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Effect of increasing amounts of olive crude phenolic concentrate in the diet of dairy ewes on rumen liquor and milk fatty acid composition

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ABSTRACT

Agro-industrial by-products contain several secondary plant metabolites, such as polyphenols, tannins, saponins, and essential oils. The effects of these compounds on animal metabolism may vary significantly according to the dose, the chemical nature of the molecules, and the overall composition of the diet. In the Mediterranean area, the olive oil extraction is associated with 2 by-products: olive pomace and wastewater, both rich in polyphenols. In particular, wastewater may be further processed to obtain olive crude phenolic concentrate (OCPC). An experiment was carried out aiming to evaluate animal performance, milk fatty acid (FA) profile, diversity of rumen microbial population, and rumen liquor FA profile in dairy ewes fed diets containing extruded linseed (EL) and increasing doses of OCPC. Twenty-eight Comisana ewes in mid lactation were allotted to 4 experimental groups. The experiment lasted 5 wk after 3 wk of adaptation. Diets were characterized by lucerne hay administered *ad libitum* and by 800 g/ewe and day of 4 experimental concentrates containing 22% of EL on dry matter and increasing dose of OCPC: 0 (L0), 0.6 (L0.6), 0.8 (L0.8), and 1.2 (L1.2) g of OCPC/kg of dry matter. Milk yield was daily recorded and milk composition was analyzed weekly. At the beginning and at the end of the experiment, samples of rumen liquor were collected to analyze FA profile, changes in rumen microbial population, and dimethylacetal (DMA) composition. The inclusion of OCPC did not affect milk yield and gross composition, whereas milk from L0.8 and L1.2 sheep

contained higher concentrations of linoleic (+18%) and α -linolenic acid (+24%) and lower concentration of the rumen biohydrogenation intermediates. A similar pattern was observed for rumen liquor FA composition. No differences were found in the diversity of the rumen microbial population. Total amount of DMA did not differ among treatments, whereas significant differences were found in the concentration of individual DMA; in the diet with a higher amount of OCPC, DMA 13:0, 14:0, 15:0, and 18:0 increased, whereas DMA 16:0 decreased. Probably the presence of polyphenols in the diet induced a rearrangement of bacteria membrane phospholipids as a response to the rumen environment stimulus. Overall, the use of OCPC allowed a significant increase in the polyunsaturated FA content of milk, probably due to a perturbation of the rumen biohydrogenation process. Further studies are needed to understand the correlation between diet composition and the pattern of DMA in rumen liquor.

Key words: biohydrogenation, milk fatty acid, dimethylacetal, olive polyphenol

INTRODUCTION

The use of extruded linseed (EL) in the diet of dairy ruminants has been extensively studied as a feeding strategy to modify milk fatty acid (FA) composition (Chilliard et al., 2007; Mele et al., 2011). The addition of EL in the ruminant diet leads to an enrichment of milk and cheese with bioactive FA, such as PUFA α -3, CLA, and vaccenic acid (VA; Mele et al., 2011; Pintus et al., 2013). However, more than 85% of PUFA contained in EL are usually biohydrogenated (Buccioni et al., 2015b). As consequence, high amounts of dietary EL are needed to obtain milk with a FA profile rich in PUFA suitable for the production of cheese with a

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proven positive effect on human health (Mele et al., 2011; Pintus et al., 2013), causing an increase in feeding costs.

Recent studies showed that feeding polyphenolic compounds to ruminants resulted in a perturbation of PUFA rumen biohydrogenation (**RBH**) with no detrimental effects on milk yield and quality (Ferlay et al., 2010; Buccioni et al., 2015a). Moreover, *in vitro* studies suggested interference between dietary polyphenols and rumen microbiome profile (Pallara et al., 2014; Costa et al., 2017).

Several agro-industrial by-products contain considerable amounts of plant secondary metabolites, including different kind of polyphenols or other compounds such as saponins and essential oils, able to modulate RBH and, consequently, FA composition of ruminant-derived products (Vasta et al., 2008).

Olive oil extraction results in 2 main by-products: olive pomace and wastewater. More than 98% of total olive polyphenols accumulate in the wastewater during the oil extraction process. Olive polyphenols may be separated from the rest of the wastewater by applying an enzymatic treatment with depolymerizing enzymes, followed by a separation with membrane filters (0.2 μm) as described in Servili et al. (2011). After this treatment, an olive crude phenolic concentrate (**OCPC**) is obtained, with a polyphenol content 4 times higher than that of the wastewater.

Olive crude phenolic concentrate is mainly composed by oleuropein-aglycone di-aldehyde (**3,4-DHPEA-EDA**) and verbascoside, whereas hydroxytyrosol (**3,4-DHPEA**) and tyrosol (**p-HPEA**) are usually present at lower amounts (Servili et al., 2011).

A previous *in vitro* study suggested that polyphenols from olive pomace were probably able to interfere with RBH of dietary PUFA (Pallara et al., 2014). However, in dairy ewes, the effect of pure olive polyphenols on milk yield and composition is only scarcely studied (Casamassima et al., 2014). To investigate the response of microbes to changes in the rumen environment as affected by specific diet ingredients, recently dimethylacetal (**DMA**) analysis of the rumen liquor has been proposed (Alves et al., 2013). Dimethylacetals derive from plasmalogen lipids and seems to play an important role in the membrane fluidity of gram-negative bacteria. In particular, DMA profile has been proposed as potential marker of the response of rumen bacteria to the environmental condition (Katz and Keeney, 1964; Minato et al., 1988; Goldfine, 2010).

Thus, the effect of the inclusion of increasing amounts of OCPC, in combination with EL as a source of PUFA, in the diet of dairy ewes on milk traits and FA composition and on DMA and FA composition of rumen liquor was evaluated. Moreover, the effect of OCPC on

overall rumen microbial community diversity was also investigated.

MATERIALS AND METHODS

Experimental Design

Twenty-eight pluriparous Comisana ewes at mid-late lactation (107 ± 9 d) and homogeneous for BW (68 ± 2.5 kg), kept at the Experimental Section of the Department of Agriculture, Food and Environmental Science, University of Perugia (Italy), were randomly allotted into 4 groups and confined in multiple pens (7 animals per pen).

The experimental trial lasted 5 wk after 3 wk of adaptation to the dietary regimen adopted for the present study. In the experimental period, the animals received 4 different diets based on chopped lucerne hay administered *ad libitum* (particle size >4 cm of length), 100 g of rolled barley, and 800 g/animal and day of a concentrate containing EL as source of PUFA, plus different amounts of OCPC to obtain different concentration of total polyphenols: 0 (**L0**), 0.6 (**L0.6**), 0.8 (**L0.8**), or 1.2 (**L1.2**) g/kg of DM. The OCPC was obtained from the filtration of fresh vegetation wastewater, according to the procedure described by Servili et al. (2011). All the concentrate ingredients were incorporated into pellets using a pelleting machine (CMS-IEM, Colognola ai Coli, Verona, Italy). Pellet diameter was 5 mm and the pelleting temperature ranged between 35 and 40°C. Ingredients and chemical composition of the experimental diets were reported in Table 1.

Diets were formulated according to the nutritional requirements of a dairy ewe weighing 68 kg and producing 1 kg of milk at 6.5% fat (Cannas et al., 2004). Four hundred grams of experimental concentrates and 50 g of rolled barley were individually fed during each milking until their consumption was completed.

The ewes were milked twice daily (0730 and 1730 h) using a milking machine (43 kPa; 150 pulsations/min) and daily milk yield was individually recorded. The handling of the animals was in accordance with the Institutional Animal Care and Use Committee of the University of Perugia.

