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# Rumen microbial community and milk quality in Holstein lactating cows fed olive oil pomace as part in a sustainable feeding strategy

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ABSTRACT

The use of alternative feed ingredients from the Agro-industry could be an efficient tool to improve the sustainability of dairy cow production. Since the richness in polyphenols, olive oil pomace (**OOP**), produced during olive oil milling, seems a promising by-product to ameliorate milk's nutritional value. The aim of this study was to test the use of OOP produced by means of a new technology (biphasic with stone deprivation) in dairy cow feeding strategy to evaluate the effect on animal performances, rumen microbiota, biohydrogenation processes and milk quality by a multidisciplinary approach. Forty multiparous Italian-Friesian dairy cows, at middle lactation, were randomly allotted into two homogenous groups and fed respectively a commercial diet (**CON**) and the experimental diet (**OOPD**) obtained by adding OOP to CON as partial replacement of maize silage. The two diets were formulated to be isoproteic and isoenergetic. The same diets were tested also in an *in vitro* trial aimed to evaluate their rumen degradability (**% DEG**). The dietary supplementation with OOP did not affect DM intake, rumen % DEG and milk production. The milk's nutritional quality was improved by increasing several important functional fatty acids (**FA**s; i.e., linoleic acid, conjugated linoleic acid, oleic acid, vaccenic acid). This finding was related to a decrease in rumen liquor biohydrogenation rate of unsaturated FAs. The stochiometric relation between volatile FA production in the rumen and methanogenesis suggested that OOP lowers the methane potential production (CON = 0.050 mol/L vs OOPD = 0.024 mol/L, SEM = 0.005, $P = 0.0011$). Rumen microbiota and fungi community did not be strongly altered by OOP dietary inclusion because few bacteria were affected at the genus level only. Particularly, *Acetobacter*, *Prevotellaceae*_UCG-004, *Prevotellaceae*_UCG-001, *Eubacterium coprostanoligenes*, *Lachnospira*, *Acetitomaulatum*, *Lachnospiraceae*_NK3A20 group were more abundant with OOPD condition ($P < 0.05$). Data reported in this study confirm that the use of OOP in dairy cow feeding can be an interesting strategy to improve milk nutritional quality increasing functional FA content without compromising the rumen degradability of the diet or causing strong perturbation of rumen ecosystem and maintaining animal performances.



## Implications

The use of alternative feed ingredients from the Agro-industry could be an efficient tool to make animal productions more sustainable. In this study, the use of olive oil pomace produced by means of a biphasic technology, with stone removal, was tested in Holstein dairy cow feeding to evaluate the effect on animal performances, rumen microbiota, biohydrogenation processes and milk quality by a multidisciplinary approach. The results of this study suggest that the inclusion of olive oil pomace in the dairy cow diet did not lower animal performances and nutrient degradability at the rumen level but increased the nutritional quality of milk.

## Introduction

Several studies focused on setting up new strategies aimed at finding a reuse for agro-industrial by-products, and animal feeding

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is one of the fields in which the possibility to reuse them has been evaluated (Wilkinson and Lee, 2018). Most of the food by-products are edible and their nutritional composition is characterised by the presence of functional molecules which can modulate rumen fermentation, reduce gas emission, and improve the quality of animal products (Ianni and Martino, 2020).

Olive oil pomace (**OOP**) produced during olive oil milling seems a promising by-product to ameliorate milk's nutritional value because is rich in soluble polyphenols (**PPs**; e.g., hydroxytyrosol and tyrosol) whose antioxidant and anti-inflammatory properties are well known in literature (Araújo et al., 2015; Medeiros-De-Moraes et al., 2018; Neofytou et al., 2020; Tzamaloukas et al., 2021). The main issue of fresh OOP use in animal feeding is its shelf-life being rich in water and strongly linked to the seasonality. Olive oil pomace produced with two-phase milling process is richer in PPs and water (75%) than that obtained with a three-phase process. The most common preservation method is the drying, but the thermal treatment induces the oxidation of PPs and polyunsaturated fatty acids (**PUFA**s) lowering the OOP nutritional value (Neofytou et al., 2021). Modern systems of milling were updated to overcome the problem of stone content making OOP more digestible for animals (Mannelli et al., 2018). Hence, the use of destoned and fresh OOP from two-phase process in ruminant diet is preferable to increase the content of PPs useful in modulating the microbial activity and preserving PUFAs by lipolysis and biohydrogenation (Cappucci et al., 2018; Mannelli et al., 2018). Since in literature, few information on the impact of dietary inclusion of destoned and fresh OOP, produced with a two-phase process, are available, the aim of this study was to evaluate the effect of this kind of OOP in dairy cow feeding strategy on animal performances, rumen microbiota (with metagenomic approach), biohydrogenation processes and milk quality.

## Material and methods

### *Experimental design*

The trial was performed in the farm of the experimental Centre "Enrico Avanzi", University of Pisa, and lasted 4 weeks, after 2 weeks of adaptation to the experimental diets. On the basis of the productive records, 40 healthy lactating cows were selected with the similar BW (598 ± 54 kg; mean ± SD), parity (parity = 1.30 ± 0.74), day in milk (148 ± 18 d) and daily milk yield (26.15 ± 2.00 kg). The sample size and the power analysis were computed by G*Power 3.1. Animals were randomly allotted to the two dietary treatments (20 animals each). The diets were administered twice daily as total mixed ration, and animals had free access to water. Once a week, the feed intake was individually recorded as the difference between offering and orts. Cows were milked twice daily (0700 am; 0700 pm) using a milking machine (42 kPa, 8-unit Herringbone automated milking parlour; Tecnozoo, Padova, Italy), and individual milk yield was daily registered. Individual milk samples (for analysis) were collected weekly (mixture of the proportional morning and evening milk yield). Then, each milk sample was divided into five aliquots for (i) microbiological analysis, (ii) proximate assays, (iii) rheological characteristic determination, (iv) FA profile characterisation, and (v) PP content and profile determination.

At the end of the trial (29th day), the rumen liquor (**RL**) was individually collected with an oro-oesophageal tube (Mannelli et al., 2018). The sampling was done from each animal after an overnight period without total mixed ration distribution and before morning feeding. Immediately after collection, each sample (about 200 mL of liquor) was immediately measured for pH (pHmeter 3310 - Jenway), divided into two aliquots (50 mL each one) and stored at − 80 °C for FA profile characterisation and microbial assays.

Moreover, two cows were used as donors with the aim to use the RL mix as inoculum for *in vitro* degradability assay.

### *Diets*

#### *Ingredients and nutritional profile*

Experimental diets (control diet, **CON**; treated diet, **OOPD**) were formulated as isoproteic and isoenergetic to meet the nutritional requirements of dairy cows at middle lactation according to the CNCPS system (NDS professional). The OOP inclusion level in this trial has been estimated on the base of previous experiments and in OOPD, it was 8% on DM, (Cappucci et al., 2018; Mannelli et al., 2018). Chemical and nutritional profile of CON and OOPD was characterised for CP, ether extract (**EE**), and ash according to the AOAC procedures (AOAC International, 1995; 976.06, 920.39, and 942.05 respectively). Neutral detergent fibre, ADF, and ADL were determined according to Van Soest and Robertson (1985) using heat-stable amylase and sodium sulphite. Results were inclusive of residual ash. Metabolisable energy and metabolisable protein were calculated according to CNCPS system ver. 6.55. Fatty acid profile was determined according to Mannelli et al. (2018). The ingredients and the composition of experimental diets and their proximate profile are reported in Table 1.

#### *Olive oil pomace characterisation*

Olive oil pomace was obtained from a two-phase technology (Cappucci et al., 2018) and provided by Olivicoltori Toscani Associati (Via Empolese, 20A, 50018 Scandicci Florence, It). The proximate profile (CP, EE, ash, NDF, ADF, ADL) of OOP was determined using the methods described above. Total PP content in OOP was determined according to the Folin-Ciocalteu method (Makkar et al., 1996) while the PP profile was determined according to Cecchi et al. (2018). The PP inclusion level was 23.058 g/Kg of OOP, corresponding to 11.091 g of hydroxytyrosol and 3.166 g of tyrosol. Olive oil pomace proximate, FA and PP profiles are reported in Table 1.

#### *Diet degradability assay*

At 28th and 29th days, rumen degradability (**% DEG**) of NDF and CP was estimated according to Tilley and Terry (1963). Each diet was fermented in triplicates. CP and NDF were determined on fermentation residuals from each bottle according to AOAC methods (1995; 976.06 and 942.05, respectively). The fermentation was replicated the day after.

Degradability was determined using the following formula:

$$\%\ \mathrm{DEG} = A_i - A_{end}/An_i\ ^{*}\ 100.$$

where $A_i$ was the concentration of CP or NDF in feed, $A_{end}$ was the concentration of CP or NDF in the RL residual after 24 h of fermentation.

