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Comparison of the Effect of Olives Leaves Extracts *(Olea europaea)* on *In vitro* Methane Production, Fermentation Efficiency and Protozoa Activity

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Abstract: The aim of this study was to investigate the effect of olive leaves extracts *(OLE)* on *in vitro* fermentation of a 60: 40 forage: concentrate diet using the *in vitro* gas production technique (IVGPT), four levels of *OLE* are tested for (0, 50,75 and 100 mg) the main fermentation parameters (pH, total gas,CH_4, truly organic matter digestibility (IVOMD), NH_3-N concentration, VFA, protozoa) were determined after 24 h of incubation. CH_4 production was significantly lower for ethyl acetate extract compared to the control ($P<0.05$). But, methanogenesis is increased for n-butanol and aqueous extracts. N- of -NH_3 production was increased for all *olive extracts* ($P<0.05$). Propionate production was increased for ethyl acetate and n-butanol extracts, whereas no effect was registered for pH and total VFA production. Ethyl acetate extract decreased IVOMD ($P<0.05$) but this parameter is comparable to control for n-butanol and aqueous extracts. Protozoa population decreased ($P<0.05$) for all extracts in comparison with the control. *OLE* exerted beneficial effects on some fermentation parameters, which might be promising to be used as an antimethanogenic additive. Moreover, the assessment of the right dosage seems to be critical to contain the environmental impact, without reducing feed nutritional value.

Key words: Olive leaves extracts · Phenolic compounds · *In vitro* gas production · Methanogenesis · Protozoa

INTRODUCTION

Antibiotic and many chemical feed additives, such as ionophore antibiotic, have been used widely in ruminant production systems 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. For this, there is an increasing interest in evaluating 'natural' alternatives to modify rumen microbial fermentation [1, 2].

Olea europaea is a plant characteristic of the Mediterranean region that is cultivated in various countries of the world due to the great interest in the production of olives and their oil, which is rich in essential fatty acids. The leaves of the olive tree are an important by-product generated by pruning of trees that have significant amount of phenolic and fatty acids important to health. Given the importance of compounds present in olive leaves such as phenolic compounds including oleuropein, tyrosol, hydroxytyrosol, caffeic acid, gallic acid, syringic acid, p-coumaric acid and luteolin, have been shown to inhibit or delay the rate of growth of a range of microorganisms [3]. Furthermore, olive

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leaf extract and its individual constituents are considered safe and non-toxic for human and animal consumption [4, 5].

Olive by-products are largely used as an alternative source of nutrients. Olive trees are usually subjected to pruning and the amount of leaves produced can be up to 30 kg per tree [6]. However, olive leaves intake levels have been shown to be low and animal performance could be limited [7]. However, the ability of olive leaf extracts to influence microbial fermentation processes in the rumen have not been evaluated and available information on this topic is scarce.

The objectives of the current study was to investigate the effectiveness of increase doses of three extracts of the olive leaves *(OLE)* rich on phenolic compounds (ethyl acetate, n-butanol and aqueous extracts) on methanogenesis and on *in vitro* ruminal fermentation parameters.

MATERIALS AND METHODS

Experimental Procedure: Three extracts (ethyl acetate, n-butanol and aqueous) of *Olea europaea* leaves with four dosages (D0 mg, D50 mg, D75mg, D100 mg) were tested using the *in vitro* gas production technique (IVGPT) in two consecutive gas runs using as substrate a diet for ruminant and as *inoculum* a buffered rumen liquor from cow. *In vitro* fermentation characteristics (i.e. gas, degradability, end-products) were determined after 24 h of incubation, as well as methane production and protozoa count. Tested diet and olives leaves were also characterised for chemical composition.

Sample Collection and Preparation: Samples of *O. Europaea* plants were collected during the flowering stage (April 2016) in Ain Beida, located in Oum El Bouaghi city, Algeria (Latitude 35°47'47″ Nord, Longitude 7°23'34″ East; 1008 m a.s.l.). The leaves were cleaned, air-dried and ground to pass through a 1-mm screen (Brabender Wiley mill, OHG Duisburg, Germany) and kept in closed jars in a dry and cool place.