Sampling and Analysis

Feed Sampling and Analysis. Feed and ort samples were collected weekly, dried at 40°C, ground using a Cyclotec 1093 mill (PBI International, Milan, Italy) with a mesh size of 1 mm, and then stored at -80°C until analysis.

The content of CP and ether extract were determined according to the AOAC methods (976.06 and 920.39,

Table 1. Ingredients, chemical composition, and fatty acid profile of the lucerne hay, flaked barley, and experimental concentrates administrated to ewes

Item	Lucerne hay	Rolled barley	Experimental diet ¹			
			L0	L0.6	L0.8	L1.2
Feeds (g/100 g of DM)						
Wheat bran			34.50	34.44	34.42	34.38
Crushed linseed			22.0	22.0	22.0	22.0
Barley			14.0	14.0	14.0	14.0
Corn			14.0	13.9	13.9	13.9
Soybean meal			10.0	10.0	10.0	10.0
Olive crude phenolic concentrate (OCPC)			0.00	0.06	0.08	0.12
Molasses			5.0	5.0	5.0	5.0
NaCl			0.20	0.15	0.15	0.15
CaCO ₃			0.10	0.15	0.15	0.15
Sodium bicarbonate			0.10	0.15	0.15	0.15
Dicalcium phosphate			0.10	0.15	0.15	0.15
Chemical composition (g/kg of DM)						
CP	128.40	121.00	175.30	180.20	183.60	176.50
Ether extract	12.90	16.00	117.60	116.10	114.90	115.50
NDF	504.00	134.10	244.30	263.00	257.00	270.20
ADF	395.70	54.20	92.40	91.40	92.51	97.10
ADL	64.01	14.90	23.70	16.70	23.10	23.40
Ash	74.20	21.00	81.00	80.40	82.10	83.30
Fatty acid (g/100 g of total fatty acids)						
C16:0	27.69	18.17	7.00	7.92	7.90	6.69
C18:0	5.40	4.58	4.62	4.02	4.02	4.48
C18:1 <i>cis</i> -9	4.05	21.18	16.80	19.11	19.03	19.46
C18:2n-6	18.93	44.97	26.14	25.65	25.68	25.89
C18:3n-3	33.95	6.06	42.96	41.13	41.24	41.16
Others	9.98	5.04	2.48	2.17	2.13	2.32

¹L0 = control diet; L0.6 = control diet plus 0.6 g/kg of OCPC; L0.8 = control diet plus 0.8 g/kg of OCPC; L1.2 = control diet plus 1.2 g/kg of OCPC.

respectively; AOAC International, 1995). The content of NDF, ADF, and ADL were determined using heat-stable amylase and sodium sulfite, in according to Van Soest et al. (1991). The results were expressed inclusive of residual ash.

Lipids of feed samples were extracted according to Folch et al. (1957) and then FAME were obtained according to Christie (1982), using 19:0 (Sigma Chemical Co., St. Louis, MO) as the internal standard. Gas chromatography procedure and methods applied to identify and quantify feed FA were the same below described for milk FA analysis.

Phenolic compounds in the experimental concentrates were determined according to Servili et al. (2011) and reported in Table 2.

Milk Sampling and Analysis. Individual milk samples were collected weekly during the morning and evening milking. Milk samples were gathered in a single sample according to the morning and afternoon yield and, subsequently, split into 2 aliquots for analysis: the first aliquot was processed to evaluate fat, lactose, protein, and urea contents by using Milkoscan 6000 FT (Foss Electric, Hillerød, Denmark) and SCC according to ISO 13366-2/IDF 148-2 (ISO-IDF, 2006), by using a

Table 2. Phenolic compounds content in the experimental concentrates administrated to ewes

Item (mg/kg of DM)	Experimental concentrate ¹			
	L0	L0.6	L0.8	L1.2
3,4-DHPEA ²	—	107.87	111.50	146.32
p-HPEA ²	—	52.07	69.64	103.74
Verbascoside	—	248.18	354.87	530.07
3,4-DHPEA-EDA ²	—	236.29	291.67	397.83
Total phenolic compounds	—	644.41	827.68	1,177.96

¹L0 = control diet; L0.6 = control diet plus 0.6 g/kg of olive crude phenolic concentrate (OCPC); L0.8 = control diet plus 0.8 g/kg of OCPC; L1.2 = control diet plus 1.2 g/kg of OCPC.

²Hydroxytyrosol (3,4-DHPEA), tyrosol (p-HPEA), and oleuropein-aglycone di-aldehyde (3,4-DHPEA-EDA).

Fossmatic 5000 (Foss Electric). Somatic cell count data were expressed as linear score (LS) where $LS = \log_2(\text{SCC}/12,500)$, according to Shook (1993).

The second aliquot of milk samples was analyzed for FA composition as follows: milk fat was extracted as reported in Mele et al. (2008). The FAME were prepared by alkali catalyzed trans-methylation procedure (Christie, 1982) with nonanoic (C9:0) and nonadecanoic (C19:0) acid methyl ester (Sigma Chemical Co.) as the internal standards.

The FA composition was determined using a GC2010 Shimadzu gas chromatograph (Shimadzu, Columbia, MD) equipped with a flame-ionization detector and a high polar fused-silica capillary column (Chrompack CP-Sil 88 Varian, Middelburg, the Netherlands; 100 m, 0.25 mm i.d.; film thickness 0.20 μm). Hydrogen was used as the carrier gas at a flow of 1 mL/min. Split/splitless injector was used with a split ratio of 1:80. An aliquot of the sample was injected under the following GC conditions: the oven temperature started at 60°C and held at that level for 1 min; it was then increased to 173°C at a rate of 2°C/min, and held at that level for 30 min, before being once again increased to 185°C at 1°C/min and held for 5 min, and then to 220°C at a rate of 3°C/min, and held for 19 min. The injector temperature was set at 270°C and the detector temperature was set at 300°C.

Individual FAME were identified and quantified according to Buccioni et al. (2015a). All FA composition results are expressed as grams per 100 g of FA.

Rumen Liquor Sampling and Analysis. Individual rumen liquor samples were collected from all animals involved in the experiment before the start of adaptation period to the experimental diets and at the end of the trial. The samples were collected by means of a stomach tube connected to an electrical pump before morning feeding, according to the procedure described by Buccioni et al. (2015a). The sampling was done from each animal after an overnight period without concentrate and hay distribution but with free access to water.

Immediately after collection, each sample was measured for pH and stored at -80°C until analysis for FA and DMA composition. Before the analysis, samples were freeze-dried and then trans-esterified by using a combined basic and acid procedure, according to Jenkins (2010) as modified by Alves et al. (2013). Briefly, 250 mg of freeze-dried rumen sample was put in a centrifuge tube and added with 1 mL of toluene and 1 mL of hexane containing internal standard (C19:0, 1 mg/mL). The basic trans-esterification was performed by the addition of 2 mL of sodium methoxide in methanol (0.5 M). The solution was vortexed and left to react for about 10 min at 50°C. Subsequently, the mixture was

cooled to room temperature and immediately added with 3 mL of HCl solution in methanol (10:90 vol:vol). The solution was allowed to react for 10 min at 80°C. Once cooled, 2 mL of a solution K_2CO_3 (6:94 wt:vol) was added, followed by the addition of 2 mL of hexane to extract FAME and DMA. The solution was vortexed, centrifuged, and finally, the supernatant phase with FAME and DMA was transferred to another tube. The extraction step was repeated twice. Identification of DMA was performed by previous purification of DMA fraction by thin-layer chromatography, as described in Alves et al. (2013). The procedures for GC analysis and FAME identification were the same above described for the analysis of milk FAME. The identification of DMA was obtained by GC/MS, according to Alves et al. (2013).

