mL) was transferred into a fresh tube and 0.2 mL of orthophosphoric acid and 0.2 mL of the internal standard (4-methylvalerian acid) were added. This mixture was again centrifuged at 20 000*g* for 20 min at 20°C in order to remove precipitated substrates. Consequently the supernatant was analysed for VFA concentrations via GC (Model 8060 MS DPFC, No.: 950713, Fisons, Rodena, Italy). The GC was equipped with a flame-ionisation detector (FID) and a 15 m × 0.530-mm capillary column (SN US46185178, J&W Scientific, Folsom, CA, USA). Temperatures of injector and detector were 170 and 190°C, respectively. Helium was used as carrier gas with a flow rate of 1 mL/min. Stratos Software (Stratos Version 4.5.0.0, Polymer Laboratories, Church Stretton, UK) was used for designing the chromatograms. Additional fermenter fluid samples were obtained on Day 9 of incubation from each Rusitec fermenter 2–3 h after the feeding procedure in order to examine concentrations of the main plant secondary compounds. The samples from all CON treatments as well as the samples containing the high dosages 500 mg/L BO or 500 mg/L FE were used for analysis.

Every day fermentation gases were collected in gas-tight aluminium bags (TECOBAG 8 L, Tesseraux Spezialverpackungen, Bürstadt, Germany) for 24 h. Gas volume was determined using the water displacement technique (Soliva and Hess 2007) and concentrations of CH_4 and CO_2 were analysed with an infrared detector (ATEX Biogas Monitor Check BM 2000, Ansyco, Karlsruhe, Germany).

After incubation for 48 h feed bags were rinsed under cold water until the water was clear and frozen at −20°C in order to determine the chemical composition of the constituents. Prior to analysis, the feed residues of the last 5 days were pooled together, oven-dried at 50°C for 48 h, and ground through a 0.75-mm sieve. The ground samples were analysed for DM by oven drying at 104°C for 4 h and ash by combustion of samples over night at 580°C. Crude protein was analysed by the Kjeldahl method (VDLUFA 2007). Contents of NDF and ADF of the feed components and the feed residues were analysed using the Fibretherm FT12 (C. Gerhardt GmbH & Co. KG, Königswinter, Germany) according to the company's protocols, whereof the method for NDF analysis is based on VDLUFA (2007) including heat-stable amylase and NDF values are reported exclusive of residual ash. The method for ADF analysis is based on VDLUFA (2007) and the official method AOAC 973.18 (AOAC International 1997). Disappearance of the nutrients was calculated from the difference between the feed and the feed residues after 48 h of incubation in the fermenters.

Black seed oil was analysed for the total amount of fatty acids and the long-chain fatty acid composition according to Suter *et al*. (1997) with slight modification in terms of internal standard. Briefly, 20 mg of oil was added to 2.5 mL of mixed internal standard of nonanoic acid methyl ester (9:0) and triundecanin (11:0) dissolved in 1.4 mL dioxane. Transesterification was carried out by adding 2.5 mL sodium methylate solution and vortexing for 3 s. The reaction lasted for 60 s before 2 mL of heptane were added followed by 5 mL of disodium citrate solution. After phase separation had occurred, the upper phase was collected for fatty acid analysis. The fatty acid composition was determined by GC (model GC-2010plus, Shimadzu, Japan) equipped with a FID and a 30 m × 0.25 mm × 0.25-µm capillary column (Zibron ZB-Wax plus, Phenomenex LTD, Germany). Helium was used as a carrier gas with a flow of 1.1 mL/min. A mixed fatty acid methyl ester standard (Supelco 37 Component FAME Mix, Supelco, Bellefonte, PA, USA) was used for the identification and the quantification of fatty acids, which was calculated from the known amounts of the internal standard fatty acids.

Analyses of plant secondary compounds of the test substances and in fermenter fluid samples

The volatile compounds of the test supplements were generated by two different methods. Black seed oil was extracted with ethanol, while the solid FE was subjected to microdistillation. Additionally the products as well as fermenter fluid samples, were analysed by a solid phase microextraction method (SPME) followed by GC and GC–mass spectrometry (MS) analysis.