### *In vivo rumen liquor analysis*

#### *Rumen liquor fatty acid and dimethylacetal profile, methane production potential*

Samples of RL (25 mL) were analysed for FA profile using C9:0 and C19:0 as internal standards and separated on Gas-chromatograph as described by Mannelli et al. (2018). The dimethylacetal (**DMA**) profile was determined according to the procedure described by Mannelli et al. (2018). Fatty acid contents are expressed as g/100 g*$10^{-3}$ of DM while DMA as g/100 g of DMA.

**Table 1**
Ingredients, chemical-nutritional profile, and main fatty acids of control diet and diet supplemented with olive oil pomace formulated for lactating Holstein cows.

| Feeds (g/100 g of DM) | Diet | | |
|---|---|---|---|
| | CON[1] | OOPD[2] | OOP[3**] |
| OOP | 0 | 8.02 | |
| Maize silage | 19.73 | 10.81 | |
| Commercial feed 195* | 21.77 | 21.05 | |
| Commercial feed CPL* | 14.48 | 14.01 | |
| Alfa-alfa Hay | 29.27 | 31.85 | |
| Grass Hay | 14.75 | 14.27 | |
| Chemical composition (g/kg of DM) | | | |
| DM | 65.15 | 60.10 | 79.53 |
| CP | 13.97 | 14.04 | 8.21 |
| EE[4] | 3.45 | 4.82 | 20.8 |
| NDF | 38.67 | 38.92 | 41.34 |
| NFC[5] | 36.18 | 34.07 | |
| ADF | 23 | 24.17 | 35.44 |
| ADL | 4.63 | 6.44 | 24.81 |
| Ash | 7.65 | 8.08 | 9.01 |
| Ca | 0.8 | 0.82 | |
| P | 0.43 | 0.42 | |
| Mg | 0.3 | 0.3 | |
| Na | 0.15 | 0.19 | |
| Sugar | 4.15 | 4.04 | |
| Starch | 22.26 | 18.93 | |
| SF[6] | 7.44 | 9.22 | |
| PS[7] | 5.09 | 4.69 | |
| ME[8] MJ/day | 237.3 | 234.7 | |
| MP[9] g/day | 2 357.40 | 2 358.20 | |
| Fatty acid (g/100 g of DM) | | | |
| C14:0 | 0.19 | 0.19 | 0.15 |
| C16:0 | 20.33 | 19.73 | 12.79 |
| C18:0 | 4.87 | 4.70 | 2.75 |
| C18:1 *cis*9 | 19.19 | 23.34 | 71.11 |
| C18:2 *cis*9*cis*12 | 32.31 | 30.70 | 12.21 |
| C18:3 *cis*9*cis*12*cis*15 | 17.85 | 16.49 | 0.99 |
| C20:5 *cis*5*cis*8*cis*11*cis*14*cis*17 | 3.56 | 3.28 | |
| Others | 1.7 | 1.57 | |
| TFA[10] | 2.71 | 3.94 | |
| Polyphenol profile (g/kg of DM) | | | |
| Hydroxytyrosol | | | 11.091 |
| Tyrosol | | | 3.166 |

* Commercial name product by Ferrero Mangimi SpA, Via Fornace, 15, 12060 Farigliano (Cuneo – CN) Italia.
** In Benincasa et al. (2021), olive oil pomace fatty acid profile was: C14:0 0.02 g/100 g DM; C16:0 13.49 g/100 g DM; C18:0 2.27 g/100 g DM; C18:1 cis9 71.11 g/100 g DM; C18:2 cis9cis12 12.21 g/100 g DM; C18:3 cis9cis12cis15 0.99 g/100 g DM.
[1] CON = control diet.
[2] OOPD = treated diet, with olive oil pomace.
[3] OOP = olive oil pomace.
[4] EE = ether extract.
[5] NFC = non-fibre carbohydrates.
[6] SF = soluble fibre.
[7] PS = soluble protein.
[8] ME = metabolisable energy.
[9] MP = metabolisable proteins.
[10] TFA = transfatty acids.

The $CH_4$ production potential (**MPP**) was stoichiometrically calculated according to Moss et al. (2000) as follows:

$$CH_4\ (mol/L) = 0.5mol_{C2:0} + 0.5mol_{C4:0} - 0.25mol_{C3:0}.$$

*DNA extraction*

The genomic DNA was extracted from 185 µl of RL, by using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following the modified protocol by Mannelli et al. (2018). The DNA extract was eluted in sterile water, and its integrity was verified by agarose gel electrophoresis (1% *w/v*). The quantity and quality of the DNA extracts were assessed by means of a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). The extracted DNA was conserved at −20 °C for further molecular analysis.

*Sequencing and bioinformatics*

Libraries were prepared (IGATech Services S.R.L., Udine, Italy) by following Illumina 16S Metagenomic Sequencing Library Preparation protocol in two amplification steps: an initial PCR amplification using locus-specific PCR primers and a subsequent amplification that integrates relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002). The primers used were Pro341F 5′-CCTACGGGNBGCAS CAG-3′/Pro805R 5′-GACTACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014) for universal bacterial and archaeal 16S rRNA, and AF-LSU-F 5′-GCTCAAAYTTGAAATCTTMAAG-3′/AF-LSU-R 5′-CTTGT TAAMYRAAAAGTGCATT-3′ for anaerobic fungal 28S rRNA genes (Dollhofer et al., 2016).

Libraries were sequenced on NovaSeq 6000 instrument (Illumina, San Diego, CA) using 250-bp paired-end mode. The Quantitative Insights Into Microbial Ecology (QIIME2) pipeline v2019.1.0 (https://qiime2.org/) method was used to process the obtained sequences (Bolyen et al., 2019). Paired-end sequences were denoised, dereplicated, and filtered for chimeras using the DADA2 plugin (Callahan et al., 2016), as implemented in QIIME2. Sequences were trimmed in order to include only bases with quality scores (median quality score higher than 30). Taxonomy was assigned to amplicon sequence variants (ASV) using the q2-feature-classifier at 97% similarity (Bokulich et al., 2018). Representative sequences were classified against SILVA database v138.1 for the Bacterial 16S rRNA gene and against LSU database (https://www.arb-silva.de/projects/ssu-ref-nr/) for Fungal 28S rRNA gene, using the function assign Taxonomy by applying a 99% identity criterion to remove highly similar sequences.

The nucleotide sequences determined in this study were deposited in the European Nucleotide Archive (ENA) database under the accession numbers PRJEB51870.

*Quantitative PCR assays*

The abundance of the bacterial and archaeal 16S rRNA genes and fungal 28S rRNA genes in RL DNA samples were determined by means of Quantitative PCR (**qPCR**) performed in an ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) by using the Blas Taq qPCR MasterMix (Applied Biological Materials Inc., Richmond, BC, Canada). In addition, the abundance of archaeal methanogens, *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* was also determined. For absolute quantification, the standard curves were generated, as described by Cucu et al. (2017) using the primer pairs presented in Table 2. A description of amplification details is given in Supplementary material S1.

*Milk composition and analysis*

*Proximate profile, microbiological assay, and rheological assay*

CP and EE were determined according to International AOAC (1995) methods. In addition, lactose and urea contents were determined by Milkoscan 6000 FT technology (Foss Electric, Hillerød, Denmark). The somatic cell count was evaluated according to ISO 13366-2/IDF 148-2 (ISO-IDF, 2006), using Fossmatic 5000 apparatus (Foss Electric). The somatic cell count value was expressed with a linear score resulting from: Linear score = $\log_2$ (somatic cell count/12 500) as reported in Shook (1993).

The second aliquot was processed immediately after sampling to determine the milk rennet characteristics at 35 °C by a Maspress apparatus (Foss Italia, Padua, Italy), according to Zannoni and Annibaldi (1981).