DNA Extraction from Rumen Liquor. Genomic DNA was extracted from 1 mL of rumen microbial suspension using the Fast DNA SPIN kit for soil (Qbiogene, Carlsbad, CA). Each sample was homogenized with a FastPrep cell disrupter instrument (Bio101, ThermoSavant, Qbiogene) for 2×40 s at speed 6.0 and then processed according to the manufacturer's guidelines. The DNA was eluted in sterile water and its integrity was verified by agarose gel electrophoresis. The amount and the purity of DNA was measured at 260 and 280 nm using a ND-1000 Spectrophotometer (NanoDrop Technologies, Labtech, Ringmer, UK).

PCR-Denaturing Gradient Gel Electrophoresis Analysis of the Total Bacterial Community. The analysis of total bacterial community was performed on a selected group of animals, according to the results obtained from FA composition of milk and rumen liquor samples. In particular, the analysis was performed on the DNA extracted from the rumen liquor of 5 animals of L0 and L1.2 groups. Moreover, DNA extracted from rumen liquor samples collected from the same animals at the sampling time before the beginning of the adaptation period was also considered in the analysis. This approach allowed evaluation of the distribution of bacterial communities before starting the experiment.

Total DNA extracted was diluted to a concentration of 5 ng/ μL and 2 μL of diluted DNA was used as template in PCR. Amplification of the V6–V8 region of the 16S rRNA gene was carried out with the primer pair F968GC (5'-CGC CCG CCG CGC GCG GCG GGC GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and R1401 (5'-CGG TGT GTA CAA GAC CC-3') for total eubacterial PCR (fragment size ~ 470 bp). Reactions were carried out using a T100 Thermal Cycler (Bio-Rad Laboratories, Hertfordshire, UK) in 25 μL volumes containing $1 \times$

Flexi PCR buffer (Promega, Madison, WI), 1.5 mM MgCl₂, 250 μM deoxynucleotide triphosphates, 400 nM each primer, and 1 U GoTaq Flexi DNA polymerase (Promega). Amplifications were performed under the following conditions: an initial denaturation of 94°C for 5 min followed by 35 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 45 s, and a final extension of 72°C for 10 min. After PCR, amplified products were verified by agarose gel electrophoresis. Subsequently, to perform PCR denaturing gradient gel electrophoresis (DGGE) analysis, amplicons were loaded on a 6% polyacrylamide gel (40% acrylamide/bis 37.5:1; Serva Electrophoresis GmbH, Heidelberg, Germany), with a 50 to 60% denaturing gradient (100% denaturant consisting of 40% vol/vol deionized formamide, 7 M urea) and electrophoresis was performed in a Dcode DGGE System (Bio-Rad Laboratories). The gels were run for 17 h at 60°C and 75 V. After electrophoresis gels were stained with SYBR Gold (Molecular Probes, Eugene, OR) and images digitalized using ChemiDoc XRS (Bio-Rad Laboratories).

The PCR-DGGE banding patterns obtained were normalized and analyzed using the software package GelCompar II Software v 4.6 (Applied Maths, Saint-Martens-Latem, Belgium). To summarize the species number (richness) of rumen bacterial communities, each band was considered as corresponding to a single microbial species and the relative abundance of each band in DGGE profile (Shannon index) was used as a proxy of bacterial diversity (Pastorelli et al., 2011).

Statistical Analysis of Data

All variables (with the exception of rumen liquor FA and DMA, and rumen bacterial diversity indices) were processed as a completely randomized design with repeated measures using the following linear mixed model (SAS 9.2, 2013, SAS Institute Inc., Cary, NC).

$$y_{ijk} = \mu + D_i + P_j + (D \times P)_{ij} + A_k[D_i] + e_{ijk}, \quad [1]$$

where y_{ijk} is the observation, μ is the overall mean, D_i is the fixed effect of diet ($i = 1$ to 4), P_j is the fixed effect of sampling time ($j = 1$ to 5), $(D \times P)_{ij}$ is the interaction between diet and sampling time, A_k is the random effect of the animal nested within the diet ($k = 1$ to 5), and e_{ijk} is the residual error.

The covariance structure was compound symmetry, which was selected based on Akaike's information criterion of the mixed model of SAS. Statistical significance of the diet effect was tested against variance of sheep nested within diet according to repeated measures design theory (Littell et al., 1998). Linear and quadratic

coefficients of polynomial orthogonal contrasts for unequally spaced OCPC doses were determined using PROC IML of SAS and then these contrasts were used to test the linear and quadratic effects of OCPC inclusion.

With regard to FA and DMA composition of rumen liquor, data were analyzed by a different model because only one sample per animal at the end of experimental period was collected.

$$y_k = \mu + b_1X_1 + (b_2X_1)^2 + e_k, \quad [2]$$

where y_k was the dependent variable (individual FA expressed as mg/g of DM), μ was the overall mean, X_1 was the independent variable (amount of OCPC supplemented: 0, 0.6, 0.8, 1.2), b was the regression coefficient, and e was the residual error.

Linear and quadratic coefficients of polynomial orthogonal contrasts for unequally spaced OCPC doses were determined using PROC IML of SAS and then these contrasts were used to test the linear and quadratic effects of OCPC inclusion.

Data of rumen liquor DMA were also processed with a stepwise discriminant analysis (SAS 9.2) to individuate further variables able to discriminate diets. Only DMA that did not show significant differences in univariate analysis were considered. The selected variables were submitted to a canonical discriminant analysis to derive canonical functions that summarize the variation between groups. The efficiency in group identification was evaluated by the test of significance of Mahalanobis distance.

Data from PCR-DGGE analysis of DNA extracted from rumen liquor samples were used to obtain richness and Shannon diversity indexes (Pastorelli et al., 2011) that were analyzed by 1-way ANOVA followed by the Tukey's test for $P < 0.05$. Data relative to the basal period were used as a covariate in the statistical model.

Band-matching data, exported as quantitative information, were standardized by calculating the relative intensity of each band (ratio of intensity of each band versus the total band intensity) and imported into PAST software (Hammer et al., 2001; <http://folk.uio.no/ohammer/past>) for multivariate statistical analysis.

One-way analysis of similarity (ANOSIM) and permutational ANOVA (PERMANOVA) were conducted to examine the statistical significance of the DGGE profiles. Nonmetric multidimensional scaling analysis was used to represent the distance between each bacterial community in a 2-dimensional space. All the analysis were conducted using the Bray-Curtis distance measure and 9,999 permutation tests.

RESULTS AND DISCUSSION

Animal Performance and Milk Composition

The main phenolic compounds contained in the wastewaters are secoiridoids. They are represented by the dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA, oleuropein aglycone) or to p-HPEA (p-HPEA-EDA, ligstroside aglycone di-aldehyde), and by the monoaldehydic forms of the same molecules (3,4-DHPEA-EA and p-HPEA-EA; Obied et al., 2008). These compounds originate during the extraction process of the olive oil, as aglycone derivatives of native secoiridoid glucosides (namely oleuropein, dimethyl oleuropein, and ligstroside) contained in the olive fruit. Additionally, verbascoside, a derivative of hydroxycinnamic acid, which contains the 3,4-DHPEA in its molecular structure, may be also present in the phenolic fraction of wastewaters from olive oil extraction (Servili et al., 1999). In the OCPC used to formulate the experimental concentrate we found 3,4-DHPEA, p-HPEA, 3,4-DHPEA-EDA, and verbascoside (Table 2). The presence of 3,4-DHPEA, p-HPEA was mainly due to the hydrolysis of 3,4-DHPEA-EDA and p-HPEA-EDA (Servili et al., 1999). Into the wastewater, all the above reported phenolic compounds are in a dynamic equilibrium and may shift between different forms very rapidly (Obied et al., 2008). Hence, we analyzed the concentrate containing increasing levels of OCPC to check the actual amounts of individual phenolic substances included in the experimental concentrates. As reported in Table 2, the total amount of polyphenols across experimental concentrates increased in accordance with the amounts of OCPC used to formulate the concentrates (Tables 1 and 2). On the other hand, the distribution of individual phenolic compounds was not fully consistent with the pattern of total polyphenols in the concentrates (Table 2), suggesting a lack of stability of the phenolic substances of OCPC during the production of the experimental concentrates (Servili et al., 2011).

