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Triterpenes from *Olea europaea* modulate in vitro ruminal fermentation

Lautaro R. Cangiano,[†] Darren D. Henry,[‖] Francine M. Ciriaco,[‖] Jose Carlos Quintela,[$] Nicolas DiLorenzo,[†,1,2] and Ignacio R. Ipharraguerre[‡,1]

[†]North Florida Research and Education Center, Department of Animal Sciences, University of Florida, Marianna, FL 32446-7906
[‡]Institute of Human Nutrition and Food Science, University of Kiel, Herrmann Rodewald Str. 6, D-24118 Kiel, Germany
[‖]Department of Animal and Dairy Science, University of Georgia, Tifton, GA 31793
[$]ProNutra Solutions S.L., Madrid, Spain

[1]The senior authors contributed equally to this work.
[2]Corresponding author: ndilorenzo@ufl.edu

ABSTRACT

Bioactive compounds present in *Olea europaea* have shown promising antimicrobial potential as an alternative to conventional coccidiostats. These effects are exerted by triterpenic acids (**TT**) present in the olive plant, namely, oleanolic acid (**OA**), ursolic acid (**UA**), and maslinic acid (**MA**). The objective of this study was to determine the effects of OA, UA, and MA on in vitro ruminal fermentation in comparison with monensin (**MON**). The study consisted of two experiments conducted as randomized complete block designs using bahiagrass hay or a high-concentrate mixed ration as basal substrates. In the first experiment (**Exp. 1**), a batch culture was performed with increasing doses of OA, UA, or MA. In Exp. 2, to increase the solubility of OA, two chemical forms were evaluated: a sodium salt (**OA-NA**) or a phyto-phospholipid complex (**OA-PHYT**) at 0, 4, 40, 100, and 200 mg/L of incubation inoculum. In both experiments, the dose 0 was used as control (**CTL**) and monensin (**MON**) as a positive control. Data were analyzed as a randomized complete block design with a factorial arrangement of treatments. For Exp. 2, orthogonal polynomial contrasts, adjusted for unequal spacing were used to determine the linear effects of increasing doses of OA-NA and OA-PHYT. In Exp. 1, OA reduced the concentration of CH_4 in the high-concentrate substrate compared with CTL ($P = 0.04$). In Exp. 2 the total gas production was linearly decreased with increasing doses of OA-NA in both substrates ($P \leq 0.02$). Furthermore, OA-NA and OA-PHYT decreased in vitro organic matter digestibility ($P < 0.01$) in the bahiagrass substrate to the same extent that MON did. However, the concentration of CH_4/g of incubated DM was only reduced by the highest doses of OA-NA ($P < 0.02$). Lastly, no effects were observed for total VFA nor the VFA profile; however, OA-NA linearly decreased the A:P ratio in the bahiagrass substrate ($P = 0.03$). In conclusion, the acid form of OA as well as the sodium salt and phyto-phospholipid complex of OA were able to modify some fermentation parameters in this study; however, the magnitude of the responses was lower compared with monensin. Future studies should test OA in vivo to determine if the effects on ruminal fermentation observed here can translate into improve production efficiency while reducing carbon emissions.

Key words: in vitro ruminal fermentation, monensin, oleanolic acid, triterpene, methane, digestibility.

INTRODUCTION

Ruminal fermentation, among other functions, allows the ruminant host to degrade fiber and utilize end-products of microbial fermentation as sources of energy and protein. This process is not 100% efficient, which also makes the degradation of organic matter in the rumen a contributor to the emission of greenhouse gases into the environment. Scientists in the field of ruminant nutrition are continuously searching for novel strategies to modulate ruminal fermentation to increase the efficiency of substrate utilization into available sources of energy and protein for the animal while reducing pollutant emissions (Calsamiglia et al., 2007). Changes in dietary formulation and the inclusion of additives can enhance or inhibit specific bacterial populations (Callaway et al., 1997; Calsamiglia et al., 2007). This is the case of ionophores, which have been extensively used to increase animal performance for the last several decades (Bohnert et al., 2000; Ipharraguerre and Clark, 2003). However, the use of ionophores has been banned for nonmedical purposes in Europe since 2006 (Directive 1831/2003/EC, 2003) and this practice is facing increasing opposition in many other regions of the world. Bioactive compounds present in *Olea europaea* have shown promising antimicrobial potential, as an alternative to conventional antibiotics and coccidiostats (Sánchez-Quesada et al., 2013). These effects are mainly exerted by triterpenes and phenolic compounds found in olive products, which differ in structure and synthetic pathways but share similarities in their mode of action (Katerere et al., 2003; Jesus et al., 2015). Triterpenes encompass one of the largest classes of plant natural products, with more than 20,000 known molecules, granting protective effects in the host plant against pathogens and pests (Thimmappa et al., 2014). The structural diversity of triterpenes is highly associated with its pharmacological effects, and is traditionally used as antimicrobial agents in some Asian countries (Hill and Connolly, 2017). Oleanolic acid (**OA**), ursolic acid (**UA**), and maslinic acid (**MA**) are the most abundant triterpenic acids in the olive plant (Sánchez-Quesada et al., 2013). Different studies have demonstrated

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the antibacterial and antiparasitic properties of OA, UA, and MA (Jiménez-Arellanes et al., 2007; De Pablos et al., 2010; Passero et al., 2011; Zhou et al., 2012), suggesting that these compounds have promising potential as natural antimicrobial molecules (Jesus et al., 2015). Both OA and UA have been reported to inhibit the synthesis of insoluble glucans, catalyzed by crude glucosyltransferase from cariogenic *Streptococcus mutans* (Kozai et al., 1987; Jesus et al., 2015). In vitro studies conducted with *Escherichia coli* demonstrated that OA can moderately affect efflux pumps, posing this mechanism as one of the potential modes of actions by which this molecule provides its antimicrobial properties (Martins et al., 2011). We hypothesized that these compounds could have modulatory effects on ruminal fermentation parameters, reducing total gas production, changing the volatile fatty acid (**VFA**) profile, with a potentially concomitant reduction in enteric methane (CH_4) production. The objective of this study was to determine the effects of olive bioactive compounds on in vitro ruminal fermentation parameters through batch culture incubations using bahiagrass hay or high concentrate mix as substrate. In addition, we decided to evaluate the impact of two different OA products with increased water solubility, on in vitro ruminal fermentation.

