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Effect of co-products from olive-oil production chain on rumen microbial communities: an *in vitro* study

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ABSTRACT

Several edible agro-industrial co-products have been studied as unconventional ingredients in ruminant diets to reduce the environmental impact of food production chains. When the chemical profile of co-products is characterised by the presence of bioactive molecules, they represent a promising tool to modulate rumen microbiota activity. In the Mediterranean area, the olive oil production chain generates olive oil pomace and olive tree leaves post-milling that are animal edible bio-waste. Two *in vitro* trials were carried out to investigate the effect of olive oil pomace and olive tree leaves as dietary ingredients on rumen fermentation and microbiome ecology. Two experimental diets, respectively containing olive oil pomace or olive tree leaves, and the related control diets, formulated to be isoproteic and isoenergetic, were fermented and then collected after 6h and 24h. Olive oil pomace increased the content of C18:1 c9 and C18:3 c9c12c15. Considering the microbial communities, the genera *Butyrivibrio*, *Fibrobacter*, and *Pseudobutyrivibrio* were less abundant, while *Christensenellaceae_R-7_group*, *Manheimia*, *Uruburuella* were more abundant in rumen liquor fermented with olive oil pomace. Similarly, the diet containing olive tree leaves increased the content of C18:1 c9 and C18:3 c9c12c15 and decreased the abundance of *Pseudobutyrivibrio* and *Rikenellaceae_RC9_gut_group*. Data reported in this study showed that the two by-products deriving from the olive oil production chain are effective in modulating microbial community in a selective manner.

HIGHLIGHTS

1. Unconventional ingredients in dairy-ruminant feeding to valorise agro-industrial co-products.
2. Unconventional feeds to improve the circularity of animal production chain.
3. Olive oil pomace and olive tree leaves as tools to modulate rumen fermentation and increase the microbial synthesis of functional molecules.

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By-products; polyphenols; rumen microbiota; ruminant feeding; sustainability

Introduction

Sustainability is a key topic in animal production and several studies have been conducted to evaluate the replacement of conventional ingredients in animal feeding strategies with alternative sources that meet animal requirements and do not compete with human nutrition (Salami et al. 2019). In this context, several edible agro-industrial by-products have been widely studied as ingredients of ruminant diets to reduce the environmental impact due to animal physiology and to the industrial production chains in agreement with the circular economy strategies (Correddu et al. 2020; Ianni and Martino 2020; FAO 2021) reducing at the same time the food *vs* feed competition. Moreover, dietary administration of co-products, especially those rich in bioactive molecules, represents a promising tool to modulate rumen microbiota activity (Patra and Saxena 2010; Vasta et al. 2019).

Olive tree farming is typical in the Mediterranean area, where the oil production chain generates several

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co-products (Molina-Alcaide and Yáñez-Ruiz 2008). Among these, olive oil pomace (OOP) obtained after olives milling, is quantitatively one of the most important. Also, the volume of olive tree leaves (OTL) resulting from the early stages of the oil production chain, is continuously increasing due to the spreading of mechanical harvesting (Khdair et al. 2018). Both are already employed as fertilisers or substrates in anaerobic digestion in biogas plants, but the presence of polyphenols (PPs) and polyunsaturated fatty acids (PUFAs) can make them promising ingredients in the ruminant diet, thus recovering a matrix rich in functional compounds and further valorising the olive-oil production chain (Romani et al. 2019; Bellucci et al. 2022). In particular, OOP is rich in water-soluble PPs (e.g. hydroxytyrosol and tyrosol), oleic acid (C18:1 c9; OA), and linoleic acid (C18:2 c9c12; LA; Benincasa et al. 2021; Difonzo et al. 2021). Moreover, the OOP produced nowadays is stone-deprived and thus more digestible, overcoming the main problem of their reuse in animal feeding (Zilio et al. 2014; Castellani et al. 2017). Olive tree leaves have a high content of oleuropein, hydrolysable PP, and of OA, and linolenic acid (C18:3 c9c12c15; LNA), but the quality of the fibre is usually worse than that of OOP (Bahloul et al. 2014; Şahin and Bilgin 2017). Considering the ability of rumen microorganisms in valorising feed nutrients and transforming them into highly digestible protein or energy, the inclusion of OOP and OTL as dietary ingredients may be hypothesised to replace conventional such as roughage (Bolletta et al. 2022; Innosa et al. 2020), or concentrate ingredients (Shdaifat et al. 2013; Chiofalo et al. 2020). Previous *in vivo* trials investigated the effect of the OOP and OTL as unconventional ingredients in ruminant diets on performances and milk quality (Bolletta et al. 2022; Scicutella et al. 2023). Olive oil pomace and OTL modulated PUFA biohydrogenation rate in rumen liquor increasing the content of functional molecules such as LA or OA in milk. Olive tree leaves led to a lower atherogenic and thrombogenic index and a smaller hypercholesterolemic potential in milk (Tsiplakou and Zervas 2008; Bolletta et al. 2022). Hence, the aim of the present study was to examine in detail the impact of OOP or OTL on rumen biohydrogenation (BH) processes and microbial communities.

Materials and methods

Two *in vitro* trials were carried out simultaneously using the same inoculum and adopting the same protocol as described below.

Feed composition

The olive tree leaves resulting from the cleaning phase of the drupes prior to milling were collected from a local oil mill and dried using a fluid-bed dryer. The temperature was constantly kept at 40 °C in order to prevent oxidative damage. Olive oil pomace was obtained from the upgraded two half phases olive milling system in which wet pomace, wastewater, and oil are generated using a moderate water quantity. The pomace was deprived of stones using stoner machines and provided by Olivicoltori Toscani Associati (*via* Empolese, 20 A, 50018 Scandicci Florence, It).

Feeds used as fermentation substrates in experiment 1 were the control feed (CONL) OTL free, and the experimental one (OTLF) in which part of conventional ingredients were replaced with OTL at the inclusion level of 9.2% of dry matter (DM) of total diet (28.0% of dietary concentrate DM).

Feeds used in experiment 2 as substrates of the fermentation were the control feed (CONP) in which the OOP was not included, and the experimental one (OOPF) in which part of conventional ingredients were replaced with OOP at the inclusion level of 8% of DM.

All experimental feeds were formulated to be isoproteic and isoenergetic with respect to their control diet and the inclusion level of OTL and OOP was chosen based on previous *in vivo* studies and literature (Castellani et al. 2017; Berbel and Posadillo 2018; Bolletta et al. 2022; Scicutella et al. 2023). The ingredients, proximate composition, and FA profile of feeds are shown in Table 1 and Table 2, respectively.

Description of fermentation trials and analysis

Feed proximate analysis

Feeds were sampled for analysis, freeze-dried, and ground to pass through a screen with a pore size of 1 mm (AOAC International 1995). The dry samples were analysed for crude protein (CP), ash, and ether extract (EE) according to the 954.01, 954.05 and 920.39 procedures of AOAC International (1995), respectively. Neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were determined by using sequential analysis, with sodium sulphite, heat stable amylase, and expressed inclusive of ash residual according to Van Soest et al. (1991). Metabolisable energy was calculated according to CNCPS system ver. 6.55.

Table 1. Ingredients, proximate composition, and fatty acid profile of the control feed without olive tree leaves (CONL) and the treated feed with olive tree leaves (OTLF).