During the experiment, the average DMI of experimental groups was 1.92 ± 0.07 , 1.93 ± 0.12 , 1.93 ± 0.06 , 1.90 ± 0.07 kg/animal and day for L0, L0.6, L0.8, and L1.2, respectively. As reported in Table 3, dietary treatments did not affect milk yield and milk composition (fat, protein, lactose, and urea). Because no data are available in literature about the use of OCPC in the diet of ruminants, comparisons may be only made with studies adopting raw materials rich in polyphenols such as olive pomace. Previous studies about the introduction of olive pomace in the diet of small ruminants are not consistent about the effect on milk yield and composition. Molina-Alcaide et al. (2010) observed no differences in milk yield including crude olive pomace in the diets of dairy goats, whereas Chiofalo et al. (2004) found an increase in productive performance of dairy ewes. However, these studies did not report the amount of phenols contained in the olive pomace neither the profile. Hence the inconsistency of results might be related to the total amount of polyphenols and to the ratio among single molecules present in the extract. The phenolic compounds contained in the olive by-products (pomace, wastewater) are in a dynamic equilibrium and may shift to different chemical forms very rapidly.

In the present study, the amount of verbascoside administrated to ewes did not significantly affect milk yield. This result did not agree with Casamassima et al. (2014) who reported a positive effect of microencapsulated verbascoside on milk yield and composition in dairy ewes at mid lactation fed with an antioxidant supplement titrated at 0.5% of verbascoside. In the trial of Casamassima et al. (2014), other components of the supplement probably contributed to enhance milk yield or preserve verbascoside by rumen degradation. In particular, microencapsulation may have preserved verbascoside from rumen degradation in the experiment of Casamassima et al. (2014). In fact, the fate of verbascoside in the rumen liquor is likely similar to that of other phenolic glycosides with similar molar weight,

Table 3. Milk yield and composition from ewes fed with lucerne hay and 800 g/ewe per day of experimental concentrates containing increasing doses of olive crude phenolic concentrate (OCPC)

Item	Experimental diet ¹				SEM	P-value	
	L0	L0.6	L0.8	L1.2		Linear	Quadratic
Milk yield (g/d)	705.3	611.1	617.1	672.4	84.2	0.546	0.465
Fat (g/100 g)	8.37	7.82	7.92	7.46	0.75	0.675	0.632
Protein (g/100 g)	6.31	6.14	6.11	6.22	0.32	0.787	0.987
Lactose (g/100 g)	4.46	4.39	4.43	4.36	0.17	0.546	0.478
Urea (mg/dL)	35.85	32.02	35.95	39.58	5.22	0.576	0.767
Linear score	3.63	3.68	5.56	4.20	0.92	0.632	0.539

¹L0 = control diet; L0.6 = control diet plus 0.6 g/kg of OCPC; L0.8 = control diet plus 0.8 g/kg of OCPC; L1.2 = control diet plus 1.2 g/kg of OCPC.

such as rutin, which are hydrolyzed in the rumen with subsequent cleavage of the heterocyclic ring (Mc Sweeney et al., 2001).

Interestingly, milk urea content (that is directly related to hematic urea) did not vary among treatments, suggesting that olive phenols did not interact in the rumen with the dietary protein metabolism (Table 3), unlike other phenolic substances such as tannins (Frutos et al., 2004; Buccioni et al., 2015a). However, in the present study, the amount of polyphenols added to the diet was very low if compared with the dose adopted in the experiment based on tannin supplementation (Frutos et al., 2004; Buccioni et al., 2015a).

Rumen Microbial Ecosystem

Many species of bacteria, archaea, and eukaryotes present in the rumen are still unknown and only a few number of them are isolated and physiologically characterized (Kong et al., 2010).

The rumen liquor samples of ewes collected before starting the experimentation and fed with L0 and L1.2 experimental diets were analyzed with PCR-DGGE. This approach allowed comparison of the extreme treatments. The 16S rRNA genes were amplified to have an overview of the diversity of total bacteria communities.

Richness and Shannon-Weiner index calculated on 16S rDNA DGGE profiles ranged from 10 to 23 and from 2.27 to 3.11, respectively, and no significant differences were found between L0 and L1.2 samples.

The analysis of total bacteria PCR-DGGE profiles highlighted a significant difference between the treatments, probably due to the influence of the olive polyphenols. The nonmetric multidimensional scaling analysis of bacterial DGGE fingerprints (Figure 1) evidenced a clear separation between community before starting the experiment and at the end of the experiment. Moreover, the pattern of bacterial communities differed according to the experimental diets. This separation was confirmed by ANOSIM and PERMANOVA analysis, which provided a significant discrimination (ANOSIM global test $R = 0.717$; $P < 0.001$; PERMANOVA global test: bacteria $F = 5.164$, $P < 0.001$) between DGGE profiles of rumen samples considering the 3 groups: basal (before the starting of the adaptation period); rumen liquor samples collected at the end of the experiment from animals fed the L0 diet (L0) or L1.2 diet (L1.2). The results of the pairwise comparisons showed significant differences (in each case $P < 0.01$) between the DGGE profiles of the 3 groups. If the rumen samples of the starting point of experimentations were considered separately, depending on the following L0 or L1.2 diets, ANOSIM and PERMANOVA did not give any significant differences between DGGE profiles

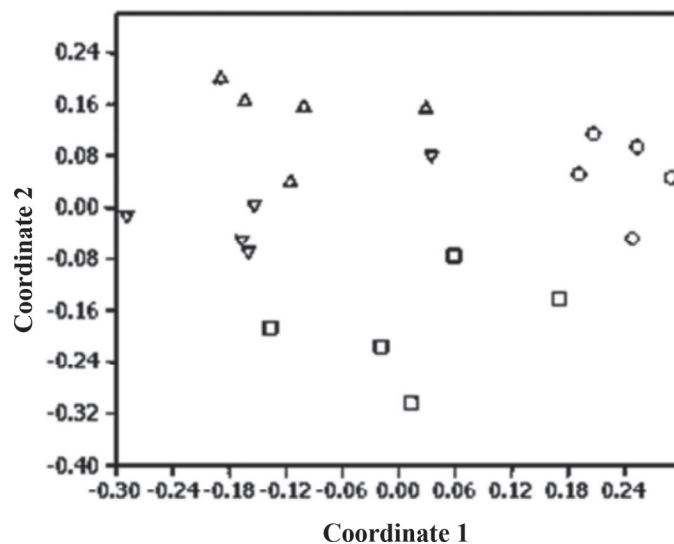


Figure 1. Nonmetric multidimensional scaling plot (2-dimensional stress = 0.2686) representing the distance between each rumen bacterial community in a 2-dimensional space according to 16S ribosomal DNA denaturing gradient gel electrophoresis profiles. Triangles represent rumen liquor samples collected before the starting of the adaptation period from animals that during the experimental period were fed the L0 diet; inverted triangles represent rumen liquor samples collected before the starting of the adaptation period from animals that during the experimental period were fed the L1.2 diet; squares represent rumen liquor samples collected at the end of the experiment from animals fed the L0 diet; circles represent rumen liquor samples collected at the end of the experiment from animals fed the L1.2 diet. L0 = control diet; L0.6 = control diet plus 0.6 g/kg of olive crude phenolic concentrate (OCPC); L0.8 = control diet plus 0.8 g/kg of OCPC; L1.2 = control diet plus 1.2 g/kg of OCPC.

of these 2 groups (ANOSIM global test $R = 0.717$; $P < 0.001$; PERMANOVA global test: bacteria $F = 5.164$, $P < 0.001$).