Extraction Procedure: The extraction of the three fractions rich in flavonoids is carried out from the aerial part of *O. europaea* plant according to the protocol of Cetkovic *et al.* [8], based on the degree of solubility of the flavonoids in organic solvents. In particular, 450 g of finely ground dry matter was macerated with methanol/water ($MeOH/H_2O$, 70:30) for three times, then a fourth maceration with hot water to renew the solvent was made. The recovered hydro-alcoholic extracts was collected and filtered through Büchner and then subjected to evaporation under low pressure at 35°C with a Rotavapor (R.215. BUCHI, Labortechnik AG, Flawil, Switzerland). Thus, the obtained final residue was added to boiling distilled water and then filtered. The filtrate was freed from waxes, lipids with hexane and chlorophyll by washing with chloroform to obtain an aqueous phase. In order to separate the flavonoids of the organic phase into aglycone, mono-glycoside, di- and tri-glycoside fractions, the remaining aqueous phase was submitted to three extractions with ethyl acetate. The remaining aqueous phase was mixed with n-butanol to recover, in particular, the di- and tri-glycoside flavonoids. The final aqueous phase mainly contains the most polar glycosylated flavonoids. Thereby, the three fractions obtained were evaporated under reduced pressure in the Rotavapor at 45°C, lyophilized (Alpha 1-4 LD plus, Bioblock Scientific, IllkirchCedex, France) and stored at 4°C until use.

Measurements and Chemical Analysis: In order to formulate a balanced diet recommended for dairy cow (forage: concentrate ratio 60:40; Net Energy for lactation 1.51 MJ/kg DM), to use as substrate in the IVGPT trial, four ingredients (alfalfa hay, ryegrasshay-silage, soya s.e. and maize) were chosen and properly combined (26.5, 36.5, 10.0 and 30.0 %, respectively). The obtained diet, as well as the *O. Europaea* samples was analysed for dry matter (DM), crude protein (CP), ether extract (EE) and ash, as reported by AOAC procedures [9] (ID number: 2001.12, 978.04, 920.39 and 930.05 for DM, CP, EE and ash, respectively). Neutral detergent fibres (NDF, with sodium sulphite and heat-stable α-amylase and expressed exclusive of residual ash), acid detergent fibres (ADF, expressed exclusive of residual ash) and acid detergent lignin (ADL, determined by solubilisation of cellulose with sulphuric acid) were analysed according to Van Soest *et al*. [10], using ANKOM 200 Technology (Fairport, New York, USA); hemicellulose and cellulose were estimated as the difference between NDF and ADF and between ADF and ADL, respectively. Starch content was measured after acid hydrolysis by polarimetric detection [11].

Incubation technique: Six dairy cows (mean body weight 680 kg) fed a total mixed ration containing corn silage, oat hay and concentrate (CP 120 and NDF 435 $g \bullet kg^{-1}$ DM) were used as rumen fluid donors. In particular, equal volume of material was collected from each cow at a

slaughterhouse authorized according to EU legislation (EU regulation No. 882/2004). The collected rumen fluids were quickly transported to the lab in pre-heated Thermos flasks, mixed and strained through four layers of cheese cloths ensuring the temperature of 39°C and the anaerobic conditions, according to the protocol suggested by Calabrò *et al.* [12]. The rumen fluid (10 ml) was then mixed to anaerobic medium (75 ml) and reducing agent (4 ml) and dispensed anaerobically into 120 ml serum flasks containing 1.0051 ± 0.022 g of substrate. Then, flasks were supplemented by 50, 75 and 100 mg of each fraction of *O. Europaea (*D50, D75 and D100 respectively); the substrate incubated without olive extract represented the control (D0). The extracts concentrations utilized in the present study were based on the result of a preliminary *in vitro* trial. The serum flasks were sealed and held in an incubator at 39°C for 24 h. Three replicates for each fraction were made and three serum flasks without extracts neither substrate were incubated as blanks to correct organic matter (OM) disappearance, gas produced and end-products.