Ingredients (% of DM)	Feed[1]	
	CONL	OTLF
Control concentrate[2]	33	0
Olive leaves concentrate[2]	0	33
Alfa-alfa hay	37	37
Pasture	30	30
Chemical profile (% of dry matter)		
Crude protein	15.9	16.2
Ether extract	2.19	2.51
Neutral detergent fibre	42.2	40.5
Acid detergent fibre	30.9	30.0
Lignin	6.52	7.34
Metabolisable energy (Mcal/Kg dry matter)	9.66	9.54
Fatty acid (g/100g fatty acids)		
C14:0	0.28	0.22
C16:0	28.3	27.5
C18:0	4.69	5.49
C18:1 c9	10.4	12.4
C18:2 c9 c12	28.8	26.4
C18:3 c9c12c15	24.7	25.1

[1]Feed, CONL control feed from Experiment 1, OTLF treated feed with olive tree leaves inclusion from Experiment 1.
[2]The control concentrate was formulated as follows: Alfalfa hay (28%), Corn (16%), Beet pulp (15%), Barley (13%), Bran (12%), Corn gluten (5%), Soybean meal (5%), Molasses (4%), Calcium carbonate (0.5%), Sodium bicarbonate (0.5%), Dicalcium phosphate (0.5%), Sodium chloride (0.5%). In the olive leaves concentrate, alfalfa hay was fully replaced by olive tree leaves.

Table 2. Ingredients, proximate composition, and fatty acid profile of the control feed without olive oil pomace (CONP) and the treated feed with olive oil pomace (OOPF).

	Feed[1]	
Ingredients (% of dry matter)	CONP	OOPF
OOP[2]	0	8.02
Corn silage	19.7	10.8
Commercial feed 195*	21.8	21.0
Commercial feed*	14.5	14.0
Alfa-alfa Hay	29.3	31.8
Grass hay	14.7	14.3
Chemical profile (g/kg of dry matter)		
Crude protein	14.0	14.0
Ether extract	3.45	4.82
Neutral detergent fibre	38.8	38.9
Acid detergent fibre	23.0	24.2
Lignin	4.63	6.44
Metabolisable energy (Mcal/Kg dry matter)	9.89	9.38
Fatty acid (g/100g fatty acids)		
C14:0	0.19	0.19
C16:0	20.3	19.7
C18:0	4.87	4.7
C18:1 c9	19.2	23.3
C18:2 c9c12	32.3	30.7
C18:3 c9c12c15	17.8	16.5

[1]Feed, CONP control feed from Experiment 2, OOPF treated feed with olive oil pomace inclusion from Experiment 2.
[2]OOP, Olive oil pomace.
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In vitro fermentation procedure

The rumen fluid was collected according to Denek et al. (2006) and Lutakome et al. (2017) using three ewes as donors. To ensure correct conditioning of rumen, animals were fed for 15 days before the slaughtering with a diet based on mixed hay *ad libitum* and 100 g of a commercial concentrate (CP = 18.2%; EE = 10.1%; NDF = 21.6%). The animals were slaughtered on empty stomach. After the slaughtering, the whole rumen of each sheep was immediately transferred to the laboratory in a thermostatic box (39° C). The rumen content was collected and filtered through a cheesecloth (pore size of 250 μm) into a flask under a flux of CO_2 at 39 °C to ensure the anaerobic condition, as described by Buccioni et al. (2006). Immediately after filtration, an aliquot of rumen liquor (RL) was sampled in triplicate and stored as blank at −80 °C until the analysis in order to verify possible artefacts. The fresh rumen fluid was immediately used to prepare the experiment as follows. Firstly, the RL inoculum for the feed fermentation was buffered by adding an artificial saliva solution (ratio 1:3, vol/vol) as reported by McDougall (1948). Feeds (1 g of DM) were incubated with 100 mL of the inoculum (3 replicates per feed) according to Buccioni et al. (2011). Following the addition of inoculum into the bottle, the headspace has been flushed continuously with CO_2 until the bottle was sealed with a cup equipped with a Bunsen valve to ensure both the escaping of the gas produced during the fermentation and the anaerobic condition for microorganisms. The incubation times were 6h and 24h. At each sampling time the fermentation was stopped by adding 1 mL of HCl 0.2 M and the whole content of each fermenter was divided into 2 aliquots for FA characterisation and microbiological assay. All samples were stored at −80 °C until the analysis. The following week, the two experiments were replicated following the same procedure.

Rumen liquor fatty acid and dimethylacetal profiles

The FA extraction and methylation were carried out using the one-step methylation described by Kramer et al. (1997), and FA profile was determined by gas-chromatography. The gas-chromatograph was equipped with a capillary column (CP-Select CB for FAMEs Varian, Middelburg, the Netherlands: 100 m x 0.25 mm i.d., film thickness 0.20 mm). The injector and FID temperature, the split ratio, the oven run, and the list of standards were according to Buccioni et al. (2011). The detection threshold for FAs was 0.01 g/100g of FA and the results were expressed in mg/100g of DM. The dimethylacetals (DMA) profile was determined according to the procedure described by Alves et al. (2013). The DMA results were expressed as g/100g of DMA.

DNA extraction, amplicons preparation and sequencing

DNA was extracted from 185 μL of each sample with Fast DNA Spin for soil kit (MP Biomedicals, Solon, OH) following the manufacturer's protocol modified as previously reported (Mannelli et al. 2018). DNA purity and quantity were checked through a spectrophotometric assay ND-1000 (NanoDrop Technologies, Labtech, Ringmer, UK). The V3-V4 region of the 16S rRNA gene was PCR-amplified with Pro341F and Pro805R primers (Takahashi et al. 2014). Sequencing was performed at BMR Genomics (Padova, Italy) by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) using a 300 bp x 2 paired-end protocol.

Bioinformatics

Bioinformatics elaborations were performed in R 4.0.3 (R Core Team 2020) with the package DADA2 (Callahan et al. 2016), version 1.16.0. Primer sequences were removed using Cutadapt (Marcel 2011). Forward reads were truncated at 280 bases and the reverse ones were truncated at 220 bases. The reads with expected errors higher than 1 were discarded. Specific error rates were estimated for the forward reads and the reverse reads and were used to infer the amplicon sequence variants (ASVs) on the dereplicated reads. The read pairs were merged with default parameters and chimeric sequences were removed. Taxonomic assignment for each ASV was performed against the SILVA 138.1 database (confidence 80%; Pruesse et al. 2007) and the ASVs with an abundance lower of 0.01% were removed.

Statistical analysis

Fatty acids and DMA data were processed as a completely randomised design using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA):

$$Y_{ijk} = m + F_i + T_j + (F \times T)_{ij} + R_k + e_{ijk}$$

Where: Y_{ijk} is the observation, m is the overall mean, F_i is the fixed effect of feed ($i = 1, 2$), T_j is the fixed effect of sampling time ($j = 1, 2$), $(F \times T)_{ij}$ is the interaction between feed and sampling time, R_k is the random effect of run nested feed ($k = 1, 2$) 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. The statistical significance of F was tested against the variance of fermenters within the feed according to the repeated measures design theory (Littell et al. 1998). Multiple comparisons among means were performed using Tukey's test.