**Table 2**
Description of the primer sets and amplification details used for the quantitative PCR and the PCR of the DNA extracts from rumen liquor of lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

| Target group | Primer | Fragment size (bp) | Amplification details |
|---|---|---|---|
| qPCR | | | |
| All bacteria | 341F (Muyzer et al., 1993) | | 40 cycles |
| (16S rRNA gene) | 5′ CCTACGGGAGGCAGCAG 3′ | | 95 °C 15 s, 60 °C 45 s, 72 °C 30 s 82 °C 10 s |
| | R806 (Caporaso et al., 2011) | | |
| | 5′ GGACTACHVGGGTWTCTAA 3′ | | |
| All archaea | ARC344F (Raskin et al., 1994) | | 40 cycles |
| (16S rRNA gene) | 5′ ACGGGGYGCAGCAGGCGCGA 3′ | | 95 °C 15 s, 61 °C 35 s, 72 °C 45 s 83 °C 10 s |
| | Arch806R (Takai and Horikoshi, 2000) | | |
| | 5′ GGACTACVSGGGTATCTAAT 3′ | | |
| Anaerobic fungi | AF-LSUF (Dollhofer et al., 2016) | | 45 cycles |
| (28S rRNA gene) | 5′ GCT CAA AYT TGA AATCTT MAA G 3′ | | 95 °C 15 s, 60 °C 45 s, 72 °C 30 s 83 °C 10 s |
| | AF-LSUR (Dollhofer et al., 2016) | | |
| | 5′ CTT GTT AAM YRA AAA GTG CAT T 3′ | | |
| Methanogens | uniMet1-F (Zhou et al., 2009) | | 40 cycles |
| | 5′ CCGGAGATGGAACCTGAGAC 3′ | | 95 °C 15 s, 60 °C 30 s, 72 °C 30 s, 82 °C 10 s |
| | uniMet1-R (Zhou et al., 2009) | | |
| | 5′ CGGTCTTGCCCAGCTCTTATTC 3′ | | |
| *Anaerovibrio lipolytica* | ALF (Tajima et al., 2001) | | 45cycles |
| | 5′ TGGGTGTTAGAAATGGATTC 3′ | | 95 °C 15 s, 60 °C 45 s, 72 °C 30 s 82 °C 10 s |
| | ALR (Tajima et al., 2001) | | |
| | 5′ CTCTCCTGCACTCAAGAATT 3′ | | |
| *Butyrivibrio fibrisolvens* | BfibF (Klieve et al., 2003) | | 40 cycles |
| | 5′ ACACACCGCCCGTCACA 3′ | | 95 °C 15 s, 60 °C 30 s, 72 °C 30 s, 81 °C 10 s |
| | BfibR (Klieve et al., 2003) | | |
| | 5′ TCCTTACGGTTGGGTCACAGA 3′ | | |
| *Prevotella ruminicola* | PRF (Tajima et al., 2001) | | 40 cycles |
| | 5′ GGTTATCTTGAGTGAGTT 3′ | | 95 °C 15 s, 60 °C 45 s, 72 °C 30 s 80 °C 10 s |
| | PRR (Tajima et al., 2001) | | |
| | 5′ CTGATGGCAACTAAAGAA 3′ | | |
| PCR | | | |
| All bacteria | | 464 | 35 cycles |
| (16S rRNA gene) | | | 95 °C 30 s, 60 °C 45 s 72 °C 45 s/72 °C 10 min |
| All archaea | | 461 | 35 cycles |
| (16S rRNA gene) | | | 95 °C 30 s, 60 °C 45 s 72 °C 45 s/72 °C 10 min |
| Anaerobic fungi | | 441 | 35 cycles |
| (28S rRNA gene) | | | 95 °C 30 s, 55 °C 45 s, 72 °C 45 s/72 °C 5 min |
| Methanogens | | 163 | 35 cycles |
| | | | 95 °C 30 s, 55 °C 45 s, 72 °C 30 s/72 °C 10 min |
| *Anaerovibrio lipolytica* | | 597 | 35 cycles |
| | | | 95 °C 30 s, 61 °C 35 s, 72 °C 60 s/72 °C 5 min |
| *Butyrivibrio fibrisolvens* | | 90 | 35 cycles |
| | | | 95 °C 30 s, 55 °C 45 s, 72 °C 30 s/72 °C 10 min |
| *Prevotella ruminicola* | | 485 | 35 cycles |
| | | | 95 °C 30 s, 57,7°C 35 s, 72 °C 60 s/72 °C 5 min |

*Fatty acid profile, and extraction and determination of the milk total polyphenol content and profile*

Fatty acid profile was determined according to Cappucci et al. (2018). All FA composition results are expressed as g/100 g of fat.

The PP content in milk samples (from animals fed CON and OOPD) collected at the beginning and at the end of the trial (1 and 4 weeks) was determined following the method described by Vázquez et al. (2014). Samples were withdrawn, transferred in Eppendorf tubes, and stored at −20 °C. The extracts were used for the determination of total content of PPs and for the characterisation of their profile.

The analysis of total PP content was carried out using the Folin–Ciocalteu method (Makkar et al., 1996). The reference curve used for spectrophotometric assay was prepared using an increasing amount (2–20 nmoles) of gallic acid (Merck Life Science S.r.l., Italy). The calculated standard-curve parameters were as follows:

$$m = 0.04928 \pm 4.26439E\text{-}4;\ b = 0.02228 \pm 0.00485.$$

The milk extracts were analysed to determine PP profile by means of HPLC and then by Chromatography–Mass Spectrometry (GC–MS) according to Genovese et al. (2022). The MassHunter data processing tool (Agilent, Santa Clara, CA, USA) and Fihen Metabolomics RTL library (Agilent G1676AA) were used to obtain a global metabolic profiling. A detailed description is reported in Supplementary material S2.

*Statistical analysis*

*Milk*

Milk data (e.g., performances or qualitative profile) recorded throughout the experiment were processed as completely randomised design with repeated measures using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA):

$$Y_{ijkl} = \mu + D_i + T_j + I_k(D) + (D \times T)_{ij} + e_{ijkl}.$$

where $Y_{ijkl}$ is the observation; $\mu$ is the overall mean; $D_i$ the fixed effect of diet (i = 1–2); $T_j$ the fixed effect of sampling time (j = 1–4); $I_k$ is the random effect of the cow within the diet (k = 1–20); $(Di \times T)_{ij}$ the interaction between diet and sampling time and $e_{ijkl}$ 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 the variance of cows within the diet according to repeated measures design theory (Littell et al., 1998). Multiple comparisons among means were performed using Tukey's test.

*Rumen liquor fatty acid and dimethylacetals*

Data of rumen FA and DMA concentration, of CP and NDF degradability and $CH_4$ prevision were processed using one-way analysis of variance SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) with a model that included diet and experimental error.

$$Y_{ij} = \mu + D_i + e_{ij}.$$

where $Y_{ij}$ is the observation; $\mu$ is the overall mean; $D_i$ the diet (i = 1–2) and $e_{ij}$ the residual error. Multiple comparisons among means were performed using Tukey's test.

*Rumen liquor microbial communities*

The data on microbial community structure were analysed using the PAST4.03 software (Hammer et al., 2001). The number of different taxa (Taxa_S) was estimated by sample coverage, and microbial α-diversity was assessed by Shannon, Evenness and Chao1 indices at the genus level (Faith, 1992). The β-diversity was estimated with a non-metric multidimensional scaling (NMDS) and a permutational multivariate analysis of variance (PERMANOVA) performed with the Bray–Curtis dissimilarity index at the genus level.

The taxa with different relative abundances between the conditions (i.e., CON, OOPD) were identified in Primer-e (Multivariate Analysis for Ecology) software (Clarke and Gorley, 2015), by ANOVA and *t*-test performed at the genus level.

To assess the significant differences between CON and OOPD, in relation to the microbial qPCR abundances and alpha diversity indices, data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$) using PAST4.03 software (Hammer et al., 2001). The normality and the variance homogeneity of the data were tested prior to ANOVA.

## Results

*Animal performances*

No animal was excluded during the whole period of the trial. Animals fed completely the daily rations and no daily orts were found, showing a comparable palatability between CON and OOPD. Indeed, no differences were observed for daily DM intake (CON = 24.10 ± 0.15 kg of DM day per head; OOPD = 24.90 ± 0.13 kg of DM day per head). Milk production did not differ between CON and OOPD groups, and only a time effect ($P = 0.0191$) was found (CON = 26.10 ± 1.96 kg of DM day per head; OOPD = 26.24 ± 1.9 5 kg of DM day per head). Moreover, the pH of RL was not different among the two groups and in an optimal range (CON 6.82 vs OOPD 6.76, SEM = 0.11, $P = 0.6372$).

*Diet degradability*

No differences among groups were found for the % DEG of CP (CON, 54.91%; OOPD, 50.15%; SEM = 2.95; $P = 0.3174$) and of NDF (CON, 88.01%; OOPD, 90.74%; SEM 1.14; $P = 0.1652$).

*Rumen fatty acid and dimethylacetal profiles, and methane potential*

Acetate content did not show significant differences between RLs from animals fed the two diets (CON *vs* OOPD) while the content of C3:0, C4:0 and C5:0 *iso* significantly decreased when OOP was added to the diet (Table 3). The C2:0/C3:0 ratio was higher in OOPD (0.097 in CON *vs* 0.193 in OOPD, SEM = 0.022, $P = 0.0029$).