Samplings and Analytical Procedures: Immediately after 24 h of incubation, total volume of gas (GP24, ml/g) accumulated in the headspace of each serum flask was recorded as reported by Calabrò *et al.* [13], using a manual pressure transducer (Cole & Parmer Instrument Co., Barringhton, IL- USA). methane (CH_4) production is determined as reported by Guglielmelli *et al.* [14], the gas phase from each serum flask was sampled (3 ml) in duplicate with a gastight syringe for the analysis carried out using a gas chromatograph (GC Trace 1310, Thermo Scientific, Waltham, MA USA) equipped with a loop TC detector and a packed column (HaySepQ SUPELCO, 3/16 inch, 80/100 mesh). The organic matter degradability (OMD, %) was determined by weight difference of the incubated OM and the undegraded residue throughout sintered glass crucibles (Schott Duran, Mainz, Germany, porosity #2) [15].

For volatile fatty acids (VFA) determination, a sample of fermenting liquor was centrifuged at 12.000 × g for 10 min at 4°C (Universal 32R centrifuge, HettichFurnTech Division DIY, Vlotho, Germany) and an aliquot (1 ml) of supernatant was mixed with 1 ml of oxalic acid (0.06 mol); VFA were measured by gas chromatography (GC Focus AI 3000, Thermo Scientific, Waltham, MA USA) equipped with a fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness), using an external standard solution composed of acetic, propionic, butyric, isobutyric, valeric and isovaleric acids, as described by Musco *et al*. [16]. The total production of volatile fatty acids (tVFA) has been considered as sum of acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, as well as acetate/propionate ratio (A/P) and branched-chain fatty acid proportion [(BCP = iso-butyrate + iso-valerate) / tVFA]. On fermenting liquor, pH was also measured (model 3030 Alessandrini Instrument, glass electrode, Jenway, Dunmow, UK) as well as ammonia nitrogen ($N\text{-}NH_3$) production, according to the colorimetric method [17].

Protozoa Quantification: In order to count the protozoa, at the end of the incubation the fermentation liquor (100 µL) of each serum flask was treated with a solution of MFS (methyl green formalin-saline), following the procedure described by Ogimoto and Imai [18] which allows the fixation of the cells due to the formaldehyde and the colouring of the nucleus cells by the methyl green. The enumeration was carried out on haemocytometer (Malassez cell, 0.200 mm, Tiefe/depth Profondeur), a thick glass plate where a chamber of count consisting of 100 rectangles is hollowed, of which 25 are subdivided into 20 small squares to facilitate the counting; the total volume of the cell is equal to 1.0 µL and 0.01 µL per rectangle, allowing the quantitative assessment of protozoa. The treated samples were stored at room temperature in darkness until counting retained. During the measurement, the sample to count was placed between slide and slide using a Pasteur pipette avoiding the formation of air bubbles. The microscopically counting was done on the rectangles subdivided into small squares using a microscope (Zeiss Binocular Microscope Axiostar, 1122-100) with a × 40 objective lens. Each sample was counted twice, if the difference between the two results was greater than 10% the count was repeated. The number of protozoa is expressed according to the following equation:

$$N = n1 \times v \times n2 \times f \times 1000$$

where N is the number of cells per ml, n1 is the number of cells counted, v is the volume of a rectangle (0.01 µl), n2 is the number of rectangles counted (25) and f is the dilution factor [19].

Calculations and Statistical Analysis: According to Blümmel *et al.* [20], the partitioning factor (PF), an indicator of the fermentation efficiency, was calculated as ratio between *in vitro* degraded DM (dDM, mg) and the volume of gas produced (G24, ml) at the end of

incubation. The microbial biomass yield (MBY, ml) was also calculated using dDM, GP24 and a stoichiometric factor (2.25) as follows Blümmel *et al.* [20]:

$$MBY = dDM + G24 \times 2.25$$

All *in vitro* data (fermentation characteristics, end-products and protozoa) were subjected to analysis of variance by two-way (ANOVA) using the General Linear Model (GLM) procedure of the IBM SPSS 22 [21], to test the effect of the extraction type (ethyl acetate, n-butanol, aqueous) and their dosages (D0, D50, D75, D100). The multiple comparisons of the averages were made using Bonferroni adjustment at the error risk of 5%.