Data from 16S rRNA gene sequencing were processed using the vegan package, version 2.5.7 (Oksanen et al. 2020) in R 4.0.3 (R Core Team 2020). A randomly rarefied dataset for each experiment was generated (9,000 sequences for experiment 1 and 10,000 sequences for experiment 2). The number of ASVs, the Chao1 index, the Simpson index, and the Shannon index were calculated to estimate the alpha-diversity and a Kruskal-Wallis test was performed to detect significant differences between the conditions. A permutational multivariate analysis of variance (PERMANOVA) based on Hellinger transformed ASV abundance data was performed using the adonis2 functions, respectively. The PERMANOVA was performed on the Bray-Curtis dissimilarity index. A Kruskal-Wallis test was performed to identify the taxa with a different relative abundance between the conditions. Spearman correlations were performed to identify the genera correlated with the content of the several FAs chosen in relation to the rumen biohydrogenation. Only genera with a relative abundance > 0.1 were correlated. Correlations were reported for $p < 0.1$ and were considered significant for $p \leq 0.05$. A trend towards significance was considered for $0.05 \leq p < 0.10$.

Results

Profile of fatty acids and dimethylacetals detected in rumen liquor fermented with the experimental feeds

Experiment 1

Considering the odd-linear chain and odd-branched chain FAs (OLCFAs; OBCFAs respectively), only sampling time influenced in statistically significant manner some of the fatty acids considered (Table 3). The content of C11:0 decreased (variation rate: CONL, 18.9% and OTLF, 36.3%) whereas the content of C13:0 iso, C13:0, and C14:0 iso increased with time regardless of the feed (variation rate: C13:0 iso CONL, 54.3% and OTLF, 34.3%; C13:0 CONL, 134.4% and OTLF, 125.5%; C14:0 iso CONL, 28.4% and OTLF, 30.3%). The C17:0 iso content showed a particular behaviour, indeed, it increased by 38.4% and decreased by 38.3% in CONL and OTLF diets, respectively.

Among the other FAs (Supplementary material Table S1), C10:1 showed a significant feed · time effect and was higher in fermenters with OTLF than in ones with CONL ($p = 0.039$) at 24h. The concentration of C6:0 and C20:0 increased from 6h to 24h (variation rate: C6:0 CONL, 264.5% and OTLF, 359.1%; C20:0 CONL, 50.3% and OTLF, 52.8%) while the

Table 3. Odd-linear and -branched chain fatty acid profile of the rumen liquor fermented in experiment 1.

	CONL[1]	OTLF[1]	CONL[1]	OTLF[1]		P value[3]		
Fatty acid mg/100g of dry matter	6h		24h		SEM[2]	F	T	F × T
C4:0 iso	4.82	4.88	11.1	11.7	1.95	0.87	0.005	0.89
C5:0 iso	0.27	0.26	0.94	0.83	0.27	0.83	0.040	0.86
C13:0 iso	1.34	1.28	2.07	1.71	0.19	0.31	0.009	0.48
C13:0 ante	0.12	0.17	0.16	0.19	0.03	0.24	0.40	0.63
C14:0 iso	0.97	0.99	1.24	1.29	0.12	0.77	0.039	0.92
C15:0	3.60	3.67	4.23	4.02	0.53	0.90	0.38	0.80
C15:0 iso	0.88	1.08	1.51	1.03	0.17	0.44	0.13	0.08
C15:0 ante	0.80	0.79	0.92	0.83	0.11	0.65	0.48	0.73
C17:0	1.66	1.70	1.96	1.98	0.25	0.92	0.29	0.96
C17:0 iso	0.18	0.20	0.25	0.12	0.03	0.92	0.035	0.11
C17:0 ante	1.75	1.67	1.70	1.73	0.16	0.86	0.96	0.75
C17:1	0.14	0.17	0.20	0.18	0.03	0.97	0.31	0.46
C18:0 iso	0.19	0.20	0.24	0.09	0.04	0.093	0.51	0.07
C20:0 ante	0.20	0.38	0.26	0.35	0.13	0.33	0.90	0.72

[1]CONL, control feed; OTLF, experimental feed formulated with olive tree leaves as ingredient.
[2]SEM, standard error mean.
[3]P value, probability of significant effect due to feed (F), sampling time (T), or to the interaction between feed and sampling time (F × T), ($p \leq 0.05$).

Table 4. Fatty acid profile involved in C18-fatty acid biohydrogenation-pathway of the rumen liquor fermented in experiment 1.

	CONL[1]	OTLF[1]	CONL[1]	OTLF[1]		P value[3]		
Fatty acid mg/100g of dry matter	6h		24h		SEM[2]	F	T	F × T
C18:0	22.0	20.2	52.9	53.7	11.0	0.97	0.012	0.91
C18:1 t4	0.05	0.03	0.11	0.07	0.04	0.39	0.24	0.82
C18:1 t5	0.04	0.03	0.06	0.16	0.05	0.40	0.19	0.33
C18:1 t6-8	0.37	0.30	0.59	0.53	0.14	0.69	0.15	0.96
C18:1 t9	0.32	0.27	0.44	0.37	0.10	0.58	0.31	0.96
C18:1 t10	0.39	0.36	0.51	0.50	0.11	0.87	0.28	0.90
C18:1 t11	5.32	7.56	7.79	7.90	2.17	0.58	0.51	0.61
C18:1 t12	0.62	0.30	0.51	0.52	0.14	0.25	0.68	0.24
C18:1 c7	2.88	0.65	0.71	0.81	1.28	0.40	0.42	0.36
C18:1 c9	12.3	19.1	9.73	13.1	2.56	0.050	0.10	0.50
C18:1 c11	0.34	0.24	0.52	0.60	0.13	0.94	0.038	0.48
C18:1 c12	2.58	2.79	2.50	2.92	0.37	0.37	0.94	0.77
C18:1 c13 + t16	0.17	0.15	0.15	0.17	0.04	0.96	0.93	0.68
C18:1 c14 + c16	0.23	0.22	0.66	0.56	0.16	0.74	0.022	0.78
C18:1 c15	0.22	0.26	0.34	0.30	0.05	0.98	0.11	0.44
C18:2 c9t12	0.26	0.14	0.12	0.09	0.03	0.024	0.006	0.16
C18:2 t9c12	0.15	0.09	0.12	0.05	0.03	0.027	0.19	0.96
C18:2 t11c15	0.22	0.09	0.13	0.79	0.30	0.37	0.30	0.19
C18:2 c9c12	42.2	47.5	20.3	25.8	4.88	0.27	<0.001	0.98
C18:2 c9t11	0.51	0.77	0.53	0.61	0.16	0.28	0.65	0.55
C18:2 t10c12	0.03	0.24	0.09	0.07	0.07	0.15	0.39	0.10
C18:3 c9t12t15	0.08	0.07	0.11	0.07	0.03	0.43	0.57	0.60
C18:3 t9c12t15	0.29	0.33	0.16	0.13	0.04	0.92	<0.001	0.28
C18:3 c9c12c15	29.4	39.8	13.5	17.7	3.54	0.044	<0.001	0.39
C18:3 c9t11c15	0.32	0.28	0.30	0.26	0.05	0.39	0.68	0.98

[1]CONL, control feed; OTLF, experimental feed formulated with olive tree leaves as ingredient.
[2]SEM, standard error mean.
[3]P value, probability of significant effect due to feed (F), sampling time (T), or to the interaction between feed and sampling time (F × T), ($p \leq 0.05$).

concentration of C12:1 and C24:1 decreased (variation rate: C12:1 CONL, 28.0% and OTLF, 49.9%; C24:1 CONL, 39.9% and OTLF, 38.9%).