Microdistillation

Distillation was carried out using the automatic microdistillation unit MicroDistiller (Eppendorf, Hamburg, Germany). About 0.2–0.3 g FE and 10 mL distilled water were filled into the sample vials. The collecting vial, which contained 1 mL of water, 0.5 g of NaCl and 300 μL of *n*-hexane with hexadecane (0.2 mg/mL) used as internal standard was connected to the sample vial with a capillary. The heating program applied to the sample vial was 15 min at 108°C and then 45 min at 112°C. The collecting vial was kept at −2°C, where the volatiles were trapped in 0.3 mL *n*-hexane.

Extraction with ethanol

As BO contained a large proportion of fatty oils, volatile compounds were extracted with ethanol as follows: 0.3 g of the original oil were added to 3 mL ethanol and kept for at least 4 h at −18°C. One millilitre of the resulting ethanol layer was taken, 100 μL of hexadecane (0.2 mg/mL) were added to hexane and subsequently analysed by GC and GC–MS.

SPME of bioactive compounds

Black seed oil, FE and fermenter fluid samples were analysed by SPME followed by GC and GC–MS. The samples were incubated in tightly sealed 10-mL vials. The SPME fibre (polydimethylsiloxane–divinylbenzene, PDMS–DVB, Supelco) was exposed for 30 min at 50°C while stirring to the headspace of the sample. Afterwards the fibre was introduced into the injection port of the GC system and desorbed for 3 min at 250°C. Camphor in methanol was used as internal standard. According to its nature the samples were prepared in the vials as follows: 20 mg of ground FE root powder and a filter paper disc soaked with 10 μL of camphor (0.1 mg/mL in methanol) were incubated in the extraction vial. Two microlitres of BO were incubated together with 1 mL of distilled water, 1 g of NaCl and 20 μL of camphor (10 μg/mL). For analysis of the fermenter fluid samples 1 g of NaCl and 20 μL of camphor (10 μg/mL) were added to 1 mL fluid. A stock solution containing 2.0 μg/mL of α-pinene, 2.2 μg/mL of limonene, 2.0 μg/mL of thymol and 22 μg/mL of thymoquinone in methanol was prepared as calibration standard in order to quantify these components in the samples. Five concentration steps were achieved by mixing 1 mL Rusitec fluid from a CON fermenter with 5–100 μL stock solution, 1 g NaCl and 20 μL camphor (10 μg/mL).

GC and GC–MS

The analyses were carried out on an Agilent Technologies 7890A GC equipped with a 5975 C quadrupole mass selective detector, a FID and a CTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). The separation was done on a 30 m × 0.25-mm fused silica column coated with 0.25 μm HP5-MS. The compounds eluting from the column were distributed with a Deans switch at equal proportions to the detector of the mass spectrometer and FID. The total ion current (m/z 40–400) from the detector of the mass spectrometer was used to identify the compounds according to their mass spectra and their retention indices (McLafferty 1989; Adams 2007). For the quantification of α-pinene, limonene, thymoquinone and thymol the instrument was operated in the selected ion modus. This allowed monitoring the ions 77 and 93, 68 and 93, 121 and 164, 135 and 150 as well as 81, 95 and 108 at the corresponding retention times for α-pinene, limonene, thymoquinone, thymol and camphor, respectively. Helium was used as a carrier gas at a constant flow rate of 1.4 mL/min, with an injector temperature of 250°C. The temperature program consisted of three different temperature levels. Temperature was held 1 min at 50°C, followed by 5°C/min up to 220°C, finally rose to 280°C with 15°C/min. For the SPME analyses the split ratio was set at 10 : 1. The injection volume of liquid samples, generated by microdistillation or extraction with methanol, was 1 μL with a split ratio of 100 : 1. The FID was operated at 250°C and supplied with 30 mL/min H_2 and 300 mL/min air.

