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Assessment of in vitro rumen fermentation patterns, gas formation and nutrient degradation of laurel oil

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Abstract: The purpose of this study was to determine the bioactive compounds of laurel oil and to evaluate the effect of different doses of laurel oil on rumen fermentation traits, gas formation and nutrient disappearance using in vitro rumen simulation technique. The trial was conducted with in vitro rumen by using Rusitec system and 2 treatments that is Laurel oil supplied at 50 mg/L and 100 mg/L. A basal diet consisted of a 0.48:0.52 ratio of hay and concentrate on a dry matter (DM) basis. The samples were analysed for fermentation parameters (pH, ammonia, volatile fatty acid (VFA), microorganism population and gas production) and the bioactive compounds of laurel oil and fermenter fluid samples were determined by gas chromatography (GC) and GC- (mass spectrophotometer) MS. Major constituents of the laurel oil was limonene (64.6%): Limonene did not affect the rumen fermentation, apparent nutrients degradation and methane emission. Total volatile fatty acid concentration was not affected but an increase was observed in isobutyrate proportion together with a quadratic influence in propionate proportion. Results showed that laurel oil supplementation did not affect negatively rumen fermentation and degradation of nutrients. Due to the data, it is considered that laurel oil could be used in ruminant diets, whilst this is the first detailed report on laurel oil affects in artificial rumen. Therefore, in vivo studies with laurel oil addition to the diets are warranted.

Keywords: Laurel oil, methane, short chain fatty acid, rusitec

Defne yağının in vitro rumen fermantasyon özellikleri, gaz formasyonu ve besin madde yıkımlanabilirliğinin belirlenmesi

Öz: Söz konusu çalışma, defne yağının biyoaktif bileşenini belirlemek ve farklı seviyelerde ilave edilen defne yağının in vitro rumen simülasyon tekniği ile fermentasyon parametreleri, gaz formasyonu ve besin madde yıkımlanabilirliği üzerine etkilerini ortaya koymak amacıyla gerçekleştirilmiştir. Çalışma, in vitro ortamda rumen sıvısı kullanılarak ve defne yağı katkısının farklı iki dozu denenerek yürütülmüştür. Söz konusu katkı maddesi rumen sıvısına 50 mg/L ve 100 mg/L olarak ilave edilmiştir. Temel rasyonun kaba yem konsantre:yem oranı 0.48:0.52 [kuru madde (KM) bazında] olarak ayarlanmıştır. Fermentasyon sonunda elde edilen rumen

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sıvısı örneklerinde fermentasyon parametreleri ölçülmüş, etken madde analizleri ise GC ve GC-MS cihazları kullanılarak gerçekleştirilmiştir. Defne yağının ana biyoaktif bileşeninin limonene (%64,6) olduğu tespit edilmiştir. Suni rumen ortamına ilave edilmiş olan Limonene fermentasyon parametrelerini, besin madde yıkımlanabilirliğini ve metan gazı emisyonunu etkilememiştir. Ayrıca toplam uçucu yağ asiti konsantrasyonu değişmezken izobütirat ve propiyonat oranının kuadratik olarak arttığı gözlemlenmiştir. Çalışmadan elde edilen sonuçlara göre defne yağının in vitro rumen fermentasyonuna ve besin madde yıkımlanabilirliğine olumsuz bir etkisi olmamıştır. Çalışmada elde edilen veriler neticesinde, defne yağının ruminant rasyonlarında kullanılabileceği düşünülmektedir ancak söz konusu çalışma defne yağının in vitro rumen fermentasyonuna etkisinde detaylı olarak gerçekleştirilen ilk çalışmadır. Bu nedenle defne yağının kullanılabilirliğinin ve etkilerinin belirlenmesi için in vivo çalışmalara ihtiyaç duyulmaktadır.

Anahtar Kelimeler: Defne yağı, kısa zincirli yağ asitleri, metan, rusitec

Introduction

One of the important greenhouse gas is methane (CH_4) and its concentration in the atmosphere has shown considerably increment in recent years (16, 19). Emissions of greenhouse gases around 5.4 billion tons derive from the agricultural sector with ruminal enteric fermentation representing a large proportion (around 39%) of emissions (11, 15). Previous studies reported that the antibiotics as feed additives improve the energy and protein availability and reduce methane emissions from ruminants. However, international legislation which has restrictions on the use of antibiotics promoted the investigate alternative feed additives, such as plant or plant extracts rich in bioactive compounds like essential oils (4, 20, 24, 28).