Thus, the effects on lipid biohydrogenation observed in the present study were probably associated with changes in the overall composition of rumen microbe population. According to literature, several bacteria strains are involved in RBH. In fact, previous *in vivo* studies on rumen bacterial diversity in cows and ewes suggest that as-yet-uncultured bacteria phylogenetically classified as *Prevotella*, *Lachnospiraceae*, *incertae sedis*, and unclassified *Bacteroidales*, *Clostridiales*, and *Ruminococcaceae* might have a relevant role in the biohydrogenation process (Boeckert et al., 2008; Belenguer et al., 2010; Huws et al., 2011; Castro-Carrera et al., 2014).

Fatty Acid Profile of Rumen Liquor

Increasing dose of OCPC in the diet resulted in a linear decrease ($P < 0.028$) of the RBH end product (C18:0) and of several RBH intermediates (C18:1 *cis*-11, C18:1 *cis*-12, C18:1 *trans*-6–8, C18:1 *trans*-11,

C18:1 *trans*-15) in rumen liquor. This result, coupled with the linear increase of C18:2n-6, C18:3n-3, and C18:3 *cis*-9,*trans*-11,*cis*-15, clearly indicated that OCPC was able to decrease the RBH, resulting in an accumulation of the substrates (C18:2n-6 and C18:2n-3) or of the first intermediates of RBH (C18:3 *cis*-9,*trans*-11,*cis*-15; Table 4). Interestingly, only a part of the RBH intermediates of C18:2n-6 and C18:3n-3 was affected by treatment. In particular, C18:1 *cis*-11, C18:1 *cis*-12, and C18:1 *trans*-11 were considered RBH

intermediates of both C18:2n-6 and C18:3n-3, whereas C18:1 *trans*-6-8 has been reported as an intermediate of RBH of C18:2n-6 or, alternatively, of C18:1 *cis*-9. Finally, C18:1 *trans*-15 has been associated with the RBH of C18:3n-3 (Shingfield et al., 2010). Because different RBH intermediates are associated with different metabolic pathways of the RBH of UFA (Shingfield et al., 2010), the lack of significance of the treatments on specific RBH intermediates could be due to a selective effect of OCPC on specific metabolic pathways.

Table 4. Fatty acid composition of rumen liquor samples at the end of the experimental period from ewes fed increasing amounts of olive crude phenolic concentrate (OCPC; data expressed as mg/g of DM)

Item	Experimental diet ¹				SEM	<i>P</i> -value ²	
	L0	L0.6	L0.8	L1.2		Linear	Quadratic
Total fatty acids	70.50	67.56	65.23	68.50	2.51	0.180	0.343
C12:0	0.21	0.21	0.20	0.19	0.028	0.940	0.826
C13:0	0.17	0.14	0.11	0.10	0.030	0.553	0.841
C13:0 <i>iso</i>	0.12	0.08	0.08	0.12	0.017	0.133	0.107
C14:0	1.15	0.98	0.91	0.95	0.170	0.555	0.667
C14:0 <i>iso</i>	0.31	0.27	0.30	0.39	0.045	0.487	0.326
C15:0	1.28	1.21	1.27	1.46	0.199	0.755	0.642
C15:0 <i>ante</i>	1.46	1.40	1.55	1.92	0.248	0.751	0.534
C15:0 <i>iso</i>	0.87	0.71	0.74	0.97	0.109	0.291	0.214
C16:0	16.14	16.14	16.67	17.71	0.905	0.894	0.677
C16:0 <i>iso</i>	0.57	0.50	0.56	0.73	0.109	0.585	0.423
C16:1 <i>cis</i> -7	0.39	0.35	0.26	0.13	0.055	0.893	0.545
C16:1 <i>cis</i> -9	0.07	0.05	0.05	0.07	0.011	0.334	0.276
C16:1 <i>trans</i> -9	0.10	0.07	0.10	0.17	0.024	0.298	0.382
C17:0	0.40	0.40	0.39	0.37	0.054	0.978	0.905
C17:0 <i>ante</i>	0.69	0.64	0.68	0.81	0.124	0.721	0.601
C17:0 <i>cyclo</i>	0.12	0.18	0.20	0.21	0.027	0.286	0.538
C17:0 <i>iso</i>	0.32	0.28	0.30	0.37	0.039	0.520	0.124
C18:0	30.69	26.39	23.21	21.15	0.928	0.001	0.389
C18:0 <i>oxo</i>	0.09	0.07	0.06	0.06	0.013	0.398	0.552
C18:1 <i>cis</i> -9	5.42	4.80	4.63	4.89	0.277	0.173	0.256
C18:1 <i>cis</i> -11	0.33	0.27	0.22	0.20	0.023	0.007	0.577
C18:1 <i>cis</i> -12	0.54	0.37	0.30	0.32	0.033	0.009	0.060
C18:1 <i>cis</i> -13	0.04	0.04	0.05	0.06	0.011	0.934	0.676
C18:1 <i>cis</i> -14+ <i>trans</i> -16	0.62	0.64	0.60	0.52	0.077	0.827	0.663
C18:1 <i>cis</i> -15	0.36	0.22	0.18	0.23	0.028	0.028	0.023
C18:1 <i>trans</i> -6, <i>trans</i> -7, <i>trans</i> -8	0.58	0.46	0.38	0.33	0.043	0.006	0.603
C18:1 <i>trans</i> -9	0.25	0.26	0.24	0.20	0.029	0.830	0.608
C18:1 <i>trans</i> -10	0.34	0.33	0.31	0.27	0.043	0.979	0.823
C18:1 <i>trans</i> -11	4.20	3.09	2.52	2.49	0.130	0.001	0.009
C18:1 <i>trans</i> -12+DMA ³ 18:2	0.61	0.60	0.56	0.50	0.059	0.982	0.745
C18:1 <i>trans</i> -15	0.91	0.75	0.66	0.63	0.070	0.004	0.507
C18:2n-6	2.12	2.19	2.28	4.21	0.351	0.006	0.210
C18:2 <i>trans</i> -11, <i>cis</i> -15	0.71	0.61	0.54	0.50	0.059	0.351	0.681
CLA <i>cis</i> -9, <i>trans</i> -11	0.30	0.25	0.24	0.29	0.011	0.650	0.080
CLA <i>trans</i> , <i>trans</i>	0.17	0.18	0.17	0.14	0.026	0.655	0.518
C18:3n-3	1.32	1.54	2.16	3.19	0.258	0.001	0.260
C18:3 <i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15	0.09	0.11	0.14	0.16	0.013	0.010	0.972
C20:0	0.36	0.32	0.32	0.35	0.020	0.175	0.176
C21:0	0.05	0.05	0.04	0.03	0.006	0.747	0.490
C22:0	0.30	0.31	0.33	0.37	0.041	0.961	0.740
C23:0	0.13	0.10	0.09	0.11	0.020	0.232	0.285
C24:0	0.32	0.33	0.35	0.37	0.033	0.937	0.910
C26:0	0.14	0.15	0.15	0.15	0.019	0.860	0.938

¹L0 = control diet; L0.6 = control diet plus 0.6 g/kg of OCPC; L0.8 = control diet plus 0.8 g/kg of OCPC; L1.2 = control diet plus 1.2 g/kg of OCPC.