Statistical analyses

Mean values of the duplicate treatments in Runs 1 and 3 were calculated, thus in total three replicates per treatment were obtained by conducting three experimental runs, each lasting for 10 days. Since the first 5 days of each experimental run were considered as an adaptation period, mean values of the last 5 days per treatment and run were submitted to ANOVA using the mixed procedure of SAS (Version 9.2, SAS Institute, Cary, NC, USA). Dietary treatment served as fixed effect, while experimental run was considered as random factor. Multiple comparisons among means were conducted using Tukey's method. To test the linear influence of dosage level the orthogonal polynomial contrasts with the CONTRAST statement of SAS were used. Differences at $P < 0.05$ were declared significant; a tendency was considered up to $0.05 \leq P < 0.10$.

Results

Chemical composition of the test substances and fermenter fluid samples

The composition of the volatile fraction of the substances is shown in Table 1. The main essential oil compound of FE was 2.17 mg/g α-pinene on a DM basis. The volatile fraction of the BO contained thymoquinone (4.83 mg/mL) and *p*-cymene (3.45 mg/mL) as main compounds followed by α-thujene. Thymoquinone showed a low affinity to the fibre and low reproducibility when assessed by SPME. It was not possible to detect concentrations below 500 ng/mL.

Fluid samples from fermenters with the CON treatment displayed traces of 1.8-cineol and α-terpineol as volatiles 2–3 h after feeding possibly originating from the hay of the basal diet. In the fermenter fluid samples with 500 mg/L BO no thymoquinone and *p*-cymene could be recovered. Instead 400–700 ng/mL thymol were detected, a compound that

originally was not present in BO. In the fermenter fluid samples, which had been supplemented with 500 mg/L FE, 3–4 ng/mL α-pinene could be measured and traces of limonene and 1.8-cineol were detected.

Fermenter fluid traits, nutrient disappearance, and CH_4 formation

Fermenter fluid samples had similar pH and redox potential values with an average of 6.93 and −211 mV, respectively (Table 2). Ammonia concentration of the fermenter fluid significantly decreased with both BO and FE compared with CON ($P < 0.001$) with a linear dosage effect ($P < 0.001$). No differences were found among treatments with regard to total VFA (69.8 mmol/L). However, dietary supplementation had an effect on propionate proportion, with a lower propionate proportion found with 50 mg/L FE compared with 500 mg/L BO ($P < 0.05$) and an in-tendency linear effect of the dosage level added. *n*-butyrate proportion of total VFA was lower with 500 mg/L BO compared with 50 mg/L FE ($P < 0.05$) but it was not different to FE at the higher dose. The contrast analysis revealed that the proportion of *iso*-butyrate tended to increase with all BO and FE supplementations compared with CON ($P < 0.1$). No treatment effects were found in numbers of ciliate protozoa, both Holotrichs and Entodiniomorphs.

Contrast analysis revealed no differences in gas production between CON vs BO and CON vs FE, but with Tukey's method CH_4 production was significantly higher ($P < 0.05$) with 500 mg/L FE compared with 50 mg/L BO (Table 3). Albeit contrast analysis showed no differences between treatments, comparison of the mean with Tukey's method revealed that DM and organic matter disappearances in tendency increased with 50 mg/L FE compared with CON ($P < 0.1$), other treatments had intermediate values.

Table 2. Effects of black seed oil and *Ferula elaeochytris* root powder on fermentation traits in the Rusitec fermenter fluid ($n = 3$)

CON, control; Dosage, linear dosage effect obtained from orthogonal polynomial contrasts; CON vs BO, contrast analysis testing control vs 50 mg/L black seed oil and 500 mg/L black seed oil; CON vs FE, contrast analysis testing control vs 50 mg/L *Ferula elaeochytris* and 500 mg/L *Ferula elaeochytris*. Within rows, means followed by the same letter are not different (Tukey, $P = 0.05$). *<0.1; **<0.05; ***0.001; n.s., not significant