Considering the FAs involved in BH processes (Table 4), OA and LNA were more abundant in RL with the inclusion of OTL, whereas the content of C18:2 c9t12 and C18:2 t9c12 was higher in fermenters with CONL regardless of the sampling time. At 24 h of fermentation, the concentration of C18:0, C18:1 c11 and C18:1 c14 + c16 increased, while the concentration of C18:2 c9c12 (linoleic acid, LA), C18:3 t9c12t15, and LNA decreased regardless of the feed (variation rate: C18:0 CONL, 140.0% and OTLF, 165.2%; C18:1 c11 CONL, 55.1% and OTLF, 153.6%; C18:1 c14 + c16 CONL, 191.6% and OTLF, 158.9%; C18:2 c9t12 CONL, 55.2% and OTLF, 38.1%; LA CONL, 51.9% and OTLF, 45.7%; C18:3 t9c12t15 CONL, 42.8% and OTLF, 60.9%; LNA CONL, 54.6% and OTLF, 55.4%).

Considering the DMA profile, few variations were found (Table S2). Only DMA 18:1 c12 showed a

Table 5. Odd-branched chain fatty acid profile of the rumen liquor fermented in experiment 2.

	CONP[1]	OOPF[1]	CONP[1]	OOPF[1]		P value[3]		
Fatty acid mg/100g of dry matter	6h		24h		SEM[2]	F	T	F × T
C4:0 iso	13.0	12.9	43.5	30.0	6.11	0.29	0.00	0.30
C5:0 iso	0.88	0.18	1.19	0.50	0.23	0.008	0.19	0.98
C13:0 iso	1.11	0.97	1.76	2.44	0.23	0.26	<0.001	0.10
C13:0 ante	0.18	0.14	0.18	0.18	0.09	0.82	0.82	0.82
C14:0 iso	0.81	0.80	0.90	1.22	0.12	0.20	0.044	0.18
C15:0	3.53	3.71	4.42	5.50	0.48	0.21	0.013	0.37
C15:0 iso	1.51	2.25	1.72	2.19	0.27	0.037	0.80	0.63
C15:0 ante	1.10	1.39	1.48	1.68	0.14	0.09	0.029	0.74
C17:0	2.01	2.22	2.55	2.68	0.28	0.56	0.10	0.88
C17:0 iso	0.35	0.21	0.23	0.37	0.10	0.95	0.84	0.19
C17:0 ante	1.90	4.72	2.06	2.73	1.15	0.16	0.45	0.37
C17:1	0.11	0.09	0.37	0.21	0.06	0.13	0.001	0.22
C18:0 iso	0.19	0.12	0.26	0.24	0.07	0.52	0.18	0.71
C20:0 ante	0.29	0.83	0.37	0.31	0.20	0.26	0.30	0.16

[1]CONP, control feed; OOPF, experimental feed formulated with olive tree leaves as ingredient.
[2]SEM, standard error mean.
[3]P value, probability of significant effect due to feed (F), sampling time (T), or to the interaction between feed and sampling time (F × T), ($p \leq 0.05$).

feed × time effect ($p = 0.046$). At 6h its content was lower in fermenters with CONL than in ones with OTLF (3.509 g/100g DMA vs 6.546 g/100g DMA) and when OTLF was fermented a higher concentration of DMA 18:1 c12 was showed at 6h with respect to the 24h (6.546 g/100g DMA vs 2.991 g/100g DMA). The content of DMA 18:1 t11 decreased with OTL dietary inclusion (CONL, 1.637 g/100g DMA at 6h, 1.515 g/100g DMA at 24h; OTDL, 0.231 g/100g DMA at 6h, 1.268 g/100g DMA at 24h, SEM = 0.367 $p = 0.05$). Instead, the concentration of DMA 15:0 and DMA 16:1 showed a sampling time effect ($p \leq 0.05$). In particular, DMA 15:0 was higher (SEM = 0.876; $p = 0.001$) and DMA 16:1 (SEM = 0.659; $p = 0.025$) was lower at 24h.

Experiment 2

The presence of OOP in the feed lowered C5:0 iso concentration regardless of the sampling time (Table 5). In contrast, the concentration of C4:0 iso increased regardless of the feed between the two sampling times (variation rate: C4:0 iso CONP, 235.5%, and OOPF 132.8%; Table 5).

Considering OLCFAs and OBCFAs (Table 5), a feed effect was observed on the concentration of C15:0 iso, which was higher in fermenters with OOPF than in ones with CONP ($p = 0.037$) regardless the sampling time. The content of C13:0 iso, C14:0 iso, C15:0 ante, C15:0, and C17:1 increased in all fermenters during the fermentation period (variation rate: C13:0 iso CONP, 58.7% and OOPF, 150.8%; C14:0 iso CONP, 11.4% and OOPF, 51.9%; C15:0 ante CONP, 34.7% and OOPF, 20.6%; C15:0 CONP, 25.2% and OOPF, 48.1%; C17:1 CONP, 230.5% and OOPF, 126.0%). A similar trend has been observed for C6:0, and C12:0 (variation rate: C6:0 CONP 218.82% and OOPF, 300.1%; C12:0 CONP, 97.7% and OOPF, 118.5%). In contrast, C14:0, C16:0, and C20:1 were affected by feed since their concentration was higher with OOPF regardless of the sampling time, and C14:0 showed also a time effect (variation rate: C14:0 CONP, 78.27% and OOPF, 70.1%) (Table S3).

The profile of FA involved in the BH pathway of LA and LNA is reported in Table 6. The RL with the inclusion of OOPF showed a higher concentration of t5, t6-8, t9, t10, and c12 C18:1 ($p \leq 0.05$), LA ($p = 0.047$), and LNA ($p < 0.001$). At the end of fermentation the concentration of C18:0 (stearic acid, SA), C18:1 t4, C18:1 t6-8, C18:1 t9, C18:1 t11 (vaccenic acid, VA), C18:1 t12, C18:1 c11, C18:1 c12, C18:1 c14 + c16 increased regardless of the feed (variation rate: SA CONP, 148.8% and OOPF, 199.9%; C18:1 t4 CONP, 235.9% and OOPF, 580.8%; C18:1 t6-8 CONP, 100.2% and OOPF, 121.5%; C18:1 t9 CONP, 74.79% and OOPF, 75.07%; VA CONP, 163.4% and OOPF, 165.44%; C18:1 t12 CONF, 43.8% and OOPF, 145.3%; C18:1 c11 CONP, 89.3% and OOPF, 151.0%; C18:1 c12 CONP, 88.8% and OOPF, 21.8%; C18:1 c14 + c16 CONP, 317.3% and OOPF, 770.9%). Conversely, OA (variation rate: CONP, 18.7% and OOPF, 41.9%), LA (variation rate: CONP, 37.0% and OOPF, 44.0%), and LNA (variation rate: CONP, 38.7% and OOPF, 54.7%) decreased from 6 h to 24 h of fermentation. Vaccenic acid and LNA content were higher in fermenters with OOPF than in ones with CONP at both sampling times (Table 6). Nor feed or time effect nor their interaction was observed for C18:2 t10c12 and C18:2 c9t11.

Considering DMA profile, only DMA 18:1 c12 showed a feed effect ($p = 0.027$), since its concentration was lower in fermenters with OOPF than in ones with CONP. The complete report of DMA data is shown in Table S4 of supplementary material.

Table 6. Fatty acid profile involved in C18-fatty acid biohydrogenation-pathway of the rumen liquor fermented in experiment 2.

