

Effects of olive leaf extract on rumen microbial fermentation in *in vitro* semi-continuous culture system (RUSITEC)

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Summary: The aim of this study was to investigate the effects of olive leaf extract (OLE) on *in vitro* fermentation of a 50 : 50 forage : concentrate diet using the rumen simulation technique (RUSITEC) and to compare its effects with monensin addition. The Rusitec system consisted of eight vessels: two received 150 mg of olive leaf extract (OLE-H) daily, two received 15 mg of olive leaf extract (OLE-L), two received 5 mg of monensin (MON, positive control), and two received no additives (CTR). After an adaptation period of 7 days, the main fermentation parameters were determined for seven consecutive days. There were no significant differences between treatments either in pH or in organic matter degradability. MON significantly increased ($p < 0.05$) propionate production and significantly reduced ($p < 0.05$) acetate and butyrate productions, acetate to propionate ratio, total protozoa count and NH₃-N concentration. Compared to CTR, the addition of both concentrations of OLE significantly increased ($p < 0.05$) total volatile fatty acid (VFA) and propionate productions. However, OLE-H decreased ($p < 0.05$) butyrate production and total protozoa count. Based on results from this study, it is reasonable to conclude that olive leaf extract exerted beneficial effects on some fermentation parameters in the rumen-simulating semi-continuous culture system, which may improve fermentation efficiency in the rumen.

Key words: Fermentation, olive leaf extract, rumen

Zeytin yaprağı ekstraktının yarı-sürekli *in vitro* kültür sisteminde (RUSITEC) rumen mikrobiyal fermantasyonu üzerine etkileri

Özet: Bu araştırmada rumen simülasyon tekniği (RUSITEC) kullanılarak zeytin yaprağı ekstraktının % 50 kaba yem % 50 konsantre yemden oluşan bir rasyonun *in vitro* fermantasyonu üzerine etkilerinin araştırılması ve bu etkilerin monensinin etkileri ile karşılaştırılması amaçlandı. Rusitec sistemde 8 fermenter kullanıldı: Bunlardan ilk ikisine günlük 150 mg (OLE-H), diğer ikisine ise günlük 15 mg (OLE-L) zeytin yaprağı ekstraktı ilave edildi. Kalan 4 fermenterden ikisine günlük 5 mg monensin (MON, pozitif kontrol) ilavesi yapılırken, son iki fermentere hiçbir ilave yapılmayıp kontrol (CTR) olarak kullanıldı. Yedi günlük adaptasyon fazından sonra, 7 gün boyunca temel fermantasyon parametreleri belirlendi. Deneme grupları arasında pH ve organik madde sindirilebilirliği açısından istatistiksel bir farklılık bulunmadı. MON propiyonat üretimini istatistiksel olarak belirgin bir şekilde artırırken ($p < 0,05$), asetat ve bütirat üretimleri, asetatın propiyonata oranı, protozoon sayısı ve NH₃-N konsantrasyonunu belirgin bir şekilde azalttı ($p < 0,05$). CTR ile karşılaştırıldığında, OLE'nin her iki konsantrasyonu da toplam uçucu yağ asidi (UYA) ve propiyonat üretimlerini istatistiksel olarak anlamlı bir şekilde artırdı ($p < 0,05$). Ancak OLE-H bütirat üretimi ve toplam protozoon sayısını azalttı ($p < 0,05$). Bu araştırmanın sonuçlarına dayanarak zeytin yaprağı ekstraktının yarı-sürekli rumen simülasyon kültür sisteminde bazı fermantasyon parametreleri üzerinde olumlu etkiler oluşturduğunu, dolayısıyla rumendeki fermantasyon verimliliğini artırabileceğini söyleyebiliriz.

Anahtar sözcükler: Fermantasyon, rumen, zeytin yaprağı ekstraktı

Introduction

Antibiotic feed additives, such as ionophore antibiotic monensin, have been used widely in ruminant production systems for many years, to improve daily gain and feed conversion rates. However, the use of antibiotics in animal nutrition has been prohibited in the European Union since January 2006 because of the potential for the selection of antibiotic-resistant bacterial strains by the risk of antibiotic residues in milk and meat products exists. Similar regulatory measures may be expected in the rest of the world. For this reason, there is

an increasing interest in evaluating 'natural' alternatives to modify rumen microbial fermentation (7, 14).