Fermentation trait	CON	Black seed oil (BO)		*Ferula elaeochytris* (FE)			*P*-value	Significance	
	0 (mg/L)	50 (mg/L)	500 (mg/L)	50 (mg/L)	500 (mg/L)	s.e.m.	Dosage	CON vs BO	CON vs FE
pH	6.95	6.91	6.92	6.94	6.93	0.085	0.068	n.s.	n.s.
Redox potential (mV)	–224	–207	–218	–209	–198	18.9	0.324	n.s.	n.s.
Ammonia (mmol/L)	18.4a	15.2b	16.0b	15.1b	14.5b	2.05	<0.001	***	***
Volatile fatty acids (mmol/L)	68.3	71.0	71.1	67.0	71.8	4.35	0.258	n.s.	n.s.
Molar proportions (% of total volatile fatty acid)									
Acetate	52.1	51.7	51.2	51.4	51.2	0.479	0.101	n.s.	n.s.
Propionate	18.0ab	18.6ab	19.3a	17.3b	18.9ab	0.819	0.091	n.s.	n.s.
iso-butyrate	1.01	1.12	1.04	1.10	1.06	0.038	0.256	*	*
n-butyrate	14.3ab	14.2ab	13.8b	15.1a	13.9b	0.985	0.225	n.s.	n.s.
iso-valerate	4.13	4.00	3.95	4.20	4.17	0.131	0.671	n.s.	n.s.
n-valerate	10.4	10.4	10.7	11.0	10.8	0.145	0.137	n.s.	n.s.
Acetate : propionate	2.91	2.79	2.66	2.97	2.73	0.125	0.056	n.s.	n.s.
Protozoal counts ($\times 10^3$/mL)									
Holotrichs	0.190	0.127	0.253	0.190	0.380	0.168	0.332	n.s.	n.s.
Entodiniomorphs	10.4	9.17	9.80	10.1	10.4	5.82	0.765	n.s.	n.s.

Table 3. Effects of black seed oil and *Ferula elaeochytris* root powder on *in vitro* gas formation and nutrient disappearance

CON, control; Dosage, linear dosage effect obtained from orthogonal polynomial contrasts. Contrast analysis between CON vs BO and CON vs FE revealed no significance. Within rows, means followed by the same lower-case letter are not significantly ($P > 0.1$) different (Tukey), and means followed by different upper-case letters tend ($0.05 < P < 0.1$) to differ (Tukey)

Parameter	CON	Black seed oil (BO)		*Ferula elaeochytris* (FE)			*P*-value
	0 (mg/L)	50 (mg/L)	500 (mg/L)	50 (mg/L)	500 (mg/L)	s.e.m.	Dosage
CH_4 (mL/day)	45.2ab	41.3b	43.6ab	43.6ab	52.3a	3.17	0.405
CO_2 (mL/day)	325	303	334	319	372	20.5	0.227
Apparent nutrient disappearance (proportionately to supply)							
Dry matter	0.519Z	0.526YZ	0.533YZ	0.542Y	0.525YZ	0.039	0.161
Organic matter	0.501Z	0.505YZ	0.514YZ	0.522Y	0.507YZ	0.041	0.527
Crude protein	0.539	0.541	0.558	0.565	0.547	0.057	0.552
Neutral detergent fibre	0.280	0.311	0.310	0.301	0.291	0.041	0.387
Acid detergent fibre	0.235	0.254	0.279	0.264	0.209	0.039	0.767

Discussion

To our best knowledge, this is the first time that BO and FE were tested in a Rusitec system for their effects on ruminal CH_4 formation and rumen fermentation traits. Other studies have demonstrated them as promising sources of bioactive compounds because of their antimicrobial and strong antioxidative properties (Burits and Bucar 2000; Ahmad *et al*. 2013), and therefore they were chosen as test substances to investigate their effects on rumen fermentation variables using the continuous culture system Rusitec.

Effects of black seed oil on in vitro *fermentation variables and CH_4 formation*

In the present experiment bioactive compounds of BO extracted from the seeds of *N. sativa* have been found to be mainly thymoquinone and *p*-cymene, which is in agreement with others (Burits and Bucar 2000; Ahmad *et al*. 2013; Piras *et al*. 2013). Kilic *et al*. (2011), who conducted an *in vitro* gas production test with the essential oil of *N. sativa*, considered long-chain fatty acids as main bioactive compounds. These included linoleic acid (51%), oleic acid (23%) and palmitic acid (16%), which is in agreement with our analysis. They also found 0.03% cavacrol, a higher proportion than found in the present experiment.