MATERIALS AND METHODS

All procedures were carried out according to the University of Florida Institutional Animal Care and Use Committee, protocol # 201709971.

Experimental Design, Animals, and Treatments

The study comprised two experiments that were conducted as a randomized complete block design with a factorial arrangement of treatments using bahiagrass hay or a high-concentrate substrate as the basal substrates. In the first experiment (**Exp. 1**), a batch culture was performed with increasing doses of 3 triterpenes (TT-types): OA, UA, or MA. Each triterpene was included at 0, 4, 25, 40, and 100 mg/L of inoculum. In a follow-up study, OA was selected to conduct the second experiment (**Exp. 2**), in which OA was used in two chemical forms, a sodium salt (**OA-NA**) or a phyto-phospholipid complex (**OA-PHYT**), at the following doses: 0, 4, 40, 100, and 200 mg/L of inoculum. The doses selected in both batch cultures were based on previous in vitro studies where effects were reported at similar concentrations (Kurek et al., 2010; Jesus et al., 2015). In both experiments, the dose of 0 mg/L was used as negative control (**CTL**) and sodium monensin at 4 mg/L of incubation fluid was used as a positive control (**MON**). Substrates were sent to a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY) for nutrient composition analyses, which are presented in Table 1.

Table 1. Composition and analyzed nutrient content (DM basis) of the substrates used for in vitro incubations

Item	High-concentrate[1]	Forage
Ingredient, % of DM		
Dry-rolled corn	84	
Bahiagrass hay	10	100
Soybean meal	5.2	
Urea	0.8	
Analyzed nutrient content, % of DM[2]		
DM	96.7	94.3
CP	13.36	9.3
Neutral detergent fiber	14.63	69.9
Acid detergent fiber	6.83	38.9
Ether extract	0.18	-

[1]All ingredients were ground to pass a 2-mm screen with the exception of urea, which was ground using a mortar and pestle.
[2]Dairy One Forage Laboratory, Ithaca, NY.

In Vitro Incubations

Ruminal fluid was collected from four ruminally cannulated crossbred steers (512.5 ± 34.6 kg of BW). For at least 2 weeks before the collection of ruminal fluid, two steers consumed a highly fermentable byproduct diet comprised of 43% soybean hulls pellets, 42% corn gluten feed pellets, and 15% peanut hulls (DM basis), whereas the other two steers received a forage-based diet comprised of bahiagrass (*Paspalum notatum* Flügge) fed ad libitum. A representative sample of digesta was collected through the ruminal cannula, ruminal fluid was strained through four layers of cheesecloth, placed in a prewarmed thermos container, and transported to the laboratory within 30 min of collection. As inoculum, a 4:1 McDougall's buffer to the ruminal fluid mixture was utilized for the forage-based incubation and a 2:1 McDougall's buffer to the ruminal fluid mixture for the high concentrate substrate. Substrates were incubated in duplicated 125-mL serum bottles for analysis of in vitro ruminal fermentation parameters and in duplicated 100-mL polycarbonate centrifuge tubes for analysis of in vitro organic matter digestibility (**IVOMD**). Each bottle or tube contained 0.7 g of the substrate (DM basis), 50 mL of inoculum, and their respective treatment. Bottles and tubes were then flushed with CO_2 while the inoculum and treatments were added. Each treatment was dissolved in 0.2 mL of dimethyl sulfoxide (**DMSO**) in Exp. 1 and ethanol in Exp. 2. Solutions delivering treatments were then pipetted into each of the bottles and tubes. The CTL treatment received the same amount of DMSO in Exp. 1 or ethanol in Exp. 2 without any treatment and blank bottles received the same volume of distilled water. This was done to ensure that every bottle and tube contained the same volume of liquid (0.2 mL organic solvent or distilled water plus 50 mL of incubation fluid) and headspace. Serum bottles were crimp-sealed with butyl rubber stoppers and polycarbonate centrifuge tubes were capped with a rubber stopper fitted with a 16-gauge needle for gas release. Bottles and tubes were incubated at 39 °C under constant agitation (60 rpm) for 24 and 48 h for high concentrate and bahiagrass hay substrates, respectively. The different incubation times are intended to simulate differences in ruminal passage rates and final pH between the two contrasting diets and to better reflect the fermentation conditions of each diet. At the end of the 24 and 48 h incubation periods, serum bottles were placed for 15 min in an ice bath to stop the fermentation and then allowed to reach room temperature for a minimum of 15 min before the beginning of gas production measurements. The 100-mL polycarbonate tubes were removed from the incubator and a pepsin-HCl solution was immediately added and continued with the determination of IVOMD.