Considering medium-chain FAs, odd- and branched-chain FAs, C11:0, C13:0 *ante* and C17:0 *iso* increased while C17:0 *ante* decreased in RL from animals fed OOPD, (Table 3). Among long-chain FAs, the content of C16:1 *cis*9 and C23:0 FAs decreased while C20:1 *cis*11, C20:3 *cis*8*cis*11*cis*17, C20:5 *cis*5*cis*8*cis*11*cis*14*cis*17 n3, C22:0, C22:6 *cis*4*cis*7*cis*10*cis*13*cis*16*cis*19 n3 and C24:1 *cis*15 increased with OOPD (Table 3).

The content of C18:1 *trans*11 (vaccenic acid), C18:1 *cis*7, C18:1 *cis*11, and C18:1 *cis*14 + *cis*16 decreased when animals fed OOPD. In contrast, C18:2 *cis*9*cis*12 (linoleic acid), C18:2 *cis*9*trans*11 (conjugated linoleic acid), C18:2 *cis*9*trans*12, C18:3 *cis*9*cis*12*cis*15 (linolenic acid) and C18:1 *cis*9 (oleic acid) increased in RL of treated OOPD group (Table 3).

The content of DMA 16:0 (CON = 40.521 vs OOPD = 32.560 SEM = 2.24 $P = 0.0184$) and DMA 18:1 *trans*11 (CON = 2.570 vs OOPD = 1.506 SEM = 0.18 $P = 0.0003$) was significantly lower while the content of DMA 16:1 (CON = 1.354 vs OOPD = 2.344 SEM = 0.25 $P = 0.0092$) increased in RL from OOPD group.

Since the $CH_4$ emission is strongly linked to stochiometric ratio among volatile FAs, using the equation of Moss et al. (2000), MPP has been calculated for each diet, considering C2:0, C3:0 and C4:0 moles. The estimated $CH_4$ production was higher in CON than in OOPD (0.050 mol/L in CON *vs* 0.024 mol/L in OOPD, SEM = 0.005, $P = 0.0011$).

*Microbial diversity*

The microbial communities for both CON and OOPD RLs were characterised by high-throughput sequencing 16S and 28S rRNA gene amplicons. The α-diversity indices (Taxa_S, Shannon, Evenness and Chao1 indices; Fig. 1, 16S and 28S) and the β-diversity (NMDS and PERMANOVA) showed no significant differences between the treatments considering both the 16S rRNA gene ($R^2 = 0.04$, $P = 0.1$) and the 28S rRNA gene ($R^2 = 0.015$, $P = 0.5$), as indicated in Supplementary Figs. S1 and S2.

*Bacterial and anaerobic fungal taxonomic assignment*

Twenty-eight bacterial phyla were identified across samples. *Firmicutes*, *Bacteroidota*, *Actinobacteriota*, *Euryarchaeota* and *Patescibacteria* were the five dominant groups representing 51.5, 28.2, 4.6, 4.0 and 3.6% in CON group and 52.0, 29.0, 3.6, 4.0, and 3.4% of the total sequences in OOPD group, respectively (Supplementary Fig. S3). No significant differences were observed at the phylum level. Then, a total of 17 classes was observed in both CON and OOPD conditions, with a relative abundance of at least 0.2% (Supplementary Fig. S4). *Clostridia* was the most abundant (40.5% in CON and 40.2% in OOPD), followed by *Bacteroidia* (28.2% in CON and 29.0% in OOPD), *Bacilli* (8.4% in CON and 8.8% in OOPD) and *Actinobacteria* (4.9% in CON and 3.6% in OOPD). However, at the class level, no differences were observed between the considered conditions. Finally, at the genera level (Fig. 2), *Acetobacter* (0.08% in CON and 0.4% in OOPD), *Prevotellaceae*_UCG-004 (0.2% in CON and 0.3% in OOPD), *Prevotellaceae*_UCG-001 (0.9% in CON and 1.2% in OOPD), *Eubacterium coprostanoligenes* (1% in CON and 1.4% in OOPD), *Lachnospira* (0.4% in CON and 0.6% in OOPD), *Acetitomaulatum* (2% in CON and 3% in OOPD), *Lachnospiraceae*_NK3A20 (3.4% in CON and 4% in OOPD) group were more abundant with OOPD condition when compared with the CON group ($P < 0.05$).

Mycobiota composition was 100% attributed to anaerobic fungi, belonging to Phylum *Neocallimastigomycota* due to primer specificity, Class *Neocallimastigomycetes*, Family *Neocallimastigaceae* in both CON and OOPD conditions. A total of six genera was observed (Fig. 3) and *Pyromices* was the most abundant (CON 37.0% and OOPD 36.0%), followed by *Neocallimastix* (CON 23.7% and OOPD 22.5%) and *Orpinomyces* (CON 16.0% and OOPD 21.0%).

**Table 3**
Rumen fatty acid profile from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

| Fatty acid (g/100 g DM*$10^{-3}$) | Diet | | SEM ($10^{-3}$) | *P* |
|---|---|---|---|---|
| | CON[1] | OOPD[2] | | |
| SCFA[3] | | | | |
| C2:0 | 0.536 | 0.601 | 0.062 | 0.4693 |
| C4:0 iso | 43.925 | 41.46 | 6.961 | 0.8039 |
| C3:0 | 10.428[a] | 4.032[b] | 1.739 | 0.014 |
| C4:0 | 132.368[a] | 61.221[b] | 14.402 | 0.0014 |
| C5:0 iso | 18.435[a] | 4.31[b] | 3.306 | 0.0049 |
| OBCFA[4] | | | | |
| C11:0 | 0.243[b] | 0.345[a] | 0.025 | 0.0074 |
| C13:0 | 15.341 | 17.155 | 3.275 | 0.698 |
| C13:0 iso | 2.478 | 2.548 | 0.279 | 0.8621 |
| C13:0 ante | 0.027[b] | 0.384[a] | 0.112 | 0.031 |
| C14:0 iso | 3.07 | 2.696 | 0.254 | 0.3066 |
| C15:0 | 16.05 | 13.767 | 1.347 | 0.2394 |
| C15:0 iso | 3.702 | 4.758 | 0.62 | 0.2376 |
| C15:0 ante | 1.422 | 1.876 | 0.178 | 0.0799 |
| C16:0 iso | 0.103 | 0.065 | 0.068 | 0.7006 |
| C17:0 | 8.787 | 6.75 | 0.929 | 0.1308 |
| C17:0 iso | 0.254[b] | 0.615[a] | 0.073 | 0.0014 |
| C17:0 ante | 9.958[a] | 7.045[b] | 0.889 | 0.0271 |
| C17:1 | 2.597 | 0.75 | 0.656 | 0.0549 |
| C18:0 iso | 0.479 | 0.479 | 0.094 | 0.9994 |
| C20:0 ante | 1.713 | 1.902 | 0.227 | 0.5598 |
| MCFA[5] | | | | |
| C6:0 | 5.826 | 5.023 | 1.442 | 0.6963 |
| C8:0 | 3.07 | 3.743 | 0.637 | 0.4603 |
| C10:0 | 2.903 | 3.573 | 0.594 | 0.4313 |
| C10:1 *cis*9 | 0.195 | 0.149 | 0.03 | 0.2813 |
| C12:0 | 2.72 | 2.388 | 0.234 | 0.3226 |
| C12:1 *cis*9 | 0.468 | 0.624 | 0.071 | 0.1306 |
| LCFA[6] | | | | |
| C14:0 | 13.421 | 11.029 | 1.161 | 0.155 |
| C14:1 *cis*9 | 7.876 | 7.125 | 0.614 | 0.3934 |
| C16:0 | 222.267 | 215.477 | 20.883 | 0.8196 |
| C16:1 *cis*9 | 4.107[a] | 2.674[b] | 0.427 | 0.0237 |
| C18:4 | 1.32 | 1.668 | 0.277 | 0.3806 |
| C20:0 | 9.696 | 21.166 | 7.497 | 0.2874 |
| C20:1 *cis*11 | 2.348[b] | 3.378[a] | 0.195 | 0.0007 |
| C20:1 *cis*16 | 2.038 | 2.579 | 0.304 | 0.2166 |
| C20:2 *cis*11*cis*14 | 0.987 | 1.252 | 0.18 | 0.3069 |
| C20:3 *cis*11*cis*14*cis*17 n3 | 1.302 | 1.268 | 0.193 | 0.903 |
| C20:3 *cis*8*cis*11*cis*17 n6 | 5.799[b] | 9.191[a] | 0.751 | 0.0032 |
| C20:4 *cis*5*cis*8*cis*11*cis*14 n6 | 6.78 | 5.124 | 0.682 | 0.0956 |
| C20:5 *cis*5*cis*8*cis*11*cis*14*cis*17 n3 | 18.232[b] | 32.162[a] | 2.699 | 0.0009 |
| C21:0 | 0.454 | 0.71 | 0.09 | 0.052 |
| C22:0 | 0.871[b] | 1.608[a] | 0.174 | 0.0052 |
| C22:1 cis13 | 2.347 | 1.872 | 0.256 | 0.1991 |
| C22:6 *cis*4*cis*7*cis*10*cis*13*cis*16*cis*19 n3 | 0.615[b] | 0.971[a] | 0.115 | 0.0358 |
| C23:0 | 7.985[a] | 5.63[b] | 0.775 | 0.0394 |
| C24:0 + C22:3 | 0.683 | 1.634 | 0.354 | 0.0664 |
| C24:1 *cis*15 | 0.741[b] | 2.328[a] | 0.185 | <0.0001 |
| FA[7] in biohydrogenation process | | | | |
| C18:0 | 442.086 | 516.121 | 125.856 | 0.6802 |
| C18:1 *trans*4 | 1.717 | 1.933 | 0.296 | 0.6092 |
| C18:1 *trans*5 | 0.923 | 1.173 | 0.173 | 0.3134 |
| C18:1 *trans*6-8 | 9.885 | 10.416 | 1.574 | 0.8129 |
| C18:1 *trans*9 | 5.972 | 5.93 | 0.784 | 0.9694 |
| C18:1 *trans*10 | 19.611 | 16.422 | 2.447 | 0.3637 |
| C18:1 *trans*11 | 61.017[a] | 39.023[b] | 5.746 | 0.0108 |
| C18:1 *trans*12 | 12.983 | 9.71 | 1.402 | 0.1085 |
| C18:1 *cis*9 | 52.562[b] | 112.692[a] | 12.354 | 0.0016 |
| C18:1 *cis*7 | 18.734[a] | 7.404[b] | 1.624 | <0.0001 |
| C18:1 *cis*11 | 12.048[a] | 6.356[b] | 1.269 | 0.0033 |
| C18:1 *cis*12 | 13.878 | 13.364 | 1.521 | 0.8129 |
| C18:1 *cis*13 + *trans*16 | 2.613 | 0.868 | 0.699 | 0.0871 |
| C18:1 *cis*14 + *cis*16 | 11.094[a] | 4.112[b] | 0.909 | <0.0001 |
| C18:1 *cis*15 | 4.053 | 3.057 | 1.08 | 0.5191 |
| C18:2 *cis*9*cis*12 | 48.953[b] | 94.077[a] | 14.216 | 0.0318 |
| C18:2 *cis*9*trans*11 | 5.594[b] | 12.865[a] | 2.045 | 0.0171 |
| C18:2 *cis*9*trans*12 | 0.222[b] | 0.458[a] | 0.072 | 0.0274 |
| C18:2 *trans*10*cis*12 | 0.563 | 0.828 | 0.178 | 0.3012 |
| C18:3 *cis*9*cis*12*cis*15 | 13.297[b] | 19.474[a] | 1.653 | 0.0126 |
| C18:3 *cis*9*trans*12*trans*15 | 0.494 | 0.364 | 0.139 | 0.5139 |