RESULTS

The chemical composition of olive leaves and control substrate is reported in Table 1. The CP content of olives leaves (101, 2 g•kg^{-1} DM) is comparable to the control substrate (P>0.05).This concentration is sufficient to ensure a good bacterial growth. But the olive leaves are rich in ash content comparatively to the control substrate (98.7g/kg of DM) (P>0.001). Concerning structural carbohydrates, it appears that olive leaves had a high level of NFC (direct source of energy). Regarding its chemical composition, olive leaves are to be considered for animal nutrition as source of ash, CP and NFC.

Parameters of gas production recorded in our experiment are mentioned in Table 2. It indicates that gas production for n-butanol extract is similar to control substrate while a slight reduction is observed for D75. Furthermore, ethyl acetate and aqueous extracts induce for all concentrations tested an increment of GP24. As expected for n-butanol extract, we noted also reduction in methane production, this later is not statistically significant (P>0.05). However, ethyl acetate extract induce an important decrease in methane production comparatively to control and the percentage of reduction was dose dependant (2.88, 3.17 and 3.53% for D50, D75 and D100, respectively).

However, in the case of this experiment, for ethyl acetate extract, for which a reduction in methane concentration in the pool gas is accompanied with an increase in propionate. The same trend is observed for n-butanol and aqueous extracts.

For aqueous extract, we noted an increase in microbial biomass yield. This observation was supported by the earlier results obtained in the same work that aqueous extract was probably rich in soluble sugars which stimulate and were used by bacteria as carbon source. However, for ethyl acetate extract microbial biomass yield is significantly reduced (P< 0.001). Beside, the n-butanol extract also reduce microbial biomass production but it is not significant.

Partitioning factor is an estimated parameter indicates the conversion of organic matter in batch system. The partitioning factor indicates that higher gas production is observed for ethyl acetate extract. At same n-butanol and aqueous extract transformed OMD towards gas production. For n-butanol and aqueous extracts, OMD is not significantly affected (P<0.05). The case of aqueous extract, this parameter is slightly increased. However, ethyl acetate extract reduce significantly OMD.

Table 1: Chemical composition (*g•kg^{-1} DM*) of the plant *Olea europaea* of and the formulated diet used as control substrate in the *in vitro* trial.

Parameters	*Olive Plant*	*Control Substrate*	S.E.M	P-Value
DM	931.2^{a}	907.5^{c}	0.43	0.001
Ash	98.7^{a}	63.8^{c}	0.82	0.001
CP	101.2^{a}	115.9^{a}	0.37	0.089
NDF	230.5^{a}	395.0^{c}	2.89	0.001
ADF	126.1^{a}	235.9^{b}	1.94	0.001
ADL	66.6^{a}	69.1^{a}	0.45	0.005
EE	20.7^{a}	37.1^{c}	0.51	0.001
Starch	ND	14.29	0.19	ND
CC	59.5^{a}	166.8^{c}	1.82	0.001
HC	106.2^{a}	159.1^{b}	1.56	0.001
NFC	549.0^{a}	388.2^{c}	2.73	0.001

DM: dry matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; hemicelluloses (HC): NDF - ADF; cellulose (CC): ADF - ADL. Non Fibrous Carbohydrates (NFC): 100 - (% NDF + % CP + % EE + % Ash) ; NRC (2001); ND : non determined.

Table 2: Effect of *OLE* at different doses on the *in vitro* fermentation characteristics after 24 h of incubation.

	Ethyl Acetate Extract				N-Butanol Extract			Aqueous Extract				
Item	D0	D50	D75	D100	D50	D75	D100	D50	D75	D100	S.E.M	P-Value
GP24,*ml/g*	106.9^{a}	110.3^{a}	124^{b}	125.8^{b}	111.8^{a}	105.5^{a}	111.5^{a}	107^{a}	120.7^{a,b}	129.7^{b}	1.58	0.001
OMD, *%*	54.65^{a}	53.70^{a,b}	47.48^{b}	48.71^{a,b}	53.36^{a}	51.25^{a}	49.05^{a}	56.69^{a}	57.16^{a}	50.80^{a}	0.69	0.022
CH_4, *ml/g*	16.95^{a}	14.07^{b}	13.78^{b}	13.42^{b}	16.84^{a}	14.85^{a}	15.46^{a}	16.96^{a}	17.92^{a}	18.42^{a}	0.296	0.001
PF, *mg/ml*	5.61^{a}	5.34^{a}	4.19^{b}	4.27^{b}	5.16^{a}	5.27^{a}	4.62^{a}	5.80^{a}	5.18 ^{a,b}	4.2^{b}	0.110	0.001
MBY, *mg*	316.8^{a}	289.3^{a,b}	203.5^{b}	215.3^{b,c}	276.7^{a}	271.3^{a}	226.2^{a}	335.1^{a}	306.2^{a,b}	231.6^{b}	9.018	0.001