In Vitro Organic Matter digestibility

For initial DM and OM of the substrates, 0.5 g of each substrate were weighed into ceramic crucibles and dried at 100

°C for 24 h. After weighing the dried samples, the crucibles were placed in an ashing oven at 550 °C for 3 h. Crucibles were then placed in a 100 °C forced-air oven for 24 h to the determine ash content and, subsequently, the OM of the samples. At the end of the initial incubation for 24 or 48 h described previously, 6 mL of 20% (vol/vol) HCl were added to each polycarbonate centrifuge tube. Subsequently, 2 mL of 5% (wt/vol) pepsin (1:3,000; Amresco Inc., Solon, OH) solution was added to the tubes, which were returned to the incubator for an additional 48-h period under constant agitation (60 rpm). Samples were then filtered (Whatman No. 541 ashless; Whatman International Ltd., Maidstone, UK), rinsed with distilled water, dried at 100 °C for 48 h, and then ashed in the oven at 500 °C for 3 h to determine IVOMD.

Total Gas, CH4, H_2S, VFA, and NH_3–N Production Analyses

Total gas produced was determined using a manometer and transforming gas pressure values measured as pounds/square inch (**psi**) into mL of gas produced in 24 or 48 h. This conversion was done considering that the 125-mL serum bottles sealed with the blue rubber stoppers had 50 mL of inoculum plus 0.7 g of substrate. The total bottle volume to the bottom of the stopper was 152.5 mL, thus the volume of the headspace was 102.5 mL. Therefore, the formula for 125-mL serum bottles was:

$$G\ (\text{mL}) = [102.5\ \text{mL}/(\text{Pa})] \times \text{Pt}$$

where G is the total gas produced in mL, 102.5 mL is the headspace volume, Pa is the mean atmospheric pressure at the University of Florida, NFREC Animal Science Laboratory (14.6399 psi), and Pt is the pressure recorded by the manometer in psi.

The concentration of CH_4 was determined as described by Henry et al. (2015). Briefly, a10 mL sample of gas was taken from the bottle headspace to determine the concentration of CH_4 by gas chromatography (Agilent 7820A GC, Agilent Technologies, Palo Alto, CA) using a flame ionization detector and a capillary column (Plot Fused Silica 25 m × 0.32 mm, Coating Molsieve 5A, Varian CP7536). Injector, column, and detector temperatures were 80 °C, 160 °C, and 200 °C, respectively, and N_2 was used as the carrier gas with a flow rate of 3.3 mL/min. The split ratio for the injected CH_4 sample was 100:1. To determine H_2S production, 5 mL of gas from the serum bottle headspace was collected and were slowly bubbled into 10-mL evacuated tubes (BD Vacutainer, Franklin Lakes, NJ) containing 5 mL of alkaline water (pH 8.5–9.0) prepared as described by Smith et al. (2010). Briefly, the tubes were shaken vigorously to ensure proper dispersion of the gas in the alkaline water. An injection of 0.5 mL of *N*,*N*-dimethyl-*p*-phenylenediamine sulfate was made into the tubes, followed by 0.5 mL of ferric chloride. Tubes were again shaken vigorously and allowed to stand at room temperature for 30 min for the reaction to occur (Smith et al., 2010). Absorbance was read at 665 nm in flat-bottom 96-well plates using a plate reader (DU-500, Beckman Coulter Inc., Palo Alto, CA).

After measurement of total gas production and collection of gases for CH_4 and H_2S analyses, bottles were opened, and the final pH of the inoculum was measured. Subsequently, 0.5 mL of a 20% H_2SO_4 solution was added to each bottle to acidify the sample to avoid VFA volatilization. Lastly, a 10-mL sample was taken and frozen for subsequent VFA and NH_3-N analyses.

Concentrations of VFA in the inoculum samples were determined in a water-based solution using ethyl acetate extraction as described by Ruiz-Moreno et al. (2015). Briefly, samples were centrifuged at 4 °C for 10 min at 10,000 × *g*. Inoculum supernatant was mixed with a metaphosphoric acid:crotonic acid (internal standard) solution at a 5:1 ratio and samples were frozen overnight, thawed, and centrifuged at 4 °C for 10 min at 10,000 × *g*. The supernatant was transferred into glass tubes and mixed with ethyl acetate in a 2:1 ratio of ethyl acetate to the supernatant. After shaking tubes vigorously, the ethyl acetate fraction (top layer) was transferred to vials. Samples were analyzed by gas chromatography (Agilent 7820A GC, Agilent Technologies) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m 0.53 mm, Varian CP7767, Varian Analytical Instruments, Walnut Creek, CA). Concentrations of NH_3-N were measured after centrifuging inoculum samples at 10,000 × *g* for 15 min at 4 °C (Avanti J-E, Beckman Coulter Inc.) following the phenol-hypochlorite technique described by Broderick and Kang (1980) with the following modification: absorbance was read at 620 nm in flat-bottom 96-well plates using a plate reader (DU-500, Beckman Coulter Inc.).

Statistical Analyses

For Exp. 1, data were analyzed as a randomized complete block design with a 4 × 3 + 2 factorial arrangement using a mixed-effect model (SAS Inst., Inc., Cary, NC). The model included the fixed effect of treatment, TT type (OA, MA, and UA; excluding monensin), TT dose (0, 4, 25, 40, 100), using dose 0 as CTL, and TT type × dose interaction (excluding monensin). The effect of the day (block) was included as a random effect. For Exp. 2, data were also analyzed as a randomized complete block design with a 4 × 2 + 2 factorial arrangement using a mixed-effect model (SAS Inst., Inc., Cary, NC). The model included the fixed effect of treatment, OA type (OA-NA and OA-PHYT; excluding monensin), OA dose (0, 4, 40, 100, 200; excluding monensin), using dose 0 as CTL, and OA × dose interaction (excluding monensin) and the random effect of the day (block). In both experiments, two bottles per treatment plus two blank bottles (without substrate) were incubated on three separate replicate days, and the average of the two bottles or tubes within the day was considered the experimental unit (n = 3/treatment). In addition, for Exp. 2 orthogonal polynomial contrasts, adjusted for unequal spacing of treatments using the IML procedure of SAS, were used to determine the linear effects of increasing doses of OA-NA and OA-PHYT on in vitro fermentation parameters and comparing both forms of OA with MON and CTL. In both Exps. 1 and 2, significance was declared at $P \leq 0.05$ and tendencies were discussed when $0.05 < P < 0.10$.