Due to the promising antimicrobial properties of the essential laurel leaf oil (LO), that compound was chosen for the present Rusitec trial. Laurel is a well-known medicinal herb and used in folk medicine in different countries for digestive disorders (e.g. impaired digestion, flatulence), rheumatic and abdominal pain (22). Crude extracts of laurel leaves possess antimicrobial properties (27), methanolic extract of *Laurus nobilis* seed oil has antioxidant (23) and the essential oil of laurel was shown to possess antifungal (2) as well as anti-inflammatory (25) characteristics.

In this study, the following hypotheses were tested: (a) laurel essential oil can be used as an effective natural dietary additive in ruminants' diets. (ii) The influence of LO on ruminal fermentation, protozoa and bacterial abundance, degradability, and CH_4 production. These hypotheses were tested with in vitro batch cultures using two levels of LO. In the current study, the degradation of nutrients in rumen and the potential antimethanogenic activity of LO were investigated.

Materials and Methods

Experimental design and diets: The trial was organized as a randomized block design with a block (Rusitec apparatus) and 2

treatments that is Laurel oil (LO) with 77.9% limonene as a main component (Table 1), supplied at 50 mg/L and 100 mg/L. A basal diet consisted of a 0.48:0.52 ratio of hay and concentrate on a dry matter (DM) basis. Concentrate was composed of barley grain (355 g/kg DM), soybean meal (152 g/kg DM) and a vitamin-mineral mixture for dairy cows[1] (15 g/kg DM). The chemical composition per kg DM of the basal diet was 908 g organic matter (OM), 195 g crude protein (CP), 163 g crude fibre (CF) and 17.9 MJ gross energy (GE). The test substances were mixed to the basal diet (12.0 g DM) shortly before the morning feeding procedure. To stimulate the result of the chewing activity of cow, the diet was dried and ground through a 2 mm screen.

Table 1: Bioactive compounds of LO and rumen fluid

Tablo 1: *Fermentasyon sonucu rumen sıvısının ve defne yağının biyoaktif bileşenleri*

	Laurel oil		
	RI	Extract	SPME
α-Pinene	939	6.6	1.9
Camphene	954		0.4
Myrcene	991		1.1
Decane	1000		0.6
α-Phellandrene	1006		traces
1,4-Cineol	1017		2.8
p-Cymene	1028	traces	1.7
Limonene	1034	77.9	64.6
1,8-Cineol	1035	9.0	4.0
γ-Terpinene	1062		1.1
α-Terpinolene	1090	6.6	7.0
Undecane	1100		1.5
Menthol	1176	traces	3.9
Allyl heptanoate	1181		2.5
Dodecane	1198		2.2
Tridecane	1300		1.8

RI = retention index; SPME = solid phase microextraction; Traces = < 0.1%

[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.

Rumen simulation technique: Rusitec apparatus equipped ten anaerobic fermenters with a volume of 800 ml which was developed by Czerkawaski and Breckenridge (9) was used in this experiment. On the first day of experiment, fermenter was inoculated with rumen fluid and solid digesta obtained from two non-lactating rumen-cannulated Brown Swiss cows fed hay *ad libitum* and maximum 0.5 kg of concentrate feed. 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) was added to the fermenter, one including the experimental diet, and the other bag containing solid rumen digesta for initial inoculation of the fermenters. On the second day of trial, bags with the digesta were replaced with a fresh bag containing the respective experimental diet. Therefore, each bag was incubated for 48 h.

Fermentation gases and end products: Fermentative gases were collected in gas-tight aluminum bags (Tecobag Tesseraux Container, Germany) for 24 h. Total gas volume was determined by using the water displacement technique. Concentrations of CH_4 and CO_2 were analyzed with an infrared detector (Atex Biogas Monitor Check BM 2000, Germany).

Rumen fluid samples were taken daily from each vessel to a plastic tube before feeding procedure. Samples were analyzed for pH-value, redox potential and NH_3 concentration by electrodes connected to a pH-meter (Mettler-Toledo, Switzerland). Bacteria and protozoa counts, distinguishing between Entodiniomorphs and Holotrichs, were determined with Bürker counting chambers (0.02-mm and 0.1-mm depths, respectively; Blau Brand®, Germany). A part of the fermenter fluid samples were collected to determine the concentration of VFA by gas chromatography (Fisons GC, Rodena, Italy). Additionally, the rumen fluid samples from fermenters were collected on the 9th day of incubation at post feeding to determine concentrations of the bioactive compounds of laurel oil. The experiment was lasted 10 days.

Degradation of nutrients: Disappearance of DM, OM and CP at 48 h were determined by Weender analysis. Feed bags of the experimental substrates were rinsed under cold water until the water was clear and frozen at −20°C. The feed residues were pooled together over the 5 days, oven dried at 50°C for 48 h and ground through a 0.75 mm screen for nutrients analysis according to the methods of the Association of Official Analytical Chemists (1). Samples were analyzed for DM by oven-drying at 105°C for 3 h, for ash by combustion of samples over night at 580°C and CP was determined by the Kjeldahl method.