**Table 3** (*continued*)

| Fatty acid (g/100 g DM*$10^{-3}$) | Diet | | SEM ($10^{-3}$) | *P* |
|---|---|---|---|---|
| | CON[1] | OOPD[2] | | |
| C18:3 *trans*9*cis*12*trans*15 | 0.163 | 0.124 | 0.046 | 0.5485 |
| C18:3 *cis*9*trans*11*cis*15 | 2.23 | 2.511 | 0.258 | 0.4464 |
| C18:2 *trans*11*cis*15 | 0.774 | 1.65 | 0.662 | 0.3564 |

[a,b] within a row, means with different letters are significantly different ($P < 0.05$).
[1] CON = control diet.
[2] OOPD = treated diet, with olive oil pomace.
[3] SCFA = rumen short-chain fatty acid profile.
[4] OBCFA = rumen odd- and branched-chain fatty acid profile.
[5] MCFA = rumen medium-chain fatty acid profile.
[6] LCFA = rumen long-chain fatty acid profile.
[7] FAs = fatty acids.

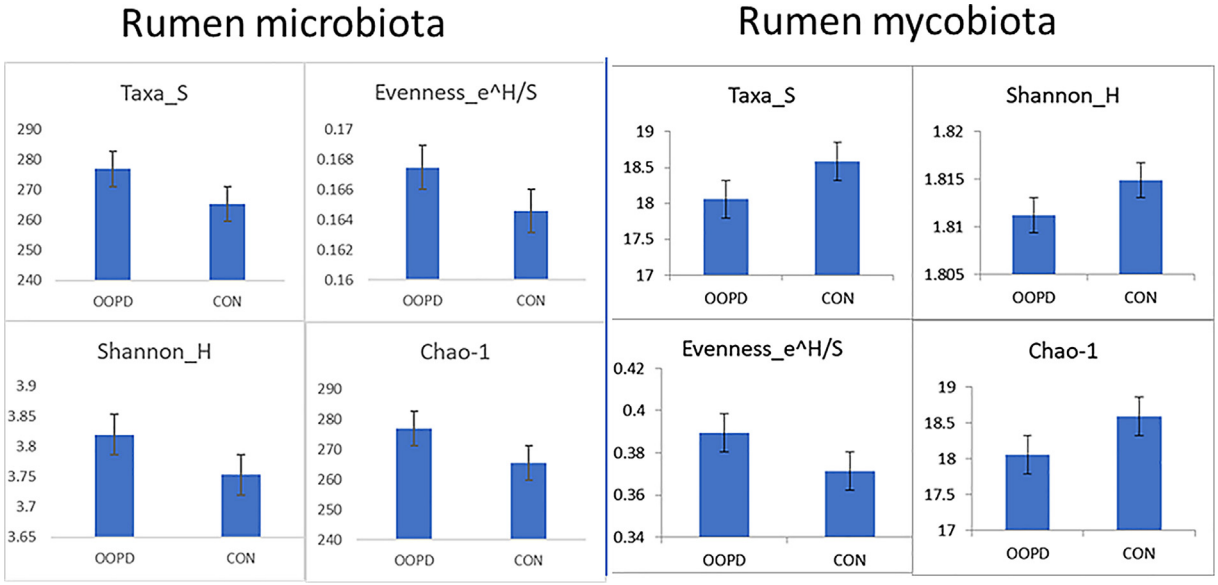


**Fig. 1.** The α-diversity indices (e.g., Taxa_S, Evenness, Shannon and Chao1) of the rumen microbiota (A) and mycobiota (B) from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace. Abbreviations: CON = control diet; OOPD = treated diet, with olive oil pomace.

*Quantitative PCR (qPCR) microbial abundance*

The abundance of bacterial 16S rRNA and anaerobic fungal 28S rRNA genes, as well as the abundance of *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* was lower in the rumen of the cows fed OOPD ($P < 0.05$). The abundance of methanogens and of total archaea communities showed no significant difference between OOPD and CON conditions (Table 4).

*Milk yield and quality and milk rennet characteristics*

Milk production, milk composition and quality parameters did not show significant differences between the two groups: EE, in CON = 4.04 vs in OOPD = 4.03 g/100 g of milk (SEM = 0.17 and $P = 0.9683$); CP, in CON = 3.41 vs in OOPD = 3.47 g/100 g of milk (SEM = 0.08 and $P = 0.5961$); Lactose, in CON = 4.71 vs in OOPD = 4.73 g/100 g of milk (SEM = 0.04 and $P = 0.8377$); log(somatic cell count), in CON = 5.01 vs in OOPD = 5.05 g/100 g of milk (with SEM = 0.10 and $P = 0.8269$); DM, in CON = 8.87 vs in OOPD = 8.95 g/100 g of milk (SEM = 0.08 and $P = 0.5210$); Urea, in CON = 25.45 vs in OOPD = 25.49 mg/100 ml of milk (SEM = 0.90 and $P = 0.9768$).

The dietary supplementation with OOP seemed to no affect clotting parameters (r, SEM = 1.499 $P = 0.1744$; k20, SEM = 1.433 $P = 0.8419$; a30, SEM = 1.584 $P = 0.1264$).