	CONP[1]	OOPF[1]	CONP[1]	OOPF[1]		P value[3]		
Fatty acid mg/100g of dry matter	6h		24h		SEM[2]	F	T	F × T
C18:0	21.8	24.5	54.2	73.6	11.6	0.37	0.003	0.50
C18:1 t4	0.05	0.04	0.16	0.25	0.06	0.53	0.018	0.43
C18:1 t5	0.11	0.15	0.11	0.35	0.06	0.037	0.12	0.12
C18:1 t6-8	0.44	0.92	0.88	2.03	0.27	0.008	0.011	0.24
C18:1 t9	0.37	0.73	0.65	1.28	0.17	0.009	0.025	0.44
C18:1 t10	0.78	1.14	0.95	1.78	0.25	0.034	0.14	0.38
C18:1 t11	3.42	4.87	9.00	12.92	2.07	0.22	0.005	0.57
C18:1 t12	0.63	0.54	0.90	1.34	0.17	0.32	0.006	0.15
C18:1 c7	0.95	0.92	0.95	1.45	0.25	0.38	0.31	0.32
C18:1 c9	18.8[c]	67.1[a]	15.3[c]	39.0[b]	4.47	<0.001	0.003	0.015
C18:1 c11	0.42	0.46	0.80	1.15	0.11	0.11	<0.001	0.19
C18:1 c12	2.36	5.04	4.46	6.14	0.45	0.000	0.002	0.29
C18:1 c13 + t16	0.17	0.10	0.23	0.17	0.05	0.24	0.24	1.00
C18:1 c14 + c16	0.22	0.09	0.91	0.76	0.18	0.44	0.001	0.95
C18:1 c15	0.42	0.14	0.34	0.30	0.10	0.11	0.71	0.23
C18:2 c9t12	0.28	0.21	0.22	0.10	0.10	0.37	0.42	0.81
C18:2 t9c12	0.11	0.26	0.07	0.15	0.06	0.09	0.22	0.57
C18:2 c9c12	40.9	63.8	25.8	35.7	7.54	0.047	0.012	0.41
C18:2 c9t11	0.72	1.26	0.59	0.73	0.27	0.23	0.25	0.46
C18:2 t10c12	0.14	0.71	0.15	0.11	0.25	0.32	0.26	0.24
C18:2 t11c15	0.18	0.14	0.15	0.30	0.05	0.31	0.22	0.09
C18:3 c9t12t15	0.05	0.44	0.10	0.14	0.11	0.08	0.28	0.15
C18:3 t9c12t15	0.12	0.64	0.18	0.13	0.18	0.21	0.23	0.13
C18:3 c9c12c15	13.2[b]	25.4[a]	8.07[c]	11.5[c]	1.54	<0.001	<0.001	0.010
C18:3 c9t11c15	0.74	1.65	0.40	0.49	0.40	0.23	0.08	0.33

[1]CONP, control diet; OOPF, treated feed with olive oil pomace as ingredient.
[2]SEM, standard error mean.
[3]P value, probability of significant effect due to feed (F), sampling time (T), or to the interaction between feed and sampling time (F × T) ($p \leq 0.05$). Means in the same row with different superscripts are significantly different.

Table 7. Alfa-diversity in microbiota from rumen liquor fermented with the control feed without olive tree leaves (CONL) or the treated feed with olive tree leaves (OTLF).

Index	CONL	OTLF	SEM[1]	P value[2]
Simpson Index	0.98	0.95	0.01	0.23
Shannon Index	4.95	4.66	0.25	0.43
Chao1 Index	428	431	48	0.97
Number of ASVs	419	418	46	0.99

[1]SEM, Standard error mean.
[2]P value, probability of significant effect due to feed ($p \leq 0.05$).

Characterisation of the microbial communities

Experiment 1

The microbial community was characterised by high-throughput sequencing of 16S rRNA gene amplicons in RL of both CONL and OTLF. The analysis of the alpha diversity showed a higher evenness in microbial community of CONL condition (Shannon and Simpson indexes) and a similar richness between the two treatments (ASV number and Chao1 indexes) (Table 7). Whilst the PERMANOVA evidenced no significant differences of the beta-diversity ($R2 = 0.26912$; $p = 0.1$). Six phyla were identified (Figure S1). The more abundant phyla were *Proteobacteria* (CONL ~37% and OTLF ~39%), *Firmicutes* (CONL ~30% and OTLF ~29%) and *Bacteroidota* (CONL ~28% and OTLF ~29%). The most abundant classes were *Gammaproteobacteria* (CONL ~37% and OTLF ~39%), *Bacteroidia* (CONL ~29% and OTLF ~30%), and *Clostridia* (CONL ~21% and OTLF ~20%) (Figure S2). Twenty-five genera were identified (Figure 1). The most abundant genera were *Prevotella* (CONL ~17% and OTLF ~13%) and *Ruminobacter* (CONL ~8.1% and OTLF ~7.7%). The relative abundance of *Pseudobutyrivibrio* ($p = 0.045$) and *Rikenellaceae_RC9_gut_group* ($p = 0.037$) was lower when OTL was included in the diet. The genus *Prevotella* correlated positively with C13:0 iso and negatively with C13:0 ante (Figure 2). The genera *Ruminococcus* and *Treponema* correlated positively with C13:0 iso and C15:0 iso (Figure 2). The genus *Manheimia* correlated negatively with C15:0 iso and C17:0 iso (Figure 2). The genus *Butyrivibrio* correlated negatively with LNA, LA, OA, C18:1 t11, C18:1 t9, SA (Figure 3). The genus *Ruminobacter* correlated positively with LNA, OA, C18:1 t11, C18:1 t9, SA (Figure 3).

Experiment 2

The microbial community was characterised by high-throughput sequencing of 16S rRNA gene amplicons in RL of both CONL and OOPF. The analysis of the alpha-diversity showed a similar evenness between the two treatments (Simpson and Shannon indexes) and a higher richness in OOPF condition (Chao1 and ASVs number) (Table 8). Whilst PERMANOVA evidenced no significant differences of the beta-diversity ($R^2 = 0.31494$; $p = 0.1$). Six phyla were identified (Figure S3). The most abundant phyla were *Proteobacteria*