RESULTS

Experiment 1

Total Gas Production, CH4, NH3–N, and H2S. There was a treatment effect for total gas production using bahiagrass substrate ($P < 0.01$; Table 3), where only MON was able to reduce gas production over the 48-h incubation period. A treatment effect for CH_4 production expressed as

mM/g of DM was observed in both substrates where MON reduced CH_4 compared with CTL ($P \leq 0.02$; Tables 2 and 3). In addition, TT type reduced the production of CH_4, but this response was substrate specific, where OA was able to reduce the production of CH_4 but only in the high-concentrate diet ($P = 0.04$; Table 2) but not in the bahiagrass substrate ($P = 0.26$; Table 3). A treatment effect was observed for the concentration of NH_3–N where MON significantly decreased NH_3–N production on the bahiagrass substrate ($P < 0.01$; Table 3), however, TT type had no effect on NH_3–N production regardless of the substrate ($P > 0.19$). Lastly, a treatment effect was observed for the concentration of H_2S because MON reduced it in both substrates ($P < 0.01$), but no effect was observed for TT type regardless of the substrate ($P > 0.31$; Tables 2 and 3).

IVOMD, pH, and VFA Profile. In vitro organic matter digestibility was affected by treatment where only MON was able to decrease IVOMD compared with CTL in both substrates ($P \leq 0.05$; Tables 2 and 3). No effect of TT-type, dose or their interaction was observed for IVOMD regardless of the substrate ($P > 0.30$; Tables 2 and 3). None of the treatments affected final pH regardless of the substrate ($P > 0.58$; Tables 2 and 3).

The concentrations of total VFA, acetate, and propionate were not affected by treatment or TT type regardless of the substrate ($P > 0.11$). In addition, concentrations of butyrate, BCVFA, and valerate were only decreased by MON in the bahiagrass substrate ($P < 0.03$; Table 5). Lastly, acetate to propionate ratio (A:P) was decreased by MON ($P < 0.01$; Tables 4 and 5) in both substrates, but no effects were observed for TT type ($P > 0.11$; Tables 4 and 5) regardless of the substrate.

Table 2. Effects of oleanolic, maslinic, and ursolic acid on batch culture in vitro ruminal fermentation using a high-concentrate substrate

Item	CTL[1]	MON[2]	TT type[3]			Dose, mg/L				SEM[4]	*P*-value[5]			
			OA	MA	UA	4	25	40	100		Trt	TT type	TT dose	TT × dose
Total Gas, mL 24 h	217.0	176.0	188.0	197.0	197.0	187.0	188.0	188.0	190.0	13.07	0.08	0.11	<0.01	0.08
IVOMD[6], %	68.2	63.9	67.0	67.0	68.1	67.7	66.7	66.8	67.6	1.29	0.05	0.30	0.50	0.99
pH	6.3	6.1	6.2	6.2	6.2	6.2	6.2	6.2	6.2	0.01	0.58	0.59	0.39	0.30
CH_4, m*M*/g of incubated DM	1.0	0.7	0.8	0.9	0.9	0.8	0.9	0.8	0.8	0.05	0.02	0.04	0.07	0.90
CH_4, m*M*/g of DM fermented	1.5	1.0	1.2	1.4	1.3	1.2	1.3	1.3	1.2	0.07	0.09	0.07	0.08	0.90
NH_3-N, m*M*	3.2	4.0	4.0	3.7	3.7	4.0	4.0	4.0	3.9	0.24	0.68	0.19	0.22	0.16
H_2S µmol/g of ferm. OM	8.1	4.8	9.6	9.4	9.4	9.1	9.7	10.5	9.0	0.66	<0.01	0.88	<0.01	0.19

[1]Control = distilled water.
[2]MON = monensin sodium at 4 mg/L of inoculum.
[3]Pentacyclic triterpene type: OA = oleanolic acid; MA = maslinic acid; UA = ursolic acid.
[4]SE of treatment means, $n = 3$ replicates/treatment (2 bottles or tubes averaged per replicate).
[5]*P*-values for the overall treatment effect (Trt), effect of pentacyclic triterpene (TT) type, dose and their interaction when analyzed as a 4 × 3 + 1 factorial arrangement (excluding the monensin treatment).
[6]IVOMD = in vitro organic matter digestibility.

Table 3. Effects of oleanolic, maslinic, and ursolic acid on batch culture in vitro ruminal fermentation using a bahiagrass substrate

Item	CTL[1]	MON[2]	TT type[3]			TT Dose, mg/L				SEM[4]	*P*-value[5]			
			OA	MA	UA	4	25	40	100		Trt	TT type	TT dose	TT × dose
Total gas, mL 48 h	177.0	110.2	157.0	164.0	162.0	158.0	158.0	155.0	157.0	4.27	<0.01	0.26	0.24	0.16
IVOMD[6], %	54.9	45.2	54.0	54.2	53.7	54.4	53.5	54.0	53.0	1.08	<0.01	0.78	0.30	0.38
pH	6.4	6.5	6.4	6.5	6.5	6.4	6.5	6.5	6.5	0.05	0.65	0.19	0.74	0.97
CH_4, mM/g of incubated DM	1.1	0.2	1.0	1.0	1.0	1.0	1.0	0.9	1.0	0.08	<0.01	0.26	0.18	0.51
CH_4, mM/g of DM fermented	2.0	0.5	1.8	1.9	1.9	1.9	1.9	1.7	1.9	0.16	<0.01	0.29	0.40	0.73
NH_3-N, m*M*	4.8	3.1	5.0	4.8	4.8	5.0	5.0	4.9	4.6	0.41	<0.01	0.67	0.82	0.75
H_2S µmol/g of ferm. OM	45.5	14.8	41.6	45.4	42.7	39.8	44.6	43.4	42.8	4.35	<0.01	0.31	0.50	0.74