*Milk fatty acid profile and milk polyphenols*

The concentration of short-chain FAs from C6:0 to C10:1 *cis*9 as well as C12:0, C14:0, C16:0, C17:0 medium-chain FAs and the related monounsaturated FAs was lower in the milk from the cows fed the diet supplemented with OOP (Table 5). Similarly, considering odd-branched chain FAs, C13:0 *ante*, C17:0 *ante,* C15:0 *iso*, and C18:0 *iso* were lower in milk from cows fed OOPD with respect to CON. Among saturated FAs, only C18:0 increased in OOPD milk samples. The content of all *trans*-C18-FA increased with OOP dietary inclusion. Specifically, C18:1 *trans*11 increased by approximately 34.0% while C18: 1 *trans*9 and C18: 1 *trans*10 by approximately 47.31 and 50.35%, respectively. Considering FAs belonging to the C18:2 series, milk from cow fed OOPD was richer in C18:2 *cis*9*cis*12, C18:2 *cis*9*trans*11 and C18:4 *cis*6*cis*9*cis*12*cis*15 but poorer in C18:3 *cis*9*cis*12*cis*15 than the milk of cow fed CON. Considering FAs belonging to the C20:0 series, a minor content of C20:5 *cis*5*cis*8*cis*11*cis*14*cis*17 and C20:4 *cis*5*cis*8*cis*11*cis*14 while a

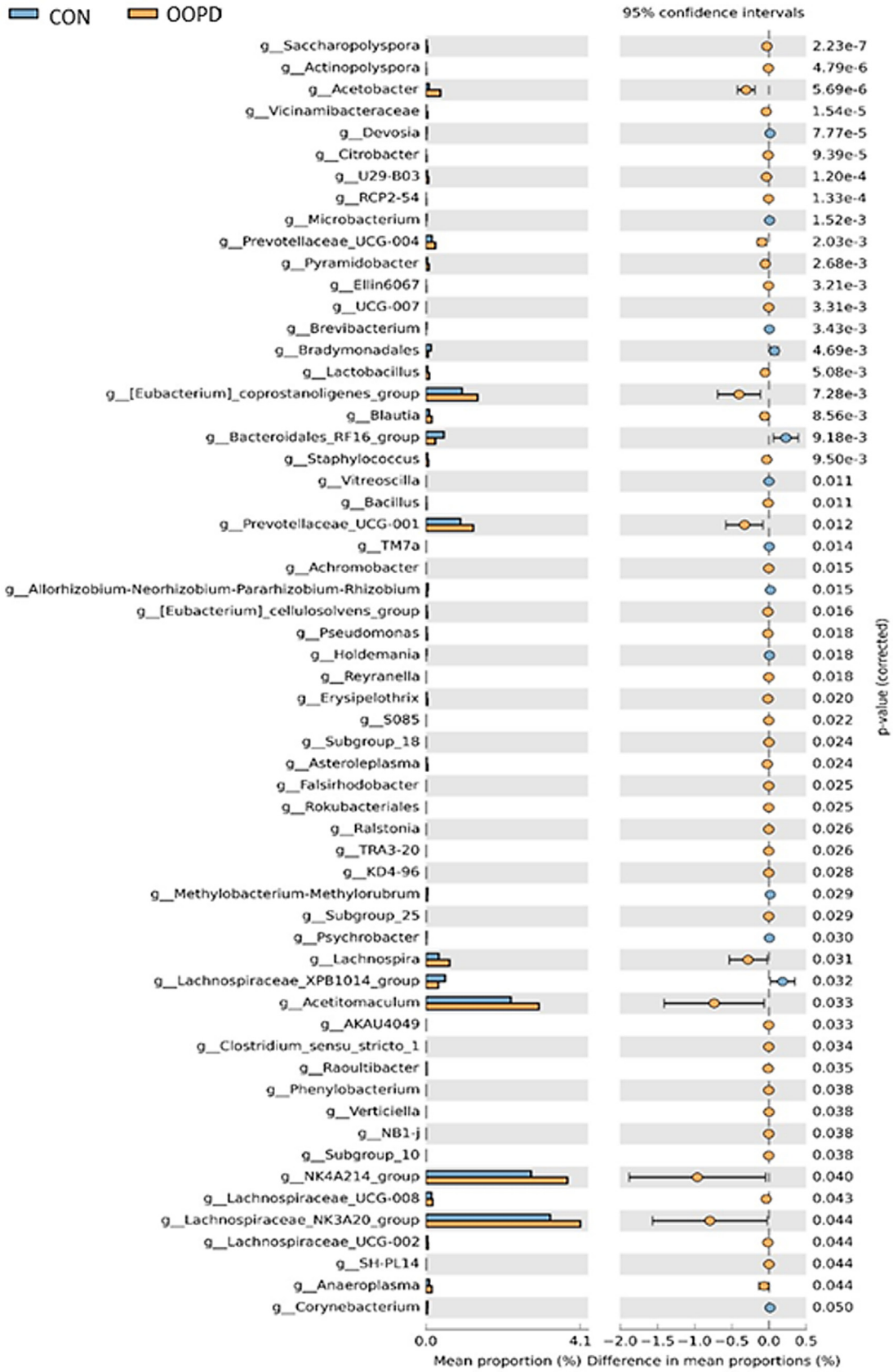


**Fig. 2.** Percent of the genera detected in rumen liquor microbiota from Holstein cows fed with the control diet or the diet supplemented with olive oil pomace and the significant differences. Abbreviations: CON = control diet; OOPD = treated diet, with olive oil pomace.

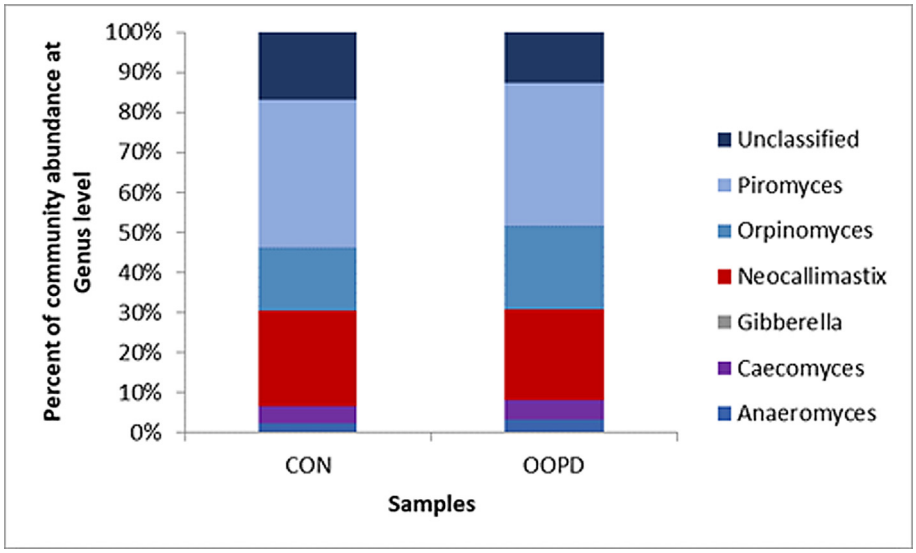


**Fig. 3.** Percent of community abundance at the genus level detected in rumen liquor mycobiota from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace. Abbreviations: CON = control diet; OOPD = treated diet, with olive oil pomace.

**Table 4**
Quantitative PCR abundance of targeted microbial communities detected in the rumen liquor from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

| Item[1] | Diet (log copies/ml$^{-1}$) | | *P* |
|---|---|---|---|
| | CON[2] | OOPD[3] | |
| Bacteria | 9.20 ± 3.60[a] | 9.06 ± 3.56[b] | 0.0022 |
| Archaea | 9.73 ± 3.89 | 9.67 ± 3.78 | 0.0853 |
| Fungi | 8.00 ± 3.14[a] | 6.94 ± 2.80[b] | 4.03E$^{-05}$ |
| Methanogens | 7.54 ± 3.04 | 7.50 ± 2.62 | 0.6338 |
| *Anaerovibrio lipolytica* | 5.27 ± 1.88[a] | 4.46 ± 1.51[b] | 7.67E$^{-05}$ |
| *Butyrivibrio fibrisolvens* | 9.20 ± 2.73[a] | 9.09 ± 2.44[b] | 0.0315 |
| *Prevotella ruminicola* | 6.93 ± 2.73[a] | 6.28 ± 2.45[b] | 0.0010 |

[a,b] Within a row, means with different letters are significantly different ($P < 0.05$).
[1] Item, targeted microbial community.
[2] CON = Control diet.
[3] OOPD = Treated diet.

major content of C20:0, C20:1 *cis*11, and C22:0 were found in OOPD milk compared to milk from animals fed CON.

Data related to total milk PP content showed no significant differences between CON and OOPD groups, either with Folin-Ciocalteu method (first CON = 10.51 ± 1.26 vs OOPD = 10.92 ± 3.25 $P = 0.5924$; second CON = 9.88 ± 2.71 vs OOPD = 10.50 ± 3.26 $P = 0.5094$) and HPLC analysis (Supplementary Figs. S5–S7). The postrun analysis did not reveal the presence of detectable PPs in any samples (data not shown).

## Discussion

### *Animal performances*

This study was carried out to evaluate the effect of OOP in dairy cow feeding on the rumen ecosystem using the metagenomic approach, productive performances, and milk quality.

The dietary supplementation with OOP did not affect the animal performances and milk proximate profile. In particular, DM intake was similar between the two groups suggesting that OOP at the 8% level of inclusion did not alter the palatability of the diet, although PPs could alter the flavour of the diet. Consequently, the two diets were equally consumed ensuring the nutrients for the milk production. In fact, yield and proximate profile of milk did not show differences among cows fed CON and OOPD.