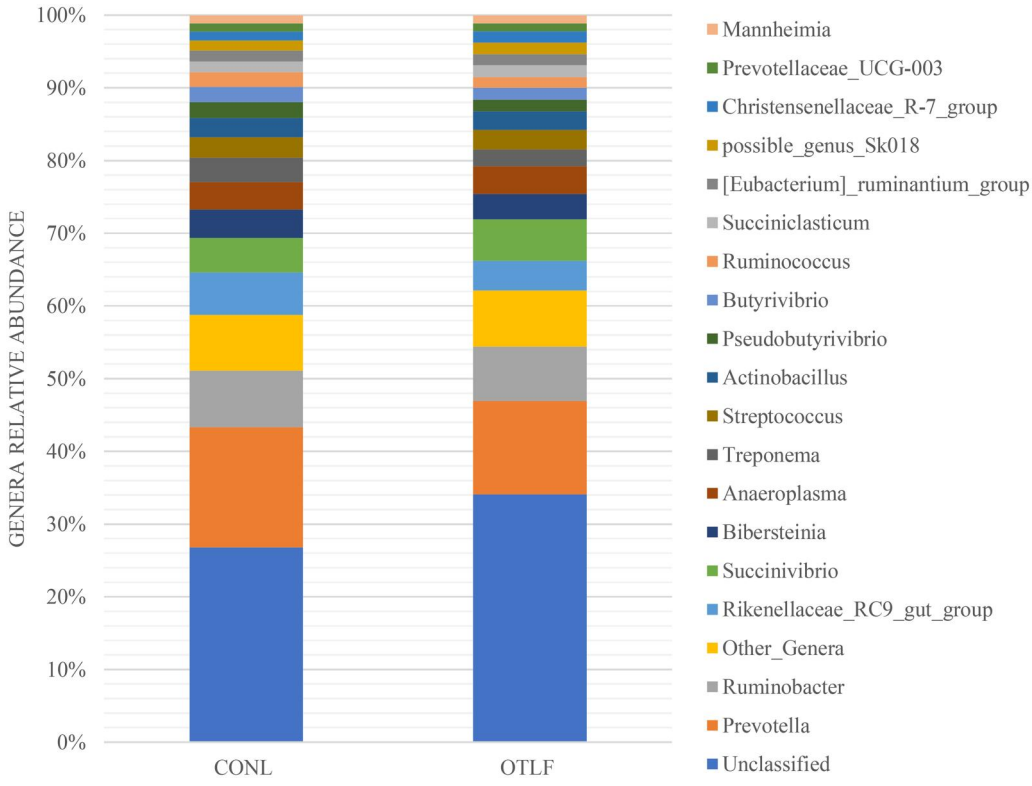

Figure 1. Genera detected in microbiota from rumen liquor fermented with the control feed without olive tree leaves (CONL) or the treated feed with olive tree leaves (OTLF). Only Genera with a relative abundance > 1% are reported.

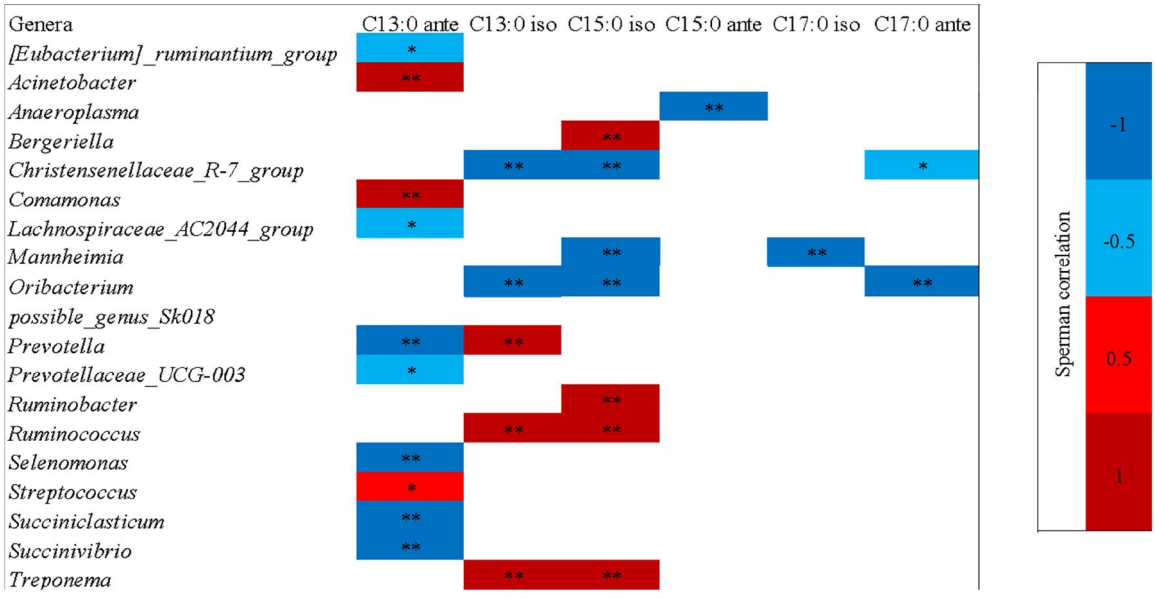

Figure 2. Correlations between odd-branched chain fatty acids and Genera in microbiota from rumen liquor fermented with the control feed without olive tree leaves (CONL) or the treated feed with olive tree leaves (OTLF). Positive correlation is indicated in red and negative correlation is indicated in blue (* for $-0.5 < p < 0.5$; ** for P < -0.5 and $p > 0.5$). Only Genera with a relative abundance > 0.1% were correlated.

(CONP ~67% and OOPF ~70%), *Bacteroidota* (CONP ~17.1% and OOPF ~17.3%), and *Firmicutes* (CONP ~12% and OOPF ~11%). The most abundant classes were *Gammaproteobacteria* (CONP ~67% and OOPF ~70%), *Bacteroidia* (CONP ~17.2% and OOPF ~17.4%), and *Clostridia* (CONP ~8% and OOPF ~7%) (Figure S4). At the genus level, 21 taxa were identified (Figure 4). The most abundant genera were *Ruminobacter*

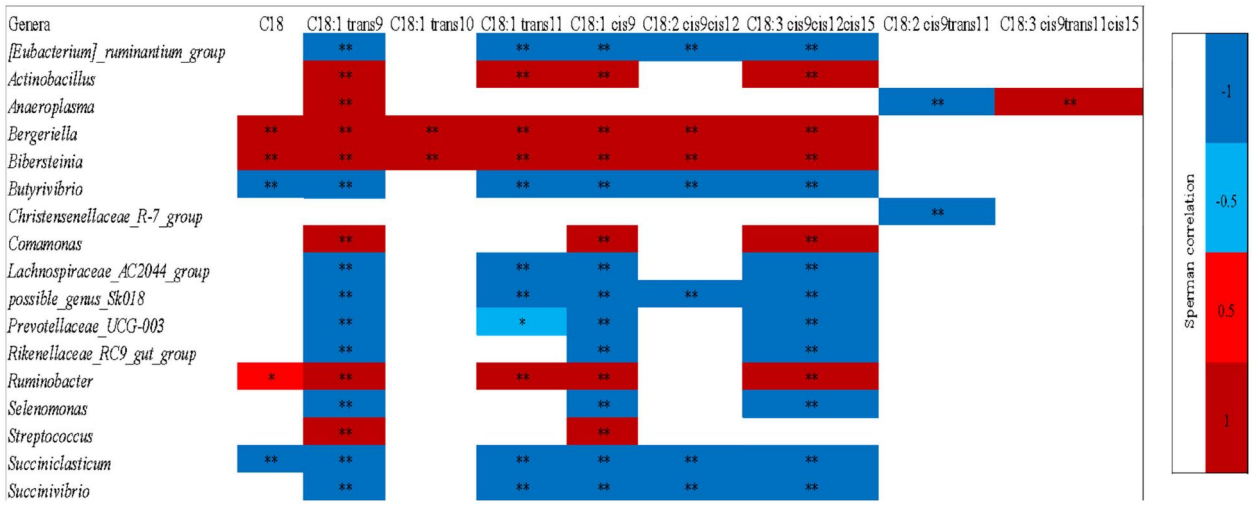

Figure 3. Correlations between C18 biohydrogenation chain and Genera detected in microbiota from rumen liquor fermented with the control feed without olive tree leaves (CONL) or the treated feed with olive tree leaves (OTLF). Positive correlation is indicated in red and negative correlation is indicated in blue (* for $-0.5 < p < 0.5$; ** for P < -0.5 and $p > 0.5$). Only Genera with a relative abundance > 0.1% were correlated.