[1]Control = distilled water.
[2]MON = monensin sodium at 4 mg/L of inoculum.
[3]Pentacyclic triterpene type: OA = oleanolic acid; MA = maslinic acid; UA = ursolic acid.
[4]SE of treatment means, $n = 3$ replicates/treatment (2 bottles or tubes averaged per replicate).
[5]*P*-values for the overall treatment effect (Trt), effect of pentacyclic triterpene (TT) type, dose and their interaction when analyzed as a 4 × 3 + 1 factorial arrangement (excluding the monensin treatment).
[6]IVOMD = in vitro organic matter digestibility.

Experiment 2

Total Gas Production, CH4, H2S. There was a treatment effect for total gas production ($P \leq 0.02$; Tables 6 and 7), where the addition of MON to the inoculum decreased total gas production in both substrates. Furthermore, there was an effect for OA inclusion in the inoculum because OA-NA linearly decreased total gas production in both substrates ($P \leq 0.02$; Tables 6 and 7). Furthermore, there was a treatment effect for CH_4 production expressed as *mM* of CH_4/per g of incubated DM due to the repressing action of MON on this trait in both the high concentrate and bahiagrass hay compared with CTL ($P < 0.01$; Tables 6 and 7). In addition, OA-NA was able reduce the production of CH_4 per g of incubated DM in the bahiagrass substrate in a dose-dependent manner ($P = 0.02$; Table 7). However, no effect was observed in the high-concentrate substrate for any of the OA forms ($P = 0.42$; Table 6). Lastly, the concentration of H_2S in the high-concentrate substrate was affected by treatment ($P < 0.01$; Table 6), and increasing concentrations of OA-PHYT linearly decreased H_2S concentration ($P < 0.01$; Table 6).

IVOMD and pH, and VFA Profile. In both substrates, MON decreased IVOMD compared with CTL ($P \leq 0.01$; Tables 6 and 7). In the bahiagrass substrate, the interaction of OA type by dose was significant for IVOMD ($P < 0.01$; Table 7), however, although a linear effect was observed ($P < 0.01$; Table 7), both OA-NA and OA-PHYT decreased IVOMD most markedly at the highest dose. Lastly, there was a treatment effect for pH in the bahiagrass substrate where the final pH of the incubation was greater ($P < 0.01$; Table 7) with MON inclusion compare with CTL. However, no effects were observed for OA on pH regardless of the substrate ($P \geq 0.17$; Tables 6 and 7).

Concentrations of total VFA, acetate, and propionate were not affected by treatment ($P \geq 0.30$; Tables 8 and 9) in both

Table 4. Effects of oleanolic, maslinic, and ursolic acid on concentration of total volatile fatty acids, and volatile fatty acids profile using a high-concentrate substrate

Item	CTL[1]	MON[2]	TT type[3]			TT Dose, mg/L				SEM[4]	*P*-value[5]			
			OA	MA	UA	4	25	40	100		Trt	TT type	dose	TT × dose
Total VFA, mM	77.4	107.2	103.3	97.8	102.0	106.8	106.9	105.5	108.4	5.60	0.46	0.45	0.06	0.09
Acetate, mM	44.8	58.4	58.1	55.2	57.2	59.9	59.6	59.0	60.8	3.14	0.54	0.43	0.09	0.06
Propionate, mM	24.3	38.7	32.8	31.0	32.7	32.1	34.2	33.7	34.4	1.86	0.14	0.52	<0.01	0.27
Butyrate, mM	6.7	8.0	10.1	9.5	9.9	10.4	10.7	10.5	10.7	0.64	0.08	0.60	0.14	0.11
BCVFA, mM [6]	0.9	1.0	1.2	1.2	1.2	1.3	1.3	1.3	1.3	0.14	0.38	0.46	0.33	0.14
Valerate, mM	0.6	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.1	0.41	0.58	0.82	0.24	0.45
A:P[7]	1.9	1.5	1.8	1.9	1.8	1.8	1.8	1.8	1.8	0.04	<0.01	0.70	0.90	1.00

[1]Control = distilled water.
[2]MON = monensin sodium at 4 mg/L of inoculum.
[3]Pentacyclic triterpene type: OA = oleanolic acid; MA = maslinic acid; UA = ursolic acid.
[4]SE of treatment means, *n* = 3 replicates/treatment (two bottles or tubes averaged per replicate).
[5]*P*-values for the overall treatment effect (Trt), effect of pentacyclic triterpene (TT) type, dose and their interaction when analyzed as a 4 × 3 + 1 factorial arrangement (excluding the monensin treatment).
[6]BCVFA= branched-chain volatile fatty acids: isobutyrate + isovalerate + 2-methylbutyrate.
[7]A:P = acetate to propionate ratio.