An important chemical property of PPs is their ability in complexing protein, and this bond occurs at pH close to neutrality (Papadopoulou and Frazier, 2004; Ishida et al., 2015). Considering the similar milk production of the two groups, it is conceivable that at the gut level, PPs did not affect the absorption of amino acid involved in the <−lactalbumin galactosyltransferase, fundamental for lactose synthesis in mammary gland, as observed also by Mannelli et al. (2018) and Cappucci et al. (2018).

### *Rumen microbiota and fatty acid profile*

The inclusion level of dietary OOP (8% on DM) did not strongly alter the rumen ecosystem. This result is consistent with data of CP and NDF rumen% DEG determined by means of the *in vitro* trial, where CON and OOPD were fermented. The OOP used in this trial was obtained by a two-phase technology, which permitted to remove olive stones reducing the undegradable fraction of the cake (Servili et al., 2011). The microbial communities in the rumen of the cows fed the two diets were very similar, as evidenced by the no significant differences in the α- and β-diversity. No taxonomic level evidenced differences among RL microbial communities of the two groups, except for the genus level. The iso-forms of odd-branched chain FAs are distinctive of the cellulolytic activity while the ante-forms for the amylolytic activity (Fievez et al., 2012). Our findings showed a different concentration of several odd-branched chain FAs when FA profiles of RL from the two groups are compared. In fact, the content of C13:0 *ante* (precursor of C17:0 *ante*) and C17:0 *iso* increased while the content of C17:0 *ante* decreased, leading to the hypothesis that OOPD made a more suitable environment for the cellulolytic bacteria than for the amylolytic bacteria. Moreover, a significant lowering of C3:0 and of C4:0 in RL of cows fed OOPD with respect to those fed CON was observed. At the same time, a comparable C2:0 concentration was found. In rumen, the fibre fermentation undergoes cellulolytic bacteria and fungi action leading to C2:0 production. In contrast, amylolytic microorganisms convert pyruvate preferentially to C3:0 or C4:0. In accordance with this hypothesis, the calculated C2:0/C3:0 ratio was higher with OOPD than CON. During the C2:0 microbial synthesis, $H_2$ is produced by cellulolytic microorganisms and used by archaea in the methanogenesis. The lower

**Table 5**
Milk fatty acid profile from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

| Fatty acid (g/100 g of fat) | Diet | | | *P* | | |
|---|---|---|---|---|---|---|
| | CON[1] | OOPD[2] | SEM | D[3] | T[4] | DxT[5] |
| C4:0 | 4.275 | 4.136 | 0.072 | 0.2104 | <0.0001 | 0.2987 |
| C6:0 | 2.466[a] | 2.146[b] | 0.043 | <0.0001 | 0.0808 | 0.5758 |
| C8:0 | 1.141[a] | 0.950[b] | 0.021 | <0.0001 | 0.0889 | 0.2714 |
| C10:0 | 4.432[a] | 3.524[b] | 0.092 | <0.0001 | 0.0335 | 0.275 |
| C10:1 *cis*9 | 0.330[a] | 0.265[b] | 0.01 | <0.0001 | 0.0074 | 0.7438 |
| C11:0 | 0.054[a] | 0.025[b] | 0.003 | <0.0001 | 0.1279 | 0.2404 |
| C12:0 | 4.483[a] | 3.429[b] | 0.107 | <0.0001 | 0.0422 | 0.1475 |
| C12:1 *cis*9 | 0.091[a] | 0.071[b] | 0.005 | 0.0037 | 0.0335 | 0.6584 |
| C13:0 | 0.138[a] | 0.098[b] | 0.004 | <0.0001 | 0.3742 | 0.5362 |
| C13:0 iso | 0.030 | 0.026 | 0.002 | 0.1385 | 0.0012 | 0.0019 |
| C13:0 ante | 0.064[a] | 0.048[b] | 0.003 | 0.0014 | <0.0001 | 0.4466 |
| C14:0 | 7.361[a] | 6.642[b] | 0.135 | 0.0004 | 0.1843 | 0.4125 |
| C14:0 iso | 0.066 | 0.066 | 0.003 | 0.8535 | 0.5982 | 0.0456 |
| C14:1 *cis*9 | 0.642[a] | 0.565[b] | 0.022 | 0.0207 | 0.1806 | 0.543 |
| C15:0 | 0.937[a] | 0.843[b] | 0.02 | 0.0017 | 0.1365 | 0.9869 |
| C15:0 iso | 0.175[a] | 0.158[b] | 0.005 | 0.0151 | 0.2625 | 0.015 |
| C15:0 ante | 0.442 | 0.435 | 0.01 | 0.6049 | 0.6707 | 0.1394 |
| C16:0 | 23.198[a] | 20.222[b] | 0.329 | <0.0001 | 0.6827 | 0.3102 |
| C16:0 iso | 0.221 | 0.202 | 0.008 | 0.0976 | 0.9885 | 0.4084 |
| C16:1 *cis*9 | 1.162[a] | 0.986[b] | 0.026 | <0.0001 | 0.9786 | 0.3961 |
| C17:0 | 0.501[a] | 0.444[b] | 0.006 | <0.0001 | 0.0002 | 0.1234 |
| C17:0 iso | 0.292 | 0.284 | 0.004 | 0.1763 | 0.1981 | 0.0832 |
| C17:0 ante | 0.479[a] | 0.447[b] | 0.006 | 0.0004 | 0.2552 | 0.1904 |
| C17:1 *cis*10 | 0.238[a] | 0.199[b] | 0.006 | <0.0001 | 0.5896 | 0.616 |
| C18:0 | 8.510[b] | 11.001[a] | 0.203 | <0.0001 | 0.0039 | 0.3436 |
| C18:0 iso | 0.036[a] | 0.023[b] | 0.002 | <0.0001 | 0.1306 | 0.042 |
| C18:1 *trans*5 | 0.020[b] | 0.069[a] | 0.005 | <0.0001 | <0.0001 | 0.0004 |
| C18:1 *trans*6-8 | 0.131[b] | 0.379[a] | 0.008 | <0.0001 | 0.0146 | 0.2139 |
| C18:1 *trans*9 | 0.216[b] | 0.410[a] | 0.01 | <0.0001 | 0.2211 | 0.1066 |
| C18:1 *trans*10 | 0.347[b] | 0.699[a] | 0.039 | <0.0001 | 0.8251 | 0.803 |
| C18:1 *trans*11 | 0.920[b] | 1.395[a] | 0.028 | <0.0001 | 0.1388 | 0.0402 |
| C18:1 *trans*12 | 0.318[b] | 0.488[a] | 0.006 | <0.0001 | 0.2391 | 0.9869 |
| C18:1 *cis*9 | 18.280[b] | 22.057[a] | 0.341 | <0.0001 | 0.0497 | 0.9065 |
| C18:1 *cis*7 | 0.240[b] | 0.267[a] | 0.005 | 0.0008 | 0.0062 | 0.2471 |
| C18:1 *cis*11 | 0.480 | 0.462 | 0.014 | 0.4078 | 0.199 | 0.3629 |
| C18:1 *cis*12 | 0.281[b] | 0.315[a] | 0.006 | 0.0001 | 0.0119 | 0.3107 |
| C18:1 *cis*13 + *trans*16 | 0.046 | 0.050 | 0.007 | 0.6672 | 0.0342 | 0.0211 |
| C18:1 *cis*14 + *cis*16 | 0.276[b] | 0.306[a] | 0.007 | 0.0055 | 0.3117 | 0.9813 |
| C18:1 *cis*15 | 0.049[a] | 0.041[b] | 0.002 | 0.0108 | 0.0002 | 0.4895 |
| C18:2 *cis*9*cis*12 | 1.617[b] | 1.851[a] | 0.04 | 0.0001 | 0.8592 | 0.309 |
| C18:2 cis9trans11 | 0.445[b] | 0.688[a] | 0.02 | <0.0001 | 0.7177 | 0.9726 |
| C18:2 *cis*9*trans*12 | 0.024 | 0.030 | 0.002 | 0.0524 | 0.0009 | 0.6671 |
| C18:2 *trans*9*cis*12 | 0.012 | 0.011 | 0.001 | 0.4304 | 0.033 | 0.0012 |
| C18:3 *trans*9*cis*12*cis*15 | 0.014 | 0.009 | 0.002 | 0.1004 | 0.3403 | 0.1129 |
| C18:3 *cis*9*cis*12*cis*15 | 0.426[a] | 0.312[b] | 0.008 | <0.0001 | 0.0062 | 0.0592 |
| C20:0 | 0.154[b] | 0.189[a] | 0.004 | <0.0001 | 0.4203 | 0.0521 |
| C20:1 *cis*11 | 0.025[b] | 0.035[a] | 0.002 | <0.0001 | 0.0006 | 0.0138 |
| C20:2 *cis*14*cis*17 | 0.013 | 0.012 | 0.002 | 0.8241 | 0.4555 | 0.3338 |
| C20:3 *cis*8*cis*11*cis*14 | 0.066 | 0.069 | 0.003 | 0.4632 | 0.4633 | 0.2756 |
| C20:4 *cis*5*cis*8*cis*11*cis*14 | 0.106[a] | 0.093[b] | 0.003 | 0.0029 | 0.0553 | 0.155 |
| C20:5 *cis*5*cis*8*cis*11*cis*14*cis*17 | 0.025[a] | 0.018[b] | 0.001 | 0.0011 | 0.001 | 0.0151 |
| C21:0 | 0.015 | 0.014 | 0.001 | 0.4117 | 0.0003 | <0.0001 |
| C22:0 | 0.036[b] | 0.048[a] | 0.002 | 0.0002 | 0.0694 | 0.3429 |
| C23:0 | 0.167 | 0.245 | 0.045 | 0.3177 | <0.0001 | 0.4251 |
| SFA[6] | 57.866[a] | 53.955[b] | 0.579 | <0.0001 | 0.1456 | 0.3637 |
| MUFA[7] | 24.304[b] | 29.223[b] | 0.373 | <0.0001 | 0.0548 | 0.9099 |
| PUFA[8] | 2.755[b] | 3.115[a] | 0.056 | <0.0001 | 0.6367 | 0.3395 |
| OIAR[9] | 0.514 | 0.501 | 0.005 | 0.1903 | 0.1158 | 0.1101 |
| DI[10] | 0.079 | 0.077 | 0.003 | 0.4864 | 0.0945 | 0.8302 |
| <16:0[11] | 27.126[a] | 23.426[b] | 0.392 | <0.0001 | 0.0487 | 0.4871 |
| >16:0[12] | 59.390[b] | 64.392[a] | 0.421 | <0.0001 | 0.0005 | 0.2512 |