Volatile compounds of laurel oil and in rumen fluid **:** Samples of LO were extracted with ethanol. Products as well as fermenter fluid samples, were analysed by a solid phase microextraction method (SPME) followed by GC-FID and GC-MS analysis (17). Samples were incubated for SPME method and the SPME fiber was exposed. Afterwards the fibre was introduced into the injection port of the GC system. Methanol was used as an internal standart. A stock solution which includes 2.0 µg/mL of α-pinene, 2.2 µg/mL of limonene, 2.0 *µg/mL of* thymol and 22µg/mL of thymoquinone in methanol was prepared as calibration standard in order to quantify these components in the samples.

Statistical analysis: Statistical analysis were performed using the procedure of the SPSS software, version 14.01 (SPSS Inc.,

Chicago, IL). Mean values of the measurement days were submitted to ANOVA. Multiple comparisons among means were conducted using Tukey test. To examine the increasing dosage of dietary LO level the polynomial (linear and quadratic) contrasts of SPSS were used. Significance was declared at $P < 0.05$; a tendency was considered for $0.05 < P \leq 0.10$.

Results

The chemical compositions of the LO are shown in Table 1. The main essential oil compound of LO was limonene accounting for about 78% as main compound followed by terpinolene, 1.8-cineol and α -pinene. In the fermenter fluid samples after 2 to 3 hour after feeding the diet with 50 mg/L LO, traces of α-pinene, limonene and 1,8-cineol could be detected.

Fermenter fluid samples had an average pH and redox potential of 6.98 and −226.03 mV, respectively (Table 2). No differences were

Table 2: Effects of graded LO on rumen fermentation in fermenter fluids

Tablo 2: *Defne yağının rumen fermentasyonuna etkisi*

	Dietary Treatment[1]				P-value[2]		
Item	CON	LO50	LO100	SEM	CON vs LO	L	Q
pH	6.98	6.94	7.01	0.03	0.461	0.394	0.288
Redox potential	-223.99	-200.63	-253.48	12.72	0.399	0.407	0.223
Ammonia (mmol/L)	13.36	12.76	14.18	0.85	0.877	0.755	0.652
Volatile fatty acids (mmol/L)	68.30	73.46	69.04	1.32	0.242	0.824	0.125
Molar proportions (%)							
acetate	35.47	37.76	35.01	0.65	0.249	0.781	0.109
propionate	12.41	13.90	11.81	0.45	0.233	0.605	0.099
iso-butyrate	0.69[b]	0.81[a]	0.77[ab]	0.02	0.018	0.064	0.039
n-butyrate	9.78	9.99	11.02	0.32	0.376	0.180	0.587
iso-valerate	2.83	2.97	2.85	0.10	0.844	0.954	0.596
n-valerate	7.11	8.03	7.60	0.24	0.282	0.452	0.234
acetate:propionate	2.90	2.72	2.96	0.08	0.568	0.783	0.305
Bacteria[3]	1.69	2.06	1.23	0.17	0.260	0.304	0.139
Holotrichs[4]	0.23	0.25	0.19	0.08	0.974	0.884	0.842
Entodiniomorphs[5]	11.23	17.06	4.01	2.82	0.307	0.346	0.165

[1]Control = basal diet (free of LO); LO50 = basal diet containing 50 mg/L LO; LO100 = basal diet containing 100 mg/L LO

[2] Tukey test and polynomial contrasts: L = linear, Q = quadratic effect of supplemental LO.

[3] x 10^9/ml; [4] x 10^3/ml; [5] x 10^3/ml

found between treatments in concentrations of ammonia (13.43 mmol/L; P=0.88) and total SCFA (70.27 mmol/l; P=0.242). However, dietary supplementation had a quadratically effect on propionate formation (P=0.099), with the lowest propionate proportion found with 100 mg/L LO (11.81% of total SCFA) and the highest with 50 mg/L LO (13.90% of total SCFA). As a percentage of total SCFA iso-butyrate had increased with the increasing dietary LO level (quadratic, P = 0.039). No treatment effects were found in numbers of bacteria 1.66×10^9/ml (P=0.260) and protozoa, including Holotrichs 0.16×10^3/ml (P=0.974) and Entodiniomorphs 10.77×10^3/ml (P=0.307). Supplements had no effect on DM degradation (on average 55.14%; P=0.962), OM (53.53%; P=0.972), and CP (57.92%, P= 0.924) (Table 3). Average CH_4 output (Table 3) was 32.73 ml/d (P=0.933). However, numerically CH_4 formation was lowest in 50 mg/L LO (−2.13% compared to control).