²*P*-value: significance from *P* < 0.05.

³DMA = dimethylacetyl.

A previous *in vitro* study showed that olive pomace was able to affect RBH of PUFA, leading to a decrease of C18:0 (SA) and to an increase of VA, the main intermediate of linoleic (LA) and α -linolenic acid (α -LNA) RBH (Pallara et al., 2014). The authors demonstrated that these changes were related to microbiome profile modification, concluding that olive polyphenols may perturb rumen microorganisms involved in the RBH of dietary FA (Pallara et al., 2014).

The perturbation of RBH has also been demonstrated for other kind of phenolic substances such as tannins. Carreno et al. (2015), comparing different kinds and doses of tannin extracts to investigate modulation of *in vitro* RBH of FA, reported that all tannin extracts were able to modulate RBH of UFA. More recently, Costa et al. (2017) reported that condensed tannins from grape seed and, to a lesser extent, from rockrose, stimulated the first steps of RBH, with an accumulation of VA and without a clear inhibition of SA production.

Hence, data from the *in vitro* study from Pallara et al. (2014) suggested that olive polyphenols reduce RBH acting on the last step of the process, similarly to what was reported for tannins, whereas results of the present *in vivo* study suggested that the first step of RBH was probably negatively affected by olive polyphenols. This lack of consistency between *in vitro* and *in vivo* studies, and among studies in general, may be due to several factors such as the rumen passage rate, interaction with basal diet, and different amount of lipids in the diet and the specific composition of phenolic substances.

Dimethylacetals in Rumen Liquor

Alves et al. (2013), recently reported that analysis of DMA [alk-1-enyl (vinyl) ether chains] in rumen liquor may be associated with bacterial plasmalogenic lipids. Dimethylacetals derive from fatty aldehydes released from plasmalogens, which are a subclass of phospholipids contained in large amounts in membranes of anaerobic bacteria (Katz and Keeney, 1964). Although it is not yet completely demonstrated, the role of DMA might be the regulation of bacterial membrane fluidity of anaerobe bacteria in response to different environmental stimuli (Goldfine, 2010; Alves et al., 2013).

As reported in Table 5, the total amount of DMA did not vary across treatments, confirming that rumen bacteria are able to adjust the composition of membrane plasmalogenic lipids without affecting the total amount of DMA (Alves et al., 2013). Interestingly, the total amount of DMA recorded in the study of Alves et al. (2013) was lower than 0.5 mg/g of whole rumen content DM, whereas in the present trial, using the liquid rumen phase, the total amount of DMA ranged

from 3.82 to 4.52 mg/g of DM for L0.8 and control diet, respectively. This large difference is probably explained by the type of rumen samples, as in the whole rumen content DM the microbial DMA concentration would be more diluted by larger amounts of undigested feed than in liquid phase rumen DM. Moreover, the rumen microbiome composition or the type of animal considered could also explain the differences in DMA content between studies. The study of Alves et al. (2013), in fact, dealt with rumen liquor from lambs fed concentrate or pelleted lucerne supplemented or not with soybean oil.

Seventeen different DMA were identified in rumen liquor. Linear and branched medium chain acetals accounted for more than 85% of total DMA. The remaining part was composed by DMA with 17 or more carbon atoms. Regardless the dietary treatment, DMA16:0 was the major component, confirming what was previously reported by Alves et al. (2013). Linear medium chain DMA (DMA 13:0, DMA 14:0, DMA 15:0) linearly increased as the percentage of OCPC in the diet increased. Conversely, the percentage of DMA 16:0 linearly decreased. Among long-chain DMA, DMA 18:0 and DMA 18:1 showed an opposite trend: the former increased, whereas the latter decreased with the increasing of OCPC percentage in the diet.

Changes in the DMA relative composition did not correspond to changes in FA composition with the only exception of C18:0, which showed the same trend in both FA and DMA composition. This did not agree with Alves et al. (2013) who found a perfect correspondence between the variation of DMA and FA composition in rumen liquor samples.

As previously suggested by Alves et al. (2013), a possible explanation of the effect of dietary treatments on DMA composition of rumen liquor may be a rearrangement of membrane phospholipids of rumen bacteria in response to changes in the rumen environment associated with dietary polyphenols. However, it is not possible to exclude that changes in bacterial population composition could also be associated with changes in DMA profile of rumen liquor. For instance, although DMA 16:0 is present in the cell membrane of several bacterial strains, some species, such as *Streptococcus bovis*, are particularly rich in DMA 16:0 (Minato et al., 1988). At the same time, high levels of DMA 13:0 and DMA 14:0 were found in the membrane of *Bacteroides succinogens* and *Lachnospira multiparous*, respectively (Minato et al., 1988). Previous studies demonstrated that subtle dietary shifts such as the substitution of one forage by another resulted in changes in the whole rumen microflora (Dehority and Orpin, 1988; Saluzzi et al., 1995).

Table 5. Total dimethylacetal (DMA; mg/g of DM) and DMA composition (% of total DMA) of rumen liquor samples at the end of the experimental period from ewes fed increasing amounts of olive crude phenolic concentrate (OCPC)

Item	Experimental diet ¹				SEM	P-value ²	
	L0	L0.6	L0.8	L1.2		Linear	Quadratic
Total DMA	4.52	3.94	3.82	4.15	0.508	0.47	0.51
DMA 12:0	0.63	0.75	0.86	0.96	0.083	0.51	0.96
DMAa 13:0	0.42	0.70	0.76	0.59	0.117	0.13	0.17
DMA 13:0	0.43	0.90	1.06	0.90	0.093	0.01	0.22
DMAi 14:0	6.28	6.27	6.46	6.88	0.392	0.88	0.68
DMA 14:0	6.55	7.42	7.47	6.72	0.236	0.02	0.68
DMAi 15:0	2.14	1.90	2.06	2.62	0.373	0.58	0.43
DMAa 15:0	13.64	15.13	15.70	15.35	0.841	0.29	0.42
DMA 15:0	5.45	6.09	6.20	5.76	0.159	0.02	0.44
DMA 16:0	47.03	44.15	43.00	43.56	0.589	0.02	0.51
DMA 16:1	3.62	3.34	2.77	1.90	0.397	0.88	0.07
DMA 17:0	0.85	0.60	0.54	0.69	0.086	0.58	0.10
DMA 17:1	2.27	2.74	2.77	2.34	0.411	0.30	0.42
DMA 18:0	2.83	3.15	3.37	3.50	0.270	0.50	0.78
DMA 18:1 <i>trans</i> -11	1.43	1.27	1.32	1.57	0.174	0.19	0.79
DMA 18:1 <i>cis</i> -9	3.10	2.47	2.44	3.02	0.219	0.39	0.06
DMA 18:1 <i>cis</i> -11	1.98	1.81	1.83	2.03	0.161	0.45	0.48
DMA 18:1 <i>cis</i> -12	0.85	0.72	0.82	1.14	0.160	0.44	0.40

¹L0 = control diet; L0.6 = control diet plus 0.6 g/kg of OCPC; L0.8 = control diet plus 0.8 g/kg of OCPC; L1.2 = control diet plus 1.2 g/kg of OCPC.

²P-value: significance from $P < 0.05$.