Table 5. Effects of oleanolic, maslinic, and ursolic acid on total volatile fatty acids, and volatile fatty acids profile using a bahiagrass substrate

Item	CTL[1]	MON[2]	TT type[3]			TT Dose, mg/L				SEM[4]	*P*-value[5]			
			OA	MA	UA	4	25	40	100		Trt	TT type	TT dose	Type × dose
Total VFA, m*M*	62.3	63.5	77.0	69.9	71.1	76.5	75.5	75.1	74.0	4.46	0.77	0.12	0.04	0.37
Acetate, m*M*	42.5	40.3	54.1	48.7	49.6	53.8	53.2	52.8	51.7	3.31	0.33	0.11	<0.01	0.31
Propionate, m*M*	13.4	19.3	16.0	14.8	14.9	15.8	15.6	15.6	15.7	0.9	0.3	0.11	0.05	0.38
Butyrate, m*M*	4.8	2.8	5.2	4.9	5.0	5.2	5.0	5.1	5.0	0.28	<0.01	0.12	0.62	0.94
BCVFA, m*M* [6]	0.9	0.6	1.0	0.9	0.9	1.0	0.9	0.9	0.9	0.03	<0.01	0.2	0.42	0.96
Valerate, m*M*	0.6	0.5	0.7	0.6	0.7	0.7	0.6	0.7	0.7	0.03	0.03	0.14	0.93	0.95
A:P[7]	3.2	2.1	3.0	3.3	3.3	3.4	3.4	3.3	3.3	0.08	<0.01	0.11	<0.01	0.06

[1]Control = distilled water.
[2]MON = monensin sodium at 4 mg/L of inoculum.
[3]Pentacyclic triterpene type: OA = oleanolic acid; MA = maslinic acid; UA = ursolic acid.
[4]SE of treatment means, *n* = 3 replicates/treatment (two bottles or tubes averaged per replicate).
[5]*P*-values for the overall treatment effect (Trt), effect of pentacyclic triterpene (TT) type, dose and their interaction when analyzed as a 4 × 3 + 1 factorial arrangement (excluding the monensin treatment).
[6]BCVFA= branched-chain volatile fatty acids: isobutyrate + isovalerate + 2-methylbutyrate.
[7]A:P = acetate to propionate ratio.

Table 6. Effects of increasing concentrations of two chemical forms of oleanolic acid (sodium salt or phyto-phospholipid complex) on in vitro batch culture fermentation using high-concentrate substrate

Item	CTL[1]	MON[2]	OA-NA[3]				OA-PHYT[3]				SEM[4]	*P*-values[5]				Linear effect[6]	
			4	40	100	200	4	40	100	200		Trt	OA	DOSE	OA × DOSE	OA-NA	OA-PHYT
Total gas, mL 24 h	155.0	144.6	155.8	152.0	147.8	146.2	153.8	148.1	150.6	152.8	1.31	0.02	0.04	<0.01	<0.01	<0.01	0.11
IVOMD[7], %	80.4	77.4	77.8	81.4	78.4	78.7	80.1	79.2	77.3	75.6	0.97	<0.01	0.40	<0.01	0.08	0.06	<0.01
pH	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7	0.12	0.80	0.90	0.68	0.80	0.63	0.67
CH_4, m*M*/ g of incubated DM	1.1	0.7	1.1	1.0	1.1	1.0	1.0	1.1	1.1	1.0	0.28	<0.01	0.42	0.35	0.39	0.13	0.82
CH_4, m*M*/g of DM fermented	1.3	0.9	1.4	1.2	1.3	1.3	1.3	1.3	1.4	1.3	0.35	<0.01	0.37	0.39	0.20	0.28	0.50
H_2S µmol/g of ferm. OM	7.5	7.8	7.8	7.8	7.8	7.9	7.3	6.8	7.1	7.0	0.83	<0.01	<0.01	0.01	0.54	0.54	0.03

[1]Control = distilled water.
[2]MON = monensin sodium at 4 mg/L of inoculum.
[3]OA-NA= sodium salt of oleanolic acid; OA-PHYT = phyto-phospholipid complex of oleanolic acid.
[4]SE of treatment means, *n* = 3 replicates/treatment (two bottles or tubes averaged per replicate).
[5]*P*-values for the overall treatment effect (Trt), and the effect of oleanolic acid (OA), dose and their interaction (OA × DOSE) when analyzed as a 4 × 2 + 1 factorial arrangement (excluding the effect of monensin).
[6]Linear effects = *P*-values of linear effect of OA-NA, OA-PHYT.
[7]IVOMD = in vitro organic matter digestibility.

Table 7. Effects of increasing concentrations of two chemical forms of oleanolic acid (sodium salt or phyto-phospholipid complex) on in vitro batch culture fermentation using bahiagrass hay substrate

Item	CTL[1]	MON[2]	OA-NA[3]				OA-PHYT[3]				*P*-values[5]				OA × DOSE	Linear effect[6]	
			4	40	100	200	4	40	100	200	SEM[4]	Trt	OA	DOSE		OA-NA	OA-PHYT
Total gas, mL 48 h	151.6	102.5	151.4	153.9	141.0	134.6	151.7	150.3	149.8	156.6	3.95	<0.01	0.03	0.22	0.03	0.02	0.49
IVOMD[7], %	50.4	46.3	48.8	49.9	49.7	45.6	50.2	48.4	50.4	46.2	0.58	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
pH	6.3	6.5	6.3	6.3	6.4	6.4	6.3	6.3	6.3	6.3	0.02	<0.01	0.17	0.82	0.16	0.11	0.22
CH_4, *mM*/ g of incubated DM	1.0	0.4	1.1	1.1	0.8	0.8	1.1	1.1	1.0	1.1	0.19	<0.01	0.04	0.24	0.02	0.02	0.19
CH_4, *mM*/g of DM fermented	2.1	0.9	2.2	2.2	1.9	1.9	2.1	2.2	2.1	2.4	0.34	<0.01	0.04	0.11	0.03	0.07	0.28
H_2S µmol/g of ferm. OM	10.8	6.9	11.5	12.4	10.1	10.0	10.8	10.5	10.4	10.0	2.30	0.47	0.57	0.70	0.88	0.34	0.72

[1] Control = distilled water.
[2]MON = monensin sodium at 4 mg/L of inoculum.
[3]OA-NA= sodium salt of oleanolic acid; OA-PHYT = Phyto-phospholipid complex of oleanolic acid.
[4]SE of treatment means, *n* = 3 replicates/treatment (two bottles or tubes averaged per replicate).
[5]*P*-values for the overall treatment effect (Trt), and the effect of oleanolic acid (OA), dose and their interaction (OA × DOSE) when analyzed as a 4 × 2 + 1 factorial arrangement (excluding the effect of monensin).
[6]Linear effects = *P*-values of linear effect of OA-NA, OA-PHYT.
[7]IVOMD = in vitro organic matter digestibility.
[8]OM = organic matter.