When tested in dosages of 50–150 mg/L (Kilic *et al*. 2011) they found no effects on *in vitro* gas production. Although BO contained a large amount of fatty acids it had no major effect on fermentation variables in the present study. Lipids, including long-chain fatty acids, have been shown to inhibit DM intake and fibre digestion when exceeding 8% in dietary DM (Beauchemin *et al*. 2008), which was not the case in the present study. Several studies have demonstrated indirect and direct anti-methanogenic effects of fatty acids due to the possible inhibition of both H_2-producing cellulolytic bacteria (Lila *et al*. 2003) and protozoa (Wina *et al*. 2006; Goel *et al*. 2008), as well as the direct inhibition of methanogenic archaea (Soliva *et al*. 2004). Beauchemin *et al*. (2007) found that already a lipid supplementation of 3.3% of dietary DM decreased CH_4 production. However, the mode of action and extent of mitigation of methanogenesis not only depends on the amount but also on the type of fatty acids as well as the feed components present (Hook *et al*. 2010). Although BO contained a large proportion of polyunsaturated fatty acids, no effects of BO on CH_4 formation were found in the present study. The chosen dosages of 50 and 500 mg/L equalling 0.178 and 1.78% total fatty acids from BO in dietary DM, even lesser considering the amount of polyunsaturated fatty acids, might have been too low to significantly inhibit methanogenesis and higher dosages might result differently.

A recent *in vitro* study with mixed ruminal bacteria by Chaves *et al*. (2008) found that 20 mg/L pure *p*-cymene decreased CH_4 formation without negative effects on VFA production, suggesting that substances rich in *p*-cymene, such as the present BO, might be a promising feed additive in terms of CH_4 mitigation. In the present study ammonia concentrations decreased with BO, which was opposite to the finding of Chaves *et al*. (2008) who found that *p*-cymene supplementation caused a significant increase in ammonia concentration by promoting the microbial deaminative activity. Several studies reported inhibitory effects of some essential oils and their bioactive compounds on bacterial peptidolysis and deamination processes depending on the dosage level (e.g. Cardozo *et al*. 2004, 2005; Busquet *et al*. 2006) but opposite effects have been shown too (Cardozo *et al*. 2004; Castillejos *et al*. 2008).

Generally CH_4 synthesis in the rumen is negatively correlated with an increase of propionate proportion and a decline of the acetate : propionate ratio (Russell 1998). Propionate formation competes with CH_4 for hydrogen in the rumen, while acetate and butyrate are considered to promote CH_4 production (Moss *et al*. 2000). However, in the present study no significant differences in propionate or in CH_4 formation between BO and CON were found.

Pure BO volatiles contained thymoquinone and *p*-cymene as major bioactive components in this study. However, in the fermenter fluid samples with 500 mg/L BO, neither thymoquinone nor *p*-cymene were detected 3 h after feeding. *Para*-cymene is a known precursor of thymol in oregano (Poulose and Croteau 1978; Jerković *et al*. 2001) and in the fermenter fluid samples 400–700 ng/mL thymol were found, although originally thymol was not present in BO. The possibility of a conversion of thymoquinone into thymol in the rumen fluid medium needs further investigations as thymol is commonly considered as precursor of thymoquinone (Rasoul-Amini *et al*. 2011). Thymol is a main essential oil component of both thyme (*Thymus vulgaris*) and oregano (*Origanum vulgaris*) and has already been tested as feed additive on rumen fermentation in several studies (Evans and Martin 2000; Castillejos *et al*. 2006; Benchaar *et al*. 2007*a*, 2007*b*). Some studies could demonstrate a CH_4 mitigating potential of thymol when administered in high dosages, although it was mostly accompanied by decreased nutrient degradation (Evans and Martin 2000; Macheboeuf *et al*. 2008). Evans and Martin (2000) found an increased acetate : propionate ratio with thymol, when applied in dosages much higher (100–400 mg/mL) than the thymol concentration found with BO in the present study. Our results support the findings of Castillejos *et al*. (2006), who found no effects of thymol on ruminal fermentation when added in a low dosage of 50 mg/L. However, effects of thymol on ruminal fermentation might also depend on dosage, diet type and its chemical composition (Klevenhusen *et al*. 2012), and ruminal pH (Calsamiglia *et al*. 2007).