Discriminant Analysis of DMA in Rumen Liquor

In the present study, several DMA were found. This aspect requires appropriate interpretation to select the best DMA that discriminate between diets, as a function of external stimuli that act on the microbial community. Twelve DMA that did not significantly vary in the univariate analysis (Table 5) were included in a discriminant analysis to reduce the number of original variables, identifying the latent variables called canonical. Eight DMA (DMAa 13:0, DMAi 15:0, DMAa 15:0, DMA 16:1, DMA 17:0, DMA 18:1 *trans*-11, DMA 18:1 *cis*-11, and DMA 18:1 *cis*-12) were selected by stepwise analysis and used for canonical discriminant analysis to evaluate the discriminant ability of these variables across the treatments. The canonical analysis extracted 3 canonical variables. Only the first 2 canonicals were considered for discriminant effect because they explained more than 92% of the variability (Table 6). Discriminant analysis separated without error and with significant Wilks' λ coefficient ($P < 0.001$). Canonical 1 was able to separate the experimental treatments, whereas canonical 2 was able to discriminate the L0 diet from all the diets containing OCPC (Figure 2). Long-chain DMA were positively associated with the L0 diet, whereas medium-chain DMA were positively associated with diet containing OCPC. The only exception was DMAa 15:0, which was negatively associated with both canonicals. Thus, discriminant analysis added more information about the potential association between

individual DMA and specific dietary treatments, suggesting a putative role of DMA as rumen biomarkers.

Milk Fatty Acid Profile

The FA composition of milk, which was rich in VA, RA, and α -LNA due to the presence of EL (Mele et al., 2011; Casamassima et al., 2014; Buccioni et al., 2015b), was only partially modified by the inclusion of OCPC in the diets (Table 7).

Among short-chain FA, the amount of C4:0 was significantly higher ($P < 0.01$) in milk fat from ewes fed L0.8 and L1.2 diets. This FA may originate either from elongation of acetate or by direct incorporation of butyrate produced during rumen fermentation of

Table 6. Discriminant dimethylacetal (DMA) by a multivariate analysis and value referring to the 2 canonicals (CAN1 and CAN2) obtained

Item	CAN1	CAN2
DMAa 13:0	0.455	-0.444
DMAi 15:0	0.259	0.122
DMAa 15:0	-0.397	-0.384
DMA 16:1	0.315	0.181
DMA 17:0	-0.098	0.551
DMA 18:1 <i>trans</i> -11	-0.216	0.151
DMA 18:1 <i>cis</i> -11	0.017	0.207
DMA 18:1 <i>cis</i> -12	0.468	0.149
Variability explained (%)	74.591	17.778
Cumulative variability (%)	74.591	92.369

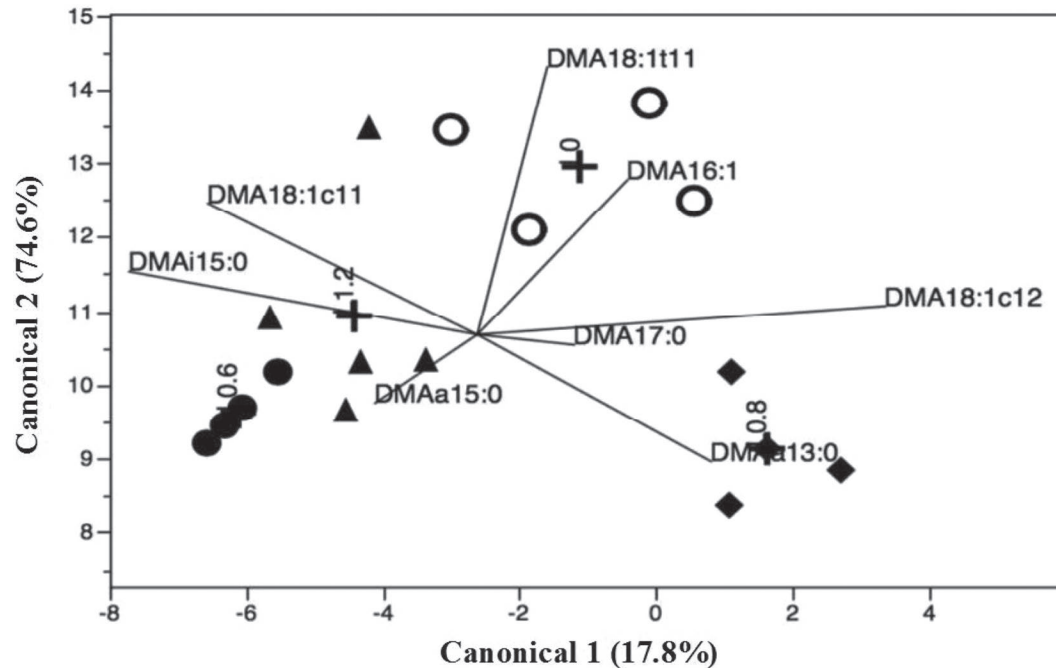


Figure 2. Plot of the dimethylacetal (DMA) and the 2 canonicals processed with a discriminant analysis. Diet profile: control (L0, empty circle); control diet plus 0.6 g/kg of olive crude phenolic concentrate (OCPC; L0.6, full circle); control diet plus 0.8 g/kg of OCPC (L0.8, full rhombus); control diet plus 1.2 g/kg of OCPC (L1.2, full triangle). The plus sign represents the centroid of each group.

carbohydrates (Chilliard et al., 2007). Pallara et al. (2014), in a previous *in vitro* study, showed that supplementing olive pomace in the substrate of fermentation also stimulated the production of C4:0. Similar results were found by Yáñez Ruiz et al. (2004) feeding Granadina and Segureña goats with diets supplemented with 2-stage dried olive pomace. The authors suggested a relationship between the production of C4:0 in rumen liquor and the amount of C4:0 in milk.

Interestingly, although SA content in rumen liquor linearly decreased as the OCPC content in the diet increased (Table 4), the percentage of SA in milk fat did not vary across treatments. Similar results were found by Buccioni et al. (2015b) in milk from grazing ewes fed a concentrate containing EL and chestnut tannins.

Milk from the L0.8 and L1.2 group contained higher levels of LA and α -LNA than milk from sheep fed the L0 and L0.6 diet ($P < 0.001$). Indeed, LA and α -LNA content was significantly increased (+18 and +24%, respectively) when the highest level of OCPC (L1.2) was fed. This result was consistent with the higher LA and α -LNA content found in the rumen liquor samples (Table 4). Previous studies about the effect of different kind of polyphenolic substances in the diet of dairy ruminants reported an increase of α -LNA similar to that observed in the present study. In particular, the milk content of α -LNA increased by almost 18% when grazing ewes were supplemented with EL and chestnut

tannin extract (Buccioni et al., 2015a). Ferlay et al. (2010), supplementing dairy cow with vitamin E, linseed, and a commercial mix of different polyphenols, reported a similar enhancement of this FA (+23%) in milk fat.

The reduction of RBH intermediates, including vacellenic acid (C18:2 *trans*-11,*cis*-15) and VA in milk from sheep fed the L0.8 and L1.2 diets was a direct consequence of the lower accumulation in rumen liquor of these FA (Table 4). Lower amount of VA escaping from the rumen, moreover, led to lower production of RA in mammary gland, being the milk fat content of RA mainly due to the mammary Δ^9 -desaturation of 18:1 *trans*-11 (Bichi et al., 2013).

Long-chain FA, such as arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3), significantly increased in milk from sheep fed L0.8 and L1.2 diets (Table 6) (+25, +22, +33, and +68%, respectively), probably as a consequence of the higher availability of the precursors (LA and α -LNA, Bionaz and Loor, 2008).