Table 8. Alfa-diversity in microbiota from rumen liquor fermented with the control feed without olive oil pomace (CONP) or the treated feed with olive tree leaves (OOPF).

Index	CONP	OOPF	SEM[1]	P value[2]
Simpson Index	0.92	0.92	0.02	0.88
Shannon Index	3.99	3.98	0.22	0.99
Chao1 Index	346	368	24	0.55
Number of ASVs	341	363	24	0.53

[1]SEM, Standard error mean.
[2]P value, probability of significant effect due to feed ($p \leq 0.05$).
ASVs: Amplicon Sequence Variants.

(CONP ~39% and OOPF ~42%) and *Prevotella* (CONP ~12% and OOPF ~11%). A lower relative abundance of the genera *Butyrivibrio* ($p = 0.016$), *Fibrobacter* ($p = 0.01$), *Pseudobutyrivibrio* ($p = 0.01$) was observed in OOPF, while the relative abundance of *Christensenellaceae_R-7_group* ($p = 0.05$), *Manheimia* ($p = 0.045$), *Uruburuella* ($p = 0.004$) was higher in OOPF. *Manheimia* and *Rikenellaceae_RC9_gut_group* correlated negatively with C15:0 iso. *Fibrobacter* and *Pseudobutyrivibrio* correlated positively with C17:0 iso (Figure 5). *Christensenellaceae_R-7_group* and *Manheimia* correlated negatively with C17:0 ante (Figure 5). The genera *Butyrivibrio*, *Fibrobacter*, and *Pseudobutyrivibrio* correlated positively with OA (Figure 6). *Christensenellaceae_R-7_group* correlated negatively with LNA, LA, OA, C18:1 t10, and C18:1 t9 (Figure 6). The genus *Uruburuella* correlated negatively with C18:1 t10 and OA.

Discussion

The reuse of by-products from agro-industrial productions for the development of sustainable feeding strategies for livestock is an increasing practice. Depending on the proximate profile, unconventional ingredients show the potential to modulate the microbial activity in the rumen and may represent interesting tools to drive rumen fermentation towards a better energy-protein balance, improving the digestibility of the diet, reducing nitrogen excretions or methane emissions, and then preserving animal welfare. This capability seems to be mainly related to the presence of bioactive molecules. Olive oil producers must dispose of tons of OTL and OOP every year as special waste with high costs for transport and disposal. The chemical composition of OTL and OOP makes them hypothetical modulators of rumen fermentation thanks to the high presence of polyphenols that can influence the activity of rumen microorganisms. Several *in vivo* trials highlighted a possible effect of OTL and OOP on rumen microbial communities (Bennato et al. 2022; Scicutella et al. 2023).

The results of this study showed that minimal changes were induced in the rumen environment by the presence of OTL and OOP in the diet, at the inclusion level adopted in these trials. In fact, both OTL and OOP influenced the relative abundance of several bacterial taxa but the overall structure of the communities (i.e. beta-diversity) was not different between controls and experimental feeds. In experiment 1, the CONL and OTLF compositions showed similar NDF, ADF and ADL content. Nevertheless, the content of C17:0 iso (a marker of cellulolytic bacteria metabolism) decreased differently during the time suggesting that changes in the activity of bacterial communities occurred (Fievez et al. 2012). It is well known that there is an interaction among cellulolytic and non-

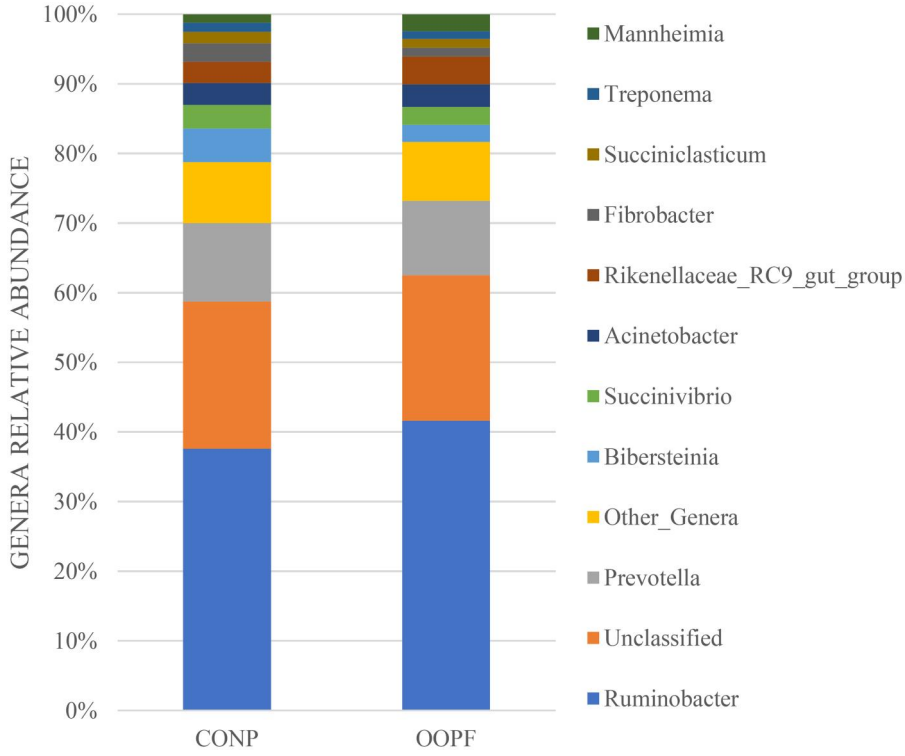

Figure 4. Genera detected in in microbiota from rumen liquor fermented with the control feed without olive oil pomace (CONP) or the treated feed with olive oil pomace (OOPF). Only Genera with a relative abundance > 1% are reported.

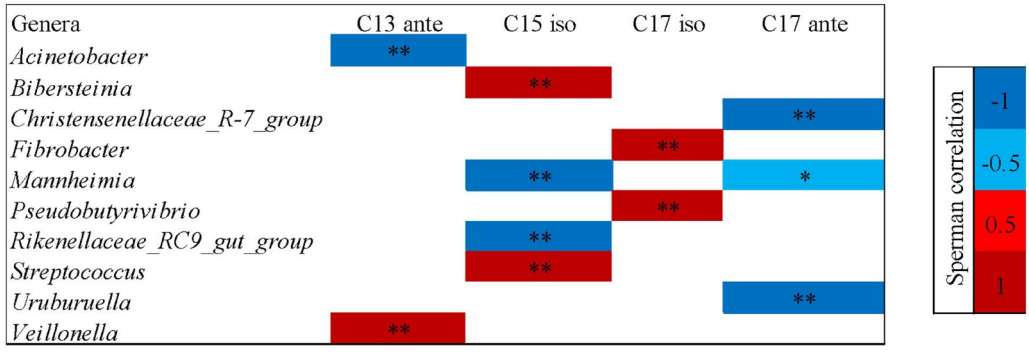

Figure 5. Correlations between odd-branched chain fatty acids and Genera in microbiota from rumen liquor fermented with the control feed without olive oil pomace (CONP) or the treated feed with olive oil pomace (OOPF). Positive correlation is indicated in red and negative correlation is indicated in blue (* for $-0.5 < p < 0.5$; ** for P < -0.5 and $p > 0.5$). Only Genera with a relative abundance > 0.1% were correlated.