Table 8. Effects of increasing concentrations of two chemical forms of oleanolic acid (sodium salt or phyto-phospholipid complex) on total volatile fatty acids and volatile fatty acids profile using a high-concentrate substrate

Item	CTL[1]	MON[2]	OA-NA[3]				OA-PHYT[3]				SEM[4]	*P*-values[5]				Linear effect[6]	
			4	40	100	200	4	40	100	200		Trt	OA	DOSE	OA × DOSE	OA-NA	OA-PHYT
Total VFA, m*M*	80.1	79.8	78.0	74.8	71.3	85.2	79.1	75.3	64.2	76.4	9.18	0.88	0.57	0.49	0.91	0.40	0.40
Acetate, m*M*	43.2	40.7	42.3	40.1	39.0	46.5	43.0	41.3	34.3	40.5	6.22	0.89	0.54	0.55	0.85	0.56	0.56
Propionate, m*M*	43.2	26.1	42.3	40.1	39.0	46.5	43.0	41.3	34.3	40.5	2.04	0.37	0.54	0.55	0.85	0.16	0.16
Butyrate, m*M*	11.7	9.1	11.3	10.6	10.1	12.0	11.3	10.5	9.3	10.8	1.41	0.70	0.52	0.48	0.95	0.65	0.65
BCVFA, m*M* [7]	1.5	1.2	1.3	1.3	1.2	1.4	1.3	1.3	1.1	1.3	0.19	0.75	0.61	0.62	0.94	0.40	0.40
Valerate, m*M*	2.2	2.4	1.9	1.8	1.8	2.0	2.0	1.9	1.8	2.0	0.49	0.49	0.92	0.86	0.99	0.04	0.04
A:P[8]	2.1	1.6	2.0	1.9	1.9	2.0	2.1	2.0	1.8	1.9	0.19	0.01	0.28	0.80	0.22	0.14	0.09

[1]Control = distilled water.
[2]MON = monensin sodium at 4 mg/L of inoculum.
[3]OA-NA= sodium salt of oleanolic acid; OA-PHYT = phyto-phospholipid complex of oleanolic acid.
[4]SE of treatment means, *n* = 3 replicates/treatment (two bottles or tubes averaged per replicate).
[5]*P*-values for the overall treatment effect (Trt), and the effect of oleanolic acid (OA), dose and their interaction (OA × DOSE) when analyzed as a 4 × 2 + 1 factorial arrangement (excluding the effect of monensin).
[6]Linear effects = *P*-values of linear effect of OA-NA, OA-PHYT.
[7]BCVFA= branched-chain volatile fatty acids: isobutyrate + isovalerate + 2-methylbutyrate.
[8]AP = acetate to propionate ratio.

Table 9. Effects of increasing concentrations of two chemical forms of oleanolic acid (sodium salt or phyto-phospholipid complex) on total volatile fatty acids, and volatile fatty acids profile, using bahiagrass hay substrate

Item	CTL[1]	MON[2]	OA-NA[3]				OA-PHYT[3]				SEM[4]	*P*-values[5]				Linear effect[6]	
			4	40	100	200	4	40	100	200		Trt	OA	DOSE	OA × DOSE	OA-NA	OA-PHYT
Total VFA, m*M*	51.4	38	46	47.3	45.2	56.7	38.9	51.8	51.3	49.8	6.36	0.51	0.85	0.34	0.55	0.88	0.875
Acetate, m*M*	37.3	24.5	33.4	34.4	32.8	40.9	28.4	33.1	37.3	36.3	4.61	0.3	0.61	0.38	0.68	0.96	0.96
Propionate, m*M*	8.9	10.2	8.1	8.1	8.1	10.3	6.6	8	9	8.4	1.19	0.3	0.39	0.28	0.51	0.22	0.22
Butyrate, m*M*	3.6	2.1	3.2	3.3	3.1	3.9	2.7	3.2	3.6	3.5	0.49	0.19	0.67	0.4	0.69	0.8	0.8
BCVFA, m*M* [7]	1.1	0.6	0.9	1.1	0.9	1.2	0.8	1	1	1.1	0.19	0.64	0.87	0.42	0.89	0.87	0.87
Valerate, m*M*	0.4	0.3	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.07	0.77	0.98	0.51	0.76	0.8	0.8
A:P[8]	4.2	2.4	4.2	4.2	4	3.8	4.3	4.2	4.2	4.3	0.18	<0.01	0.13	0.45	0.11	0.03	0.28

[1]Control = distilled water.
[2]MON = monensin sodium at 4 mg/L of inoculum.
[3]OA-NA= sodium salt of oleanolic acid; OA-PHYT = phyto-phospholipid complex of oleanolic acid.
[4]SE of treatment means, *n* = 3 replicates/treatment (two bottles or tubes averaged per replicate).
[5]*P*-values for the overall treatment effect (Trt), and the effect of oleanolic acid (OA), dose and their interaction (OA × DOSE) when analyzed as a 4 × 2 + 1 factorial arrangement (excluding the effect of monensin).
[6]Linear effects = *P*-values of linear effect of OA-NA, OA-PHYT.
[7]BCVFA = branch chain volatile fatty acids: isobutyrate + isovalerate + 2-methylbutyrate.
[8]AP = acetate to propionate ratio.

the high concentrate and the bahiagrass substrate. However, the A:P ratio was decreased by MON in both substrates ($P \leq 0.01$; Tables 8 and 9). Furthermore, OA-NA linearly decreased the A:P ratio in the bahiagrass substrate ($P = 0.03$; Table 9) and OA-PHYT tended to linearly decrease the A:P ratio in the high-concentrate substrate ($P = 0.09$; Table 8).