CONCLUSIONS

Increasing doses of OCPC in the diet of dairy ewes supplemented with EL did not have any detrimental effect on milk yield and composition, and resulted in a

Table 7. Fatty acid composition of milk from sheep fed increasing amounts of olive crude phenolic concentrate (OCPC)

Fatty acid	Experimental diet ¹				SEM	Linear	Quadratic
	L0	L0.6	L0.8	L1.2			
C4:0	3.34 ^b	3.41 ^b	3.79 ^a	3.67 ^a	0.07	0.004	0.765
C6:0	1.79	1.93	2.04	2.05	0.09	0.741	0.897
C8:0	1.36	1.48	1.56	1.56	0.09	0.311	0.567
C10:0	3.76	4.20	4.29	4.25	0.27	0.593	0.637
C10:1 <i>cis</i> -9	0.12	0.13	0.13	0.13	0.01	0.564	0.768
C11:0	0.03	0.04	0.04	0.04	0.003	0.803	0.785
C12:0	2.39	2.47	2.55	2.44	0.11	0.314	0.921
C13 <i>iso</i>	0.02	0.02	0.02	0.02	0.001	0.498	0.891
C13 <i>ante</i>	0.01	0.01	0.01	0.01	0.002	0.961	0.546
C12:1 <i>cis</i> -9	0.02	0.02	0.02	0.02	0.002	0.711	0.657
C13	0.05	0.05	0.05	0.05	0.002	0.881	0.687
C14 <i>iso</i>	0.08	0.09	0.09	0.09	0.007	0.920	0.893
C14	8.10	7.90	8.22	7.86	0.22	0.561	0.741
C15 <i>iso</i>	0.21	0.19	0.22	0.22	0.01	0.678	0.598
C15 <i>ante</i>	0.38	0.37	0.39	0.38	0.015	0.773	0.639
C14:1 <i>cis</i> -9	0.14	0.13	0.13	0.13	0.01	0.877	0.622
C15	0.86	0.86	0.93	0.88	0.037	0.569	0.887
C16 <i>iso</i>	0.2	0.19	0.21	0.21	0.009	0.647	0.966
C16	19.9	19.84	20.62	19.95	0.39	0.765	0.454
C16:1 <i>trans</i> -9	0.27	0.37	0.21	0.21	0.02	0.428	0.644
C16:1 <i>cis</i> -7	0.33	0.32	0.30	0.30	0.009	0.776	0.556
C17 <i>iso</i>	0.35	0.35	0.36	0.34	0.01	0.850	0.778
C16:1 <i>cis</i> -9	0.67	0.62	0.64	0.65	0.03	0.934	0.997
C17 <i>ante</i>	0.34	0.31	0.36	0.32	0.01	0.761	0.888
C17	0.52	0.53	0.61	0.54	0.03	0.825	0.676
C18 <i>iso</i>	0.03	0.03	0.03	0.03	0.002	0.747	0.882
C17:1 <i>cis</i> -9	0.14	0.15	0.16	0.14	0.008	0.675	0.665
C18:0	12.44	11.72	12.49	13.61	0.48	0.432	0.345
C18:1 <i>trans</i> -5	0.04	0.04	0.04	0.04	0.002	0.798	0.771
C18:1 <i>trans</i> -6-8	0.73 ^a	0.71 ^{ab}	0.62 ^b	0.64 ^b	0.025	0.001	0.344
C18:1 <i>trans</i> -9	0.61 ^a	0.62 ^a	0.54 ^{ab}	0.54 ^b	0.023	0.007	0.232
C18:1 <i>trans</i> -10	0.79 ^a	0.75 ^a	0.69 ^b	0.72 ^{ab}	0.033	0.015	0.441
C18:1 <i>trans</i> -11	4.05 ^{ab}	5.10 ^a	3.30 ^b	3.15 ^b	0.32	0.001	0.265
C18:1 <i>trans</i> -12	1.14 ^a	1.04 ^{ab}	0.92 ^b	1.01 ^{ab}	0.04	0.032	0.339
C18:1 <i>cis</i> -9	22.32	22.15	21.48	21.39	0.55	0.636	0.691
C18:1 <i>cis</i> -11+ <i>trans</i> -15	0.99	0.89	0.91	0.88	0.04	0.693	0.599
C18:1 <i>cis</i> -12	1.03 ^a	0.76 ^b	0.77 ^b	0.87 ^{ab}	0.05	0.001	0.211
C18:1 <i>trans</i> -16	0.91	0.90	0.87	0.96	0.04	0.576	0.669
C18:1 <i>cis</i> -15	0.64 ^a	0.50 ^{ab}	0.44 ^b	0.26 ^c	0.04	0.001	0.728
C18:2 <i>cis</i> -9, <i>trans</i> -12	0.16 ^a	0.13 ^b	0.13 ^b	0.13 ^b	0.006	0.019	0.336
C18:2 <i>trans</i> -11, <i>cis</i> -15	1.52 ^a	1.29 ^{ab}	1.22 ^b	1.25 ^b	0.08	0.001	0.321
C18:2n-6	2.08 ^b	2.23 ^{ab}	2.41 ^a	2.45 ^a	0.08	0.001	0.221
C20:0	0.32	0.29	0.33	0.33	0.01	0.879	0.883
C18:3n-3	1.74 ^{bc}	1.67 ^c	2.02 ^{ab}	2.16 ^a	0.08	0.001	0.459
CLA <i>cis</i> -9, <i>trans</i> -11	1.87 ^{ab}	2.27 ^a	1.56 ^{bc}	1.39 ^c	0.13	0.001	0.434
C21:0	0.16	0.19	0.15	0.15	0.01	0.768	0.775
C18:4n-3	0.08	0.09	0.07	0.07	0.006	0.824	0.882
C20:2n-6	0.06	0.07	0.05	0.04	0.01	0.719	0.799
C18:3 <i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15	0.26 ^a	0.23 ^{ab}	0.23 ^{ab}	0.20 ^b	0.01	0.031	0.899
C22	0.15	0.17	0.16	0.14	0.01	0.658	0.433
C20:4n-6	0.12 ^b	0.12 ^b	0.14 ^{ab}	0.15 ^a	0.006	0.023	0.478
C23	0.09	0.08	0.11	0.11	0.008	0.845	0.886
C20:5n-3	0.04 ^b	0.05 ^a	0.06 ^a	0.06 ^a	0.003	0.022	0.523
C24	0.03	0.05	0.07	0.06	0.006	0.693	0.665
C22:4n-6	0.02	0.02	0.02	0.02	0.004	0.998	0.911
C22:5n-3	0.10 ^b	0.12 ^{ab}	0.14 ^a	0.14 ^a	0.006	0.035	0.665
C22:6n-3	0.04 ^b	0.06 ^a	0.06 ^a	0.06 ^a	0.004	0.028	0.443

^{a-c} $P < F$: significant differences at $P < 0.05$ indicated by different superscript letters.

¹L0 = control diet; L0.6 = control diet plus 0.6 g/kg of OCPC; L0.8 = control diet plus 0.8 g/kg of OCPC; L1.2 = control diet plus 1.2 g/kg of OCPC.

linear increase of α -LNA content in milk. Conversely, the concentration of some RBH intermediates in rumen liquor and milk fat decreased with the increasing concentration OCPC in diet. This result suggested that OCPC can negatively affect the RBH of α -LNA, allowing an accumulation of this FA in milk fat. This result may be conveniently transferred to feeding practice to reduce the amount of linseed in the diet of dairy ewes and to obtain significant enrichment of α -LNA content in milk fat. This may lead to a decrease of feeding cost for farmers, taking into consideration that EL is an expensive dietary ingredient. However, the production of OCPC as a dietary ingredient must be improved to obtain a stable content of individual polyphenols in the OCPC. Data about DMA composition and the effects on total bacterial diversity suggested that olive polyphenols probably interfere with rumen microbiome and some DMA could be markers of this phenomenon. However, further studies are needed to find specific associations between DMA composition and individual rumen bacterial strains.

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