Effects of F. elaeochytris *root powder on* in vitro *fermentation variables and CH_4 formation*

Ferula (*Apiaceae*) is a genus with ~170 species of which many are applied in the traditional medicine in central and south-western Asia because of health promoting effects (Sahebkar and Iranshahi 2010). Essential oils of various *Ferula* species, containing as major component α-pinene, have been shown to exert selective antimicrobial effects (Kose *et al*. 2010). So far there is a lack in literature about the bioactive compounds in *F. elaeochytris* root powder. Başer *et al*. (2000) analysed the bioactive compounds in the essential oil of *F. elaeochytris* fruits and found nonane, α-pinene and germacrene B as main compounds. The majority of bioactive compounds in the essential oil of fruits, seeds, roots etc. of various *Ferula*

species were found to be terpenoid compounds with α-pinene, β-pinene, myrcene and limonene (among monoterpene hydrocarbons), linalool, terpineol and neryl acetate (among oxygenated monoterpenes), β-caryophyllene, germacrene B, germacrene D and δ-cadinene (among sesquiterpene hydrocarbons) and caryophyllene oxide, α-cadinol, guaiol and spathulenol (among oxygenated sesquiterpenes) being the major ones (Sahebkar and Iranshahi 2010). In the present experiment the main bioactive compound of FE was identified as the monoterpene α-pinene.

No significant effect of FE on CH_4 formation was observed, which is in agreement with Crane *et al*. (1957), who found no CH_4-mitigating effect of α-pinene *in vitro*. They even observed a depression of fibre degradation, which is in contradiction to the promoting effect on microbial activity observed by Oh *et al*. (1967). In the present experiment supplementation with 50 mg/L FE tended to increase *in vitro* DM and organic matter disappearance compared with CON, which supports the observations of Oh *et al*. (1967). In the present study FE supplementation decreased NH_3 concentration in the fermenter fluid samples. According to published literature (Calsamiglia *et al*. 2007; Hart *et al*. 2008; Benchaar and Greathead 2011), the effects of bioactive compounds on VFA production and ammonia concentrations are highly variable, but both seem to decrease with increasing level of bioactive compounds (Klevenhusen *et al*. 2012). Indeed, a recent meta-analysis indicated that responses of total VFA and particularly ammonia were more pronounced with dosages of bioactive compounds exceeding 100 mg/g (Klevenhusen *et al*. 2012), which, however, are unrealistic for *in vivo* conditions. The study by Malecky *et al*. (2009) investigated effects of a blend of monoterpenes, including linalool, *p*-cymene, α-pinene and β-pinene, in dairy goats. They observed no influence on rumen fermentation, probably due to intensive microbial degradation of the terpenes in the rumen. However, Broudiscou *et al*. (2007) observed strongly promoted maltose fermentation by the addition of α-pinene in batch culture, although α-pinene seemed to be easily degraded by ruminal microbes. In a different study, α-pinene and limonene showed low recovery when incubated for 24 h with rumen fluid (Haider 2004) supporting the theory of rapid microbial degradation. In the present study low concentrations of α-pinene were still detectable after 3 h of incubation. As mentioned by Malecky and Broudiscou (2009) monoterpenes are not only solubilised in the aqueous phase but likely also attach to feed particles or are volatised into the gas phase. Both alternatives have not been investigated in the present experiment but should be taken into consideration for interpretation of the results.

Conclusions

Overall, effects of BO and FE were minor, as no significant effects on nutrient degradation and CH_4 formation were observed. Nevertheless, supplementation of FE tended to increase nutrient degradation *in vitro*, when added at 50 mg/L. The lower NH_3 concentrations in the fermenter fluid samples in comparison to CON indicated some microbial manipulation by both substances. Although BO contains considerable amounts of long-chain fatty acids, known to possibly inhibit ruminal fibre degradation, no negative effects on fibre degradation, VFA concentration and protozoal counts were found with the low dosages provided. Further research testing higher doses of BO is warranted to investigate their potential as ruminant feed additive, and further studies need to show if the beneficial effect of FE on nutrient degradation is consistent when it is supplemented to different diet types and *in vivo*.

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