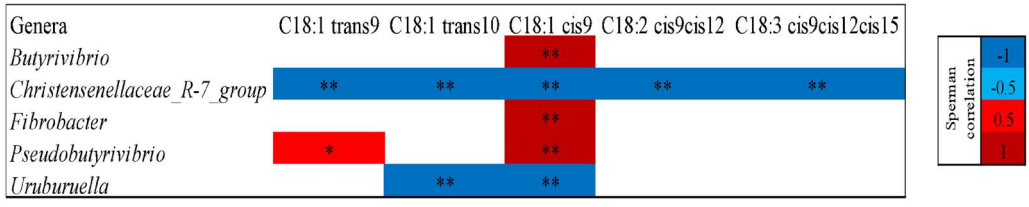

Figure 6. Correlations between C18 biohydrogenation chain and Genera in microbiota from rumen liquor fermented with the control feed without olive oil pomace (CONP) or the treated feed with olive oil pomace (OOPF). Positive correlation is indicated in red and negative correlation is indicated in blue (* for $-0.5 < p < 0.5$; ** for P < -0.5 and $p > 0.5$). Only Genera with a relative abundance > 0.1% were correlated.

cellulolytic bacteria in the fibre degrading chain that allows the employment of the intermediate products derived from soluble cellulose hydrolysis in other rumen metabolic pathways. In the rumen, cellulolytic bacteria provide non-cellulolytic microorganisms with cellodextrins and cellobiose produced during cellulose catabolism ensuring complete fibre digestion (Suen et al. 2011).

The involvement of *Prevotella* in fibre degradation is documented as well as the role in producing FA iso by cellulolytic and FA ante by amylolytic bacteria activities (Dehority 2003; Fievez et al. 2012). This is in accordance with our experiment 1 data that indicated *Prevotella* positive correlation with C13:0 iso and negative correlation with C13:0 ante, regardless of the feed. Furthermore, *Pseudobutyrivibrio* and *Rikenellaceae_RC9_gut_group*, which are microorganisms involved in the VFAs production (C4:0 and C2:0, respectively) during fibre fermentation (Van Gylswyk et al. 1996; Tavella et al. 2021), were less abundant in fermenters with OTLF. It could be supposed that other microorganisms could be engaged in VFA production and fibre degradation. Moreover, it cannot be ruled out an OTL selective action on rumen microflora due to the antimicrobial properties of bioactive compounds contained in olive leaves, such as PPs, previously reported in several studies (Patra and Saxena 2010; Anter et al. 2014; Redondo et al. 2015; Kholif and Olafadehan 2021).

The relative abundance of the genus *Butyrivibrio*, another cellulolytic bacterium, was not different between the treatments. This genus is also involved in the BH of LA and LNA (Jenkins et al. 2008) as suggested by our data, which highlighted a negative correlation between this microbial species and the concentrations of the FAs mentioned above, regardless of the dietary treatment.

In our experimental conditions, an accumulation of OA and LNA occurred as a response to OTL inclusion in the feed, probably due either to the high content of OA and LNA in OTLF and to a lowering of BH rate of the unsaturated FA.

Considering that the OA hydrogenation in the rumen leads preferentially to C18:1 t9 isomer (Mosley et al. 2002) and that C18:1 t9 concentration was similar between fermenters of both CONL and OTLF at each sampling time despite the highest content of OA in OTLF, it is conceivable that OTL dietary inclusion affected the BH process, decreasing the isomerisation rate of c9 double bound to t9 configuration. Alternatively, the hydrogenation process to the saturated form could be enhanced. Moreover, the BH seems to be affected even during LNA and LA hydrogenation, specifically for the t11 double bound one which is the rate-determining step as in RL fermenting OTLF, it was slower than in control treatment (CONL 31.69% and OTLF 4.29%).

Considering the effect of OOP on rumen microbial communities, similar to OTL, the changes in the microbiome were minimal compared to the control treatment. However, our data suggest a possible selective effect on microorganisms. In fact, the relative abundance of *Fibrobacter* and *Butyrivibrio,* the main cellulolytic bacteria present in the rumen, probably decreased as a consequence of OOP inclusion in the feed. More investigations using a meta-transcriptomic approach are needed to identify the potential role of certain enzymes and/or to determine genes up or down regulation. However, the lower concentration of C5:0 iso in the RL fermenting OOPF could be due to the increase of the content of C13:0 iso and C15 iso FA synthesis. In fact, C5:0 iso is derived by the degradation of Leucine and it is used to produce odd iso FA by carbon chain elongation (Dherbécourt et al. 2008). The content of C13:0 iso increased from 6h to 24h fermentation regardless of feed but doubled with OOPF treatment, whereas the content of C15:0 iso, the derivative of C13:0 iso elongation, was higher in fermenters with OOPF than in CONP ones at each sampling time.

The greater content of OA in OOP can contribute to explain the higher concentration of this FA in RL fermented with OOPF respect with CONP either at 6h and 24h, as well as the greater concentration of C18:1 t9 and SA in OOPF treatment. However, the similar variation of C18:1 t9 concentration in fermenters with CONP and OOPF between 6h and 24h could be consistent with a low isomerisation rate of c9 double bound to t9. A perturbative effect of OOP on microbial communities involved in the lipolysis or BH processes is deduced by the accumulation of LA and LNA in RL fermented with OOPF and by the lack of feed, time, or feed· time effect on C18:2 c9t11 and of C18:3 c9t11c15. The higher content and the lower variation of C18:1 c12 in fermenters with OOPF (CON, 47.04% and OOPF, 17.87%) could suggest that the isomerisation of c12 double bound to t11 is lower when OOP is added in the feeds due to a probable selective effect on this BH step. Despite the accumulation of LA and LNA with OOPF fermentation, SA did not show significant differences in RL for all fermenters because of the higher concentration of OA in OOP condition that is hydrogenated to the corresponding saturated FA. Mannelli et al. (2018) found a decrease in the relative

abundance of *Anaerovibrio lipolytica* when OOP is included in the diet. *Anaerovibrio lipolytica* is involved in the lipolysis process which is fundamental for the BH of FA because bacteria need the free carboxylic moiety to isomerise or reduce the double bonds of unsaturated FA. Thus, these authors hypothesised that LNA accumulation could be related to a decrease in microbial lipolytic activity. In our trial, no significant differences were found for lipolytic bacteria abundances suggesting the involvement of other microorganisms or that OOP could affect also bacteria operating the first steps of the BH. Interestingly, OOP increased the content of C18:1 t10 indicating that a high dietary supplementation with this by-product in a feeding strategy for ruminants could favour the production of an isomer responsible for milk fat syndrome. However, the ratio C18:1 t11/C18:1 t9 was largely >1 at each sampling time.

Conclusion

The growing demand for more sustainable processes imposes a new use of plant by-products generated mainly by agrifood industries. Olive processing by-products can be reused as novel ingredients in the diet of ruminants so that to improve the sustainability of both production chains. By this strategy, the food *vs* feed competition is lowered and by-products from one of the typical Mediterranean productions alternatively destined for the disposal are valorised. Data reported in this study confirmed that OTL and OOP from olive post-milling can be considered good unconventional ingredients in ruminant feeding strategy and that their impact on the microbial community is minimal. Both OTL and OOP showed similar effects on rumen BH, selectively modulating the microbial activities without negative effects on rumen ecology.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, M.P., upon reasonable request.

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