The olive (*Olea europaea* L.) leaf is known to be resistant in nature to microorganisms and insect attack, and much research has focused on the antimicrobial activity of compounds contained in olives and olive oil. Phenolic compounds including oleuropein, tyrosol, hydroxytyrosol, caffeic acid, gallic acid, syringic acid, p-coumaric acid and luteolin, isolated from olive leaves, have been shown to inhibit or delay the rate of growth of a range of microorganisms (11). Furthermore, olive leaf

extract and its individual constituents are considered safe and non-toxic for human and animal consumption (1, 17). However, the ability of olive leaf extract to influence microbial fermentation processes in the rumen have not been evaluated. The aim of this study was to evaluate the effects of two doses of olive leaf extract on ruminal fermentation in a long-term *in vitro* study. Ionophore antibiotic monensin was also added as a positive control to compare its effects with those of the olive leaf extract in the same *in vitro* conditions.

Materials and Methods

Incubation technique: The rumen simulation technique as described by Czerkawski and Breckenridge (9) was used. Eight 750-ml fermentation vessels were available. The inoculum was obtained from two freshly slaughtered beef cattle (400 kg mean body weight) at a commercial slaughter facility and transferred in warm (39°C) insulated flasks to the *in vitro* system within 30 min. Each animal was fed about 1.8 kg barley straw and 7.2 kg of a commercial mineral- and vitamin-supplemented concentrate for beef cattle (DM basis). The same diet was also used for *in vitro* incubation trial. The chemical composition of experimental diets is shown in Table 1. Ruminal fluid was filtered through four layers of cheesecloth to partition into liquid and solid (digesta) fractions. To begin the experiment, each fermentation vessel was filled with 750 ml of filtered ruminal fluid and two nylon bags (80×120 mm; 150 µm pore size), one containing 80 g of solid digesta and the other containing 10 g of feed (5 g barley straw and 5 g concentrate on a DM basis). After 24 h, the solid digesta bag was replaced by a fresh feed bag. Thereafter, one feed bag was replaced daily, so that each feed bag remained in the fermentation vessel for 48 h. Fermentation vessels received a continuous infusion of a buffer (pH 7.4) at a rate of 750 ml/d. The chemical composition of the buffer solution was presented in Table 2.

Table 1. Chemical composition of experimental diets
Tablo 1. Deneysel rasyonun kimyasal bileşimi

	Concentrate	Barley straw
DM (g/kg)	910	920
Composition (g/kg DM)		
OM	820	846
Ash	90	74
CP	148	38
NDF	284	688
ADF	95	467
ADL	31	91

DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral-detergent fibre; ADF = acid-detergent fibre; ADL = acid-detergent lignin

Table 2. Chemical composition of the buffer solution (mmol/l)
Tablo 2. Tampon solüsyonun kimyasal bileşimi (mmol/l)

Ingredient	
NaCl	28.00
KCl	7.69
CaCl ₂ .2H ₂ O	0.22
MgCl ₂ .6H ₂ O	0.63
NH ₄ Cl	5.00
Na ₂ HPO ₄ .12H ₂ O	10.00
NaH ₂ PO ₄ .H ₂ O	10.00
NaHCO ₃	97.90

Experimental procedure: The incubation trial consisted of a 7-day adaptation period (to achieve steady state conditions) followed by a 7-day collection period. At the start of the collection period, two vessels received 15 mg of olive leaf extract powder (OLE-L) daily, another two vessels received 150 mg of olive leaf extract powder (OLE-H) daily, another two vessels received 5 mg of monensin (MON) (sodium salt, Fluka) daily, and another two vessels received no additives (control, CTR). Olive leaf extract powder was provided by Kale Naturel Herbal Products Company, Ltd., Balıkesir, Turkey. Previous composition investigation studies have shown that olive leaf extract contained 18% of oleuropein as active ingredient.

Analytical procedures and samplings: The pH values were measured daily in each fermentation vessel at the time of feeding using an epoxy body pH electrode (WD-35801-00, Oakton) connected to a pH-meter (Ion 6, Acorn series, Oakton). Liquid effluent was collected daily and samples were taken and frozen at - 20°C for volatile fatty acids (VFA) and NH₃-N determination. Volatile fatty acids were quantified by the method of Oetzuerk et al. (15) using HPLC (Dionex Summit P680, ASI100) with a Rezex ROA-Organic Acid column (7.8 × 300 mm) at 60°C, isocratic elution with 0.005 M H₂SO₄, and UV detection at 210 nm. Daily production rates of VFA were estimated by multiplying the respective concentration by the volume of effluent collected.