Table 3: Effects of LO on gas formation and nutrients degradation

Tablo 3: *Defne yağının gaz formasyonu ve besin madde sindirilebilirliğine etkisi*

	Dietary Treatment[2]				P-value[3]		
Item[1]	CON	LO50	LO100	SEM	CON vs LO	L	Q
Total volume (ml)	1424.73	1394.00	1552.46	47.51	0.540	0.360	0.423
O_2 (ml/d)	59.0	39.8	48.8	0.50	0.269	0.440	0.226
CO_2 (ml/d)	230.6	227.9	229.9	0.63	0.986	0.971	0.887
CH_4 (ml/d)	32.9	32.2	33.1	0.08	0.933	0.950	0.719
Degradation of nutrients (%)							
DM	55.07	54.52	55.82	1.43	0.962	0.866	0.807
OM	53.39	53.03	54.17	1.47	0.972	0.864	0.846
CP	57.91	56.39	59.45	2.39	0.924	0.834	0.716

[1] DM = Dry Matter; OM = Organic Matter; CP = Crude Protein

[2]Control = basal diet (free of LO); LO50 = basal diet containing 50 mg/L LO; LO100 = basal diet containing 100 mg/L LO

[3] Tukey test and polynomial contrasts: L = linear, Q = quadratic effect of supplemental LO.

Discussion and Conclusion

This experiment is the first to evaluate the influence of LO on rumen fermentation traits, gas production and microbial population by using the Rusitec system. The test substances dosages were 50 and 100 mg/L, which are equivalent to 2.92 and 5.84 mg/g DM. There were no differences were detected in concentrations of ammonia and total VFA indicating that no major disturbances of ruminal fermentation were caused by the test supplements. According to the literature reviews of Calsamiglia et al. (8), Hart et al. (14) and Benchaar and Greathead (3) influence of essential oils on VFA production and ammonia concentrations are highly variable, however both seem to reduce with increasing level of essential oil dosage. Thus, in the current trial, the dosages chosen might have been below the limit which negatively affects microbial nutrient degradation and, hence, VFA production. However, differences were found regarding the individual VFA iso-butyrate as well as the propionate.

In a previous study of García-Gonźalez et al. (12) leaves of *Laurus nobilis* were incubated in 24 h batch cultures and analyzed for possible antimethanogenic effects. However, no promising effects on CH_4 formation were found, which is in agreement with the present results. Limonene was the major constituent of pure LO, but in the fermenter fluid samples only traces were found of this monoterpen 3 h post feeding. This was probably a result of the continuous buffer flow and fermenter liquid dilution, as Broudiscou et al. (5) found limonene to be poorly degradable by ruminal microbes in batch culture with no effects on hexose degradation. Multiple terpens extracted from douglas fir needles, including also limonene, numerically showed little or no influence on rumen microbial activity (21). In the present study 50 mg/L of LO has already not effected ruminal bacterial abundance.

In 2006, Castillejos et al. (7) reported that decreased total VFA with limonene dosages above 50 mg/L indicating disturbed microbial fermentation. High concentrations of limonene were also associated with a decreased ruminal ammonia production in vitro (7) when applied at 5000 mg/L, a high dosage without practical relevance, and no effects on ammonia were found in the present study with LO. Limonene tended to reduce propionate proportions with 100 mg/L LO in the current study which reported similar value (26), whereas tended to higher with 50 mg/L LO. An increasing in propionate production might indicate the inhibition of CH_4 production due to hydrogen sink in the rumen after CH_4 (18). It could be the reason for the lowest methane concentration with 50 mg/L LO in this experiment. The tendency for quadratic increase in the molar proportion of isobutyrate reported herein has been previously observed in vitro rumen batch cultures with CTO *(Cymbopogon winterianus)* rich in d-limonene (6, 13).

There is lack of evidence concerning the effect of LO on antimicrobial activity resulted to ruminal fermentation traits, degradation of nutrients and methane formation can be explained by the fact that just maintained in

vitro method in the present study. One reason might be the conditions during the trial such as bag characteristics for degradation, incubation condition in the rumen. The possible effect that there is no in vitro investigation that will support the effects of LO can not be excluded. Dorman and Deans (10) previously indicated that the selective antimicrobial activity of limonene. However, in current study the amount of limonene applied to the fermenters was considerably lower.

In conclusion, GC-FID and GC-MS analyses of laurel essential oil showed the presence of limonene as a major bioactive compound (64,6%). Hence, this essential oil can also be used as a natural source of limonene in animal nutrition. In addition to composition of LO, because of no negative effects on rumen fermentation traits and degradation, it can be used as a feed additive in ruminant diets. Further in vivo and in vitro studies are warranted to understand the presence antimicrobial activity in ruminants.

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