GP24: cumulative gas production related to incubated organic matter; OMD: *in vitro* organic matter degradability; CH_4: methane production related to incubated OM; PF: partitioning factor; MBY: microbial biomass yield.

D0, D50, D75 and D100: 0 (control), 50, 75 and 100 mg of added extract in the culture flasks, respectively.

Values in the same row and extract subtable not sharing the same subscript are significantly different at P<0.05.

Tests are adjusted for all pair wise comparisons within a row of each extract subtable using the Bonferroni correction.

Table 3: Effect of *OLE* at different doses on fermentation end-products after 24 h of *in vitro* incubation.

	Ethyl Acetate Extract				N-Butanol Extract			Aqueous Extract				
Item	D0	D50	D75	D100	D50	D75	D100	D50	D75	D100	S.E.M	P-Value
pH	6.47^{a}	6.63^{b}	6.60^{b,c}	6.56^{c}	6.53^{a}	6.53^{a}	6.53^{a}	6.47^{a}	6.44^{a}	6.50^{a}	0.010	0.001
$N\text{-}NH_3$, *mg/l*	53.9^{a}	75.72^{b}	53.01^{a}	42.58^{a}	60.75^{a}	55.03^{a}	59.35^{a}	61.66^{a,b}	68.30^{b}	51.4^{a}	1.561	0.001
tVFA, *mmol/g*	77.53^{a}	69.97^{a}	70.86^{a}	73.64^{a}	77.61^{a}	74.57^{a}	76.93^{a}	84.48^{a}	90.28^{a}	88.18^{a}	0.985	0.333
Acetate, *mmol/g*	48.72^{a}	45.77^{a}	43.97^{a}	48.98^{a}	15.28^{b}	14.20^{b}	17.01^{b}	55.33^{a}	56.83^{a}	56.51^{a}	0.780	0.001
Propionate, *mmol/g*	14.32^{a}	13.96^{a}	16.77^{a}	17.46^{a}	15.28^{a}	14.20^{a}	17.01^{a}	16.22^{a}	17.49^{a}	18.50^{a}	0.334	0.343
Butyrate, *mmol/g*	10.91^{a}	7.62^{a,b}	7.81^{a,b}	4.50^{b}	10.92^{a}	9.66^{a}	9.93^{a}	10.23^{a}	12.51^{a}	10.31^{a}	0.165	0.001
Iso-butyrate, *mmol/g*	0.68^{a,b}	0.52^{a}	0.49^{a}	0.79^{b}	0.58^{a,b}	0.73^{a}	0.43^{b}	0.54^{a}	0.69^{a}	0.52^{a}	0.016	0.017
Valerate, *mmol/g*	1.53^{a}	1.21^{a,b}	1.09^{b}	1.13^{b,c}	1.31^{a,b}	0.95^{b}	1.17^{a,b}	1.22^{a,c}	1.60^{b}	1.15^{c}	0.042	0.007
Iso-valerate, *mmol/g*	1.37^{a}	0.89^{b}	0.74^{b}	0.79^{b}	1.21^{a,c}	0.73^{b}	0.79^{b,c}	0.95^{a,b}	1.15^{a,b}	0.86^{b}	0.040	0.009
A/P ratio	3.49^{a}	3.42^{a,c}	2.68^{b}	2.81^{b,c}	3.17^{a,b}	3.00^{a,b}	2.89^{b}	3.41^{a}	3.25^{a}	3.06^{a}	0.044	0.018
BCP	0.03^{a}	0.02^{a,b}	0.02^{b}	0.02^{a,b}	0.02^{a}	0.02^{a,b}	0.02^{b}	0.02^{b}	0.02^{a,b}	0.02^{b}	0.001	0.001

$N\text{-}NH_3$: ammonia nitrogen; tVFA: total volatile fatty acids = acetate + propionate + butyrate + iso-butyrate + valerate + iso-valerate; A/P: acetate/propionate ratio; BCP: (branched-chain fatty acid proportion) = (iso-butyrate + iso-valerate) / tVFA.