Ruminal NH₃-N samples were allowed to thaw completely at 4°C before analysis. Ammonia-N concentration was measured using the indophenol blue method (8). Ammonia and phenol were oxidised by sodium hypochlorite in the presence of sodium nitroprusside to form a blue complex. Absorbance was measured colorimetrically at 546 nm using a Double Beam UV Spectrophotometer (Shimadzu, UV-150-02). Intensity of the blue is proportional to the concentration of ammonia present in the sample.

For protozoa counting, rumen fluid samples of fermentation vessels were taken daily immediately before substrate exchange. 1 ml of sample was carefully mixed

with 1 ml of a solution of 0.6 g methyl green, 6 g NaCl and 100 ml formaldehyde (37%) filled up to 1000 ml aqua dest. Portions of the samples were then pipetted into a counting chamber (Fuchs-Rosenthal: 0.0625 mm²; 0.2 mm deep; Marienfeld, Germany). Total numbers of protozoa, without quantifying different types, were determined using a light microscope (Leica CME).

Dry matter was determined by drying at 65°C for 48 h. Ash concentration was determined after ignition at 600°C for 12 h in a muffle furnace and used to calculate organic matter. The digestibility of organic matter at 48 h was calculated as original organic matter sample weight minus organic matter residue weight divided by the original sample weight. This value was then multiplied by 100 to derive the digestibility of organic matter percentage.

Statistical analyses: Results are given as mean \pm standard deviation (SD); n designates the number of fermentation vessels run in parallel. Statistical analysis was performed by a one-way analysis of variance (ANOVA) using the SigmaStat 3.1 software (Systat Software, Erkrath, Germany). In case of a significant ANOVA result, post hoc Duncan tests were performed to evaluate the statistical differences between the groups. P values < 0.05 were considered significant.

Results

Mean values of analysed parameters are summarised in Table 3. There were no significant differences between treatments either in pH or in organic matter degradability. MON caused ($p < 0.05$) the lowest C2:C3 ratio (-33% , compared to CTR), without changing total VFA production. This decrease was accompanied by changes in the VFA produced, producing more propionate (27%), less acetate (-17%) and butyrate (-24%). However, compared with CTR, addition of OLE-L and OLE-H resulted in a 9% and 4% increases ($p < 0.05$)

in total VFA production, respectively. In general, these increases were mediated by respective change in the production rate of propionate ($p < 0.05$). Nevertheless, due to the non-significant increase in acetate production, the acetate to propionate ratio was not significantly affected by both levels of olive leaf extract. OLE-H treatment decreased ($p < 0.05$) butyrate production. Compared to CTR, addition of MON and OLE-H reduced ($p < 0.05$) the number of total protozoa by about 71% and 18%, respectively. NH₃-N concentration was also decreased ($p < 0.05$) in the presence of MON by 13%. However, OLE-L and OLE-H treatments did not have a significant effect on NH₃-N concentration.

Discussion and Conclusion

Olive leaf extract was evaluated for its potential application as a modifier of rumen fermentation. There is very limited published information on effects of olive leaf extract or its major compound oleuropein on microbial activity in the rumen. In the present study, no differences were observed between treatments either in pH or in organic matter degradability. Similarly, Newbold et al. (13) and Castillejos et al. (6) also reported that plant essential oil mixtures did not affect rumen pH and organic matter digestibility. MON changed the VFA profile as expected, by decreasing acetate and butyrate productions, increasing propionate production, and diminishing the ratio of acetate to propionate. These changes are in concordance with those usually reported in the literature (23). Shifts in the VFA pattern by olive leaf extract, on the other hand, were clearly different from MON treatment. Both OLE-L and OLE-H increased the daily production of total VFA without affecting the acetate to propionate ratio. VFA are important energy substrates for ruminants and approximately 70% of the metabolizable energy of ruminants has been reported to be supplied by VFA (21). Total VFA productions have