DISCUSSION

We showed that triterpenic acids from *O. europaea* are able to modify ruminal fermentation parameters in a compound and substrate-dependent manner. Specifically, OA as free acid, sodium salt, and phyto-phospholipid complex was able to reduce the concentration of CH_4 and H_2S and similarly decrease the A:P ratio to MON, although the magnitude of the responses was almost always lower for OA than MON. Different studies have demonstrated the antimicrobial effects of OA, when added to in vitro incubations (Jiménez-Arellanes et al., 2007; Passero et al., 2011; Zhou et al., 2012). Certainly, it has been suggested that the antimicrobial activity of OA entails inhibition of the synthesis of insoluble glucans on bacterial cell walls

(Kozai et al., 1987; Jesus et al., 2015) and efflux pumps (Martins et al., 2011), ultimately compromising bacterial viability.

The effects of these compounds were evaluated using two different substrates, either bahiagrass hay or a high concentrate mix. The purpose of this approach was to investigate the dependency of the response to triterpenes on the substrate and characterize their effects on ruminal fermentation parameters. Total gas production has been previously reported to decrease with MON inclusion on in vitro batch culture incubations at the same dose used in this study (4 mg/L), both with high-concentrate and bahiagrass hay as substrates (Henry et al., 2015). The authors observed a reduction of the asymptotic maximal gas production in both high- and low-concentrate substrates (Henry et al., 2015). In this study, we observed that MON decreased total gas production across experiments and substrates. In Exp 1, the only effect observed for all the tested triterpenic acids (OA, UA, or MA), was a tendency for OA to decrease total gas production and reduce CH_4 concentration in the high concentrate substrate. Because triterpenic acids from *O. europaea* are rather insoluble in aqueous media (Guinda et al., 2010), we speculated that these marginal effects resulted from the poor solubility of such compounds in the inoculum. In fact, using the sodium salt and phytosome forms of OA, which are expected to increase its solubility in the inoculum, appeared to increase its antimicrobial activity as denoted by the partly depressed fermentation found in Exp 2. The decrease in IVOMD and gas production observed with the highest doses of OA-NA and OA-PHYT (100 mg, and 200 mg/L of inoculum) was comparable to the effect of MON. However, it is important to note that to achieve a similar effect to that observed by MON it required a dose 50 times higher. Furthermore, the effects observed for OA in the second experiment were more pronounced when bahiagrass hay rather than concentrate was used as the substrate, mirroring what we observed for MON. This substrate-dependent effect might be attributed to an inhibitory action on cellulolytic bacteria, similar to the well-established effects of MON on this particular bacterial population of the rumen (Chen and Wolin, 1979). Therefore, the depression in fermentation is exacerbated with substrates that contain more structural carbohydrates. The inhibition of cellulolytic bacteria activity in turns reduces the digestion of fiber, ultimately reducing the amount of substrate that is fermented (Bogaert et al., 1990; Anassori et al., 2012). Furthermore, the reduction in substrate fermentation depresses total gas production, which includes CH_4.

The observed reduction in CH_4 production cannot be explained only by a reduction in IVOMD and gas production. This is because when CH_4 concentration was expressed as *mM*/g of DM fermented to account for the change in IVOMD, we still observed a linear decrease in this trait in response to the increasing doses of OA-NA. This reduction of CH_4 can be partly explained by the shift in the acetate:propionate observed. Propionate captures more H_2 when a glucose molecule is fermented into this VFA compared with acetate and butyrate (Russell et al., 1998). Therefore, increasing propionate production at the expense of acetate reduces free H_2 and thereby the availability of precursors for methanogenic archaea to reduce CO_2 to CH_4. In this study, the reduction in CH_4 concentration caused by OA-NA was correlated with a shift in the acetate to propionate ratio (A:P), even though the molar concentrations of propionate and acetate were only numerically different. This interaction between the profile of VFA and the reduction potential of the fermentation media is also observed with H_2S, where in the second experiment OA-PHYT tended to decrease the A:P ratio in the high concentrate substrate while reducing H_2S concentration by 6%, suggesting that propionate acted as a hydrogen sink, reducing free hydrogen available for sulfur reduction into H_2S (Smith et al., 2010). This finding is important because diets with a sulfur content greater than 0.50% (DM basis) have been reported to induce toxicity (Gould 1998; Smith et al., 2010). Accumulation of H_2S is the result of the reduction of S in the rumen combined with low ruminal pH, which increases the amount of sulfide in the gaseous state that ultimately reaches the bloodstream and increases the risk of cytotoxicity.

In conclusion, both the acid form of OA as well as the sodium salt and phyto-phospholipid complex of OA were able to modify some fermentation parameters in this study such as the concentrations of CH_4, H_2S, as well as A:P ratio; however, the magnitude of the responses was lower for the triterpenic acids compared with monensin. In addition, the effect of OA on in vitro rumen fermentation appears to be substrate-dependent, with more prominent effects in incubations with forage-based substrates. Future studies should expand on these results to understand if these findings can be replicated in vivo. If proved effective, the inclusion of OA in the diet as a feed additive could prevent H_2S accumulation in the rumen and reduce the risk of feeding byproducts with high sulfur content, such as distillers grains, while contributing to control carbon emissions in the context of high-fiber diets.

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Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

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