D0, D50, D75 and D100: 0 (control), 50, 75 and 100 mg of added extract in the culture flasks, respectively.

Values in the same row and extract subtable not sharing the same subscript are significantly different at P<0.05.

Tests are adjusted for all pair wise comparisons within a row of each extract subtable using the Bonferroni correction.

Table 4: Effect of*OLE*on total protozoa counts (No. x 10^5) after 24 h *in vitro* incubation.

	Ethyl Acetate Extract				N-Butanol Extract			Aqueous Extract				
Item	D0	D50	D75	D100	D50	D75	D100	D50	D75	D100	S.E.M	P-Value
Protozoa ($\times 10^5$Cell/ml)	7.73^{a}	3.30^{b}	1.92^{c}	1.58^{c}	7.42^{a,b}	7.48^{a}	6.92^{b}	7.42^{a}	6.33^{b}	6.50^{b}	0 .27	0.001

D0, D50, D75 and D100: 0 (control), 50, 75 and 100 mg of added extract in the culture flasks, respectively.

The *in vitro* fermentation end-products and pH values are reported in Table 3.Only ethyl acetate extract affect pH values in the fermentation liquor at the end of the *in vitro* incubation in comparison to control (D0) (P<0.001) (Table 3), a slight increase (P<0.05) in pH values was noted for n-butanol extract but it's not significant. For aqueous extract pH values are compared to control.

For both concentrations and for the three extracts ammonia nitrogen production increased comparatively to control except for D100 of ethyl acetate and aqueous extract.

All the Olive extracts did not affect (P>0.05) total VFA production in comparison to the control. However, it is important to note a slight decrease of total VFA values for ethyl acetate and n-butanol extracts compared to the control, except in aqueous extract for which an increment was observed for the three concentrations.

In parallel, the addition of all extracts increase propionate production comparatively to the control. Regarding butyrate, only the inclusions of ethyl acetate extract decrease its production, for n-butanol and aqueous extracts the values is compared to the control. The ratio of acetate to propionate (A/P) is affected by ethyl acetate and n-butanol extract and this later is significantly lower than control value (P<0.05).

As reported in Table 4, results of the current experiment illustrate that all olive leaves extracts induce a decrease in protozoa population after 24 h of incubation:

protozoa population for ethyl acetate extract is considerably lower than in control (D0) and this reduction is dose dependant. The greatest reduction is recorded (79.6%) with the 100 mg dose. Due to the addition of the others two extracts, the protozoa count also was reduced, but less compared to ethyl acetate extract.

DISCUSSION

Olive leaves content was evaluated for its potential application as modifier of rumen fermentation, firstly, the present study has conducted to determine chemical composition of olive leaf. This part has demonstrated that olive leaf have an appreciate protein and ash contents, these results were consistent with that obtained by Delgado-Pertõanaez *et al*. [22]. However, the same results are different for those noted by Martin-Garcia *et al.* [23], these inconsistence were probably due to several conditions such as origin, proportion of branches on the tree, storage conditions, climatic conditions, moisture content and degree of contamination with soil and oils.

Additional, we have recorded in this study that NFC concentration is higher comparatively to control substrate. Effectively; the concentration of this component depends on factors such as the variety of the olive tree, climatic conditions, year and proportion of wood.

For these reasons, Molina-Alcaide and Yanez-Ruiz [24], in their report stated that olive leaves provide half of the energy and amino acid requirements of sheep and goats at maintenance level if it adequately supplemented, it can be used as part of the forage in diets.

There is limited information about the effect of olive leaves extracts on *in vitro* fermentability and methane production in the rumen. However, some reports have identified a significant potential of natural plant extracts as a novel source for methane mitigation from ruminants [25,26,27].