Table 3. Effects of olive leaf extract and monensin on ruminal fermentation in the Rusitec system
Tablo 3. Zeytin yaprağı ekstraktı ve monensinin Rusitec sisteminde ruminal fermantasyona etkileri

Item	Treatment			
	CTR	MON	OLE-L	OLE-H
pH	6.75 \pm 0.02	6.75 \pm 0.01	6.74 \pm 0.02	6.73 \pm 0.01
Total VFA (mmol/d)	19.27 \pm 2.43 ^a	18.30 \pm 1.95 ^a	21.08 \pm 1.82 ^b	20.13 \pm 1.50 ^b
Acetate	10.53 \pm 1.24 ^a	8.75 \pm 1.58 ^b	11.46 \pm 0.93 ^a	10.99 \pm 0.78 ^a
Propionate	5.67 \pm 0.90 ^a	7.20 \pm 1.00 ^b	6.45 \pm 0.62 ^c	6.31 \pm 0.59 ^c
Butyrate	3.07 \pm 0.35 ^a	2.34 \pm 0.33 ^b	3.17 \pm 0.31 ^a	2.82 \pm 0.32 ^c
C2:C3	1.87 \pm 0.11 ^a	1.25 \pm 0.33 ^b	1.78 \pm 0.07 ^a	1.75 \pm 0.07 ^a
Total protozoa (x10 ³ /ml)	3.44 \pm 0.88 ^a	0.98 \pm 1.27 ^b	3.80 \pm 1.16 ^a	2.81 \pm 0.84 ^c
NH ₃ -N (mmol/l)	5.94 \pm 0.63 ^a	5.14 \pm 0.68 ^b	6.09 \pm 0.70 ^a	5.68 \pm 0.41 ^a
OMD (%)	40.71 \pm 4.24	39.57 \pm 4.99	42.75 \pm 4.80	42.16 \pm 4.50

Values are means \pm SD; Means within the same row with different letter (a, b, c) differ ($p < 0.05$); C2:C3: acetate-to-propionate ratio; OMD: organic matter degradability

been reported as lower (2), higher (5), and not different (4, 6, 13) when plant extracts or plant essential oils were tested. These differing results may be partially explained by the experimental conditions of these studies, including type of diets, plant species and/or their active substances used, and pH value of rumen fluid. Calsamiglia et al. (3) reported that effects of plant essential oils are pH- and diet-dependent, and their use may be beneficial only under specific conditions.

In the current study, addition of MON and OLE-H significantly reduced protozoa numbers. Rumen ciliate protozoa play diverse roles in rumen metabolism and, in their absence, the numbers of bacteria and starch degradation increase, and NH₃-N concentration decreases (19). Monensin reduced the total protozoa count, consistent with its antiprotozoal effects (10). In agreement to the present study, Yáñez Ruiz et al. (20) reported that protozoa concentrations in the rumens of animals fed olive leaf were lower than in animals fed standard diets.

MON decreased NH₃-N concentration, a wasteful end-product of protein degradation. MON supplementation has been reported to inhibit growth and activity of both rumen protozoa and proteolytic bacteria including obligate amino acid-fermenting bacteria, thus decreasing deamination of amino acid and the rate of NH₃-N production in the rumen (12, 19). Wallace et al. (22) suggested that the anti-microbial properties of plant essential oils can be exploited to modulate activities of rumen microbial populations by reducing dietary protein degradation, thereby enhancing rumen N escape. In the present study, however, olive leaf extract did not affect NH₃-N concentration, suggesting that at the levels evaluated; this plant extract had no dramatic impact on the deaminase activity of rumen microorganisms.

Based on our data, it is reasonable to conclude that olive leaf extract exerted beneficial effects on some fermentative processes in the rumen-simulating semi-continuous culture system (RUSITEC), which may improve fermentation efficiency in the rumen. Shifts in the fermentation pattern by olive leaf extract were quite different from monensin, suggesting that the mode of action of olive leaf extract is not the same as that of monensin. In fact, monensin is thought to act primarily by selective inhibition of Gram-positive bacteria (18), while olive leaf extract inhibits both Gram-positive and Gram-negative bacteria (16). Future *in vitro* and *in vivo* researches are required to identify the selection of optimal dose and types of diets that confer positive effects of olive leaf extract on microbial population and fermentation in the rumen.

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