In the present work, all olive extracts induce an increment of gas production except for n-butanol extract for D75; these results were different to those observed by Jafari *et al*. [28]. As known, gas production is an index of microbial fermentative activity, although change in VFA proportion may cause a small variation on gas volume, Therefore ours results on gas production would suggest an inspecific activity of our extracts content on ruminal microbial fermentation.

Our results have shown that methane production in the rumen could be reduced by 2.88 to 3.53% when ethyl acetate extract was added to the *in vitro* incubation compared with control without compromising concentrations of VFA.

According to McDonald *et al.* [29], the fermentation of foods rich in starch tends to produce more propionate, while fibrous substrates results in the increased formation of acetate. In accordance also with Orskov *et al*. and Wolin equation [30, 31], reduction in methane is synonym to an increase in propionate production. The addition of ethyl acetate extract stimulated some favorable pathways such as propionate production, which caused a significant reduction in acetate to propionate ratio. For n-butanol and aqueous extract this increase of propionate production is probably due to its content higher in soluble sugars.The decrease in the C2 / C3 ratio with increasing propionate proportion are reported by many authors. This confirms that the reduction of CH4 is the result of the redirection of hydrogen molecules from the path of methanogenesis to the production of propionate [32].

These findings were consistent with the result obtained by Molina-Alcaide *et al.* [33], who reported lower acetate to propionate ratio in continuous culture fermenters fed a diet containing olive leaves in comparison to alfalfa hay. The decrease in acetate to propionate ratio observed with olive leaves extract might be due to a reduced growth of cellulolytic bacteria in the rumen, which is in line with a previous study [33].

In the present study, For all the treatments, the pH values ranged between values (6.47 and 6.63). Under normal conditions, the healthy and functional rumen is characterized by a slightly acidic pH. The pH of the ruminal environment is the resultant of acid production, salivary buffers and buffers own ration. Although it is difficult to define with certainty a precise range, it is generally accepted that in normal operating conditions of the rumen, the pH is between 6.0 and 7.0 [34], these values considered within the optimal range to maintain normal growth and cellulolytic activity of ruminal microbiota [35].

NH3-N is a wasteful end-product of protein degradation, aaccording to Wallace *et al.* [36], who 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. Contrary in our study, the increase of ammonia

production with the olive leaves extracts compared with control could probably attributed to a selective inhibition of microorganisms that are users of ammonia N and suggesting also that the levels of this plant extracts had no dramatic impact on deamination of amino acids by hyper-ammonia producing bacteria [37, 38]. Differently, Jafari *et al*. [28], who tested Papaya leaf fractions rich on phenolic compounds (chloroform and ethyl acetate), suggested that the decrease in *vitro* NH_3N concentration compared with that in control may due to phenolic hydroxyl groups in plant extracts (e.g., tannin) that can react with proteins, forming tannin-protein complexes and thus preventing the degradation by proteases and binding proteins at ruminal pH, from these previous studies we can suggested that our extracts either contains low amount of tannin or totally absent. This situation is accompanied also with decrease in microbial biomass yield, which can suggest that ammonia is not used for biomass synthesis except for aqueous extract.

For protozoa population, only ethyl acetate extract causes a significant decrease in protozoa population related to a decrease in methane production which is in accordance with previous *in vitro* studies on the effect of plant extracts rich on polyphenol carried out by several authors [28, 39].

It's well known that ciliate protozoa are an important key in methanogenesis in the rumen as methanogens attach to their surface and it is expected that reducing protozoa would also reduce the methanogen population because 10 to 20% of methanogens live in association with protozoans, thus decreasing CH4 production.

CONCLUSION

OLE especially ethyl acetate extract exerted beneficial effects on some fermentative processes in the rumen, Ethyl acetate extract appears to have those attributes for use in ruminant feeding to suppress methanogenesis. Which may improve fermentation efficiency in the rumen, suggesting that the mode of action of olive leaves extracts is not well. 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 *OLE* on microbial population and fermentation in the rumen. Therefore, our results need to be confirmed further, over a range of samples and conditions and validated with *in vivo* testing, before these products can be advanced further in animal nutrition.

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