[a,b] within a row, means with different letters are significantly different ($P < 0.05$).
[1] CON = Control diet.
[2] OOPD = Treated diet.
[3] D = diet effect.
[4] T = time effect.
[5] DxT = diet × time effect.
[6] SFA = Saturated fatty acid.
[7] MUFA = Monounsaturated fatty acid.
[8] PUFA = Polyunsaturated fatty acid.
[9] OIAR = ratio odd-iso to ante-iso FA: (iso 15:0 + iso 17:0)/(ante-iso 15:0 + ante-iso 17:0).
[10] DI = desaturation index (*cis*9 14:1/14:0 + *cis*9 14:1).
[11] <16:0, de novo fatty acid.
[12] >16:0, preformed fatty acids.

value observed for the animals fed OOPD seemed to confirm the inhibitory effect of PPs contained in OOP (e.g., hydroxytyrosol and tyrosol) on the biomethane generation as described by Chen et al. (2008). In our trial, neither methanogen nor cellulolytic microorganism abundance decreased when OOP is added to the diet. Hence, the lowering of MPP could be related to a decrease in the microorganism activity after the inhibition of several enzymes involved in methanogenesis since PPs can differently bind proteins (Vasta et al., 2019). Considering that NDF % DEG and C2:0 production were comparable when the two diets were fermented, $H_2$ synthesis was supposed unvaried. Thus, methanogen competitors in using $H_2$ as a substrate could be advantaged by OOP dietary inclusion. At the genus level, OOP positively affected the abundance of the genera *Acetobacter* and *Eubacterium* that normally are thermodynamically less efficient in $H_2$ utilisation as consequence of a less favourable Gibbs free energy (−32.68 kj/mol $H_2$ vs −24.94 kj/mol $H_2$; pH = 7). *Eubacterium* is an ammonia-hyperproducing genus that uses $H_2$ to produce ammonia during amino acid fermentation while *Acetobacter* uses $H_2$ as a substrate during the acetogenesis (Wallace et al., 2003; Kersters et al., 2006). Several authors hypothesised that the decrease in $CH_4$ production could be related to the C18 unsaturated FA (C18:3 *cis*9*cis*12*cis*15, C18:2 *cis*9*cis*12 and C18:1 *cis*9) content, acting directly against the microorganisms involved in $CH_4$ formation (Rico et al., 2016). However, our results did not completely confirm this hypothesis. In fact, OOPD increased C18:3 *cis*9*cis*12*cis*15, C18:2 *cis*9*cis*12, and C18:1 *cis*9 concentrations, the abundances of methanogens and archaea, determined with a DNA-based approach, were not different between RL from animals engaged in this trial. Indeed, this is consistent with the unvaried abundance of methanogens observed in other studies in which OOP was fermented (Mannelli et al., 2018).

The absence of alteration of RL ecosystem by OOP dietary inclusion is also confirmed by the results observed after the characterisation of fungal community. Fungi are able to concur in $H_2$ production during the cellulose degradation contributing to acetate synthesis (Dehority, 2003). In this trial, the addition of OOP to the diet did not change the composition of microbiome community at any taxonomic level. This finding is consistent with the data of NDF %DEG and C2:0 production that are comparable for both diets.

Considering long-chain FA profile, C22:6 *cis*4*cis*7*cis*10*cis*13*cis*16*cis*19 and C20:5 *cis*5*cis*8*cis*11*cis*14*cis*17 percentage was significantly higher when OOP was added to the diet of animals, probably due to a lowering of biohydrogenation processes at the first step of the pathway. In fact, no differences were found in the concentration of the other putative biohydrogenation intermediates of C22:6 *cis*4*cis*7*cis*10*cis*13*cis*16*cis*19 and C20:5 *cis*5*cis*8*cis*11*cis*14*cis*17, identified on the base of Toral et al. (2018).

Among the rumen C18 PUFA, C18:3 *cis*9*cis*12*cis*15, C18:2 *cis*9*cis*12, and C18:2 *cis*9*trans*11 were found increased with OOP dietary inclusion, associated with a C18:1 *trans*11 decrement. Specifically, C18:2 *cis*9*cis*12 biohydrogenation seems to be slowed down either during the *cis*12 isomerisation to C18:2 *cis*9*trans*11 (first step) and during the *cis*9 saturation to C18:1 *trans*11 (second step). Instead, C18:3 *cis*9*cis*12*cis*15 biohydrogenation undergone a slowing down at the *cis*15 double-bound saturation to C18:1 trans11 (third step).

The concentration of C18:1 *cis*9 was higher in RL of animals fed OOPD than CON, as expected because it is the main FA in OOP. However, C18:1 *cis*9 accumulation is probably due also to a decrease in biohydrogenation process rate, in which a wide range of C18:1 *trans* isomers are formed, as observed by Mosley et al. (2002). In fact, no differences were found in *trans*-C18:1 isomer profile between CON and OOPD groups except for C18:1 *trans*11 that decreased.

Several studies showed that PPs act in a different manner on biohydrogenation processes, influencing microbial communities or interfering with enzyme activities (Vasta et al., 2019). However, several authors highlighted the toxic effect of long-chain FAs, such as C20:5 *cis*5*cis*8*cis*11*cis*14*cis*17, C22:6 *cis*4*cis*7*cis*10*cis*13*cis*16*cis*19, C18:2 *cis*9*cis*12, C18:3 *cis*9*cis*12*cis*15, and C18:1 *cis*9 on bacteria. Specifically, C20:5 *cis*5*cis*8*cis*11*cis*14*cis*17 and C22:6 *cis*4*cis*7*cis*10*cis*13*cis*16*cis*19 seem to increase the duodenal flux of PUFAs and long-chain FAs changing the profile of microbial community (Carreño et al., 2019) while C18:2 *cis*9*cis*12, C18:3 *cis*9*cis*12*cis*15, and C18:1 *cis*9 determine a slowing down of microorganism growth (Enjalbert et al., 2017). All these findings about long-chain FAs confirm the selective action of OOP in modulating specific steps of PUFA biohydrogenation. In fact, in the present study, the isomerisation in the 12 positions and the saturation in the 9 and 15 positions of the carbon chain seems to be affected by OOP dietary inclusion.

This hypothesis is consistent with the lower *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* abundances determined by qPCR, both involved in the biohydrogenation process (Dehority, 2003). Another condition that could have affected the biohydrogenation rate was the decrease of *Anaerovibrio lipolytica* abundance. In fact, *Anaerovibrio* genus is responsible of the triglycerides lipolysis that is a prerequisite since this process needs free carboxyl group (Jenkins et al., 2008).

Considering DMA profile, no great variation was observed confirming that OOP did not strongly perturb the rumen ecosystem. The main changes were observed for DMA-C18:1 *trans*11 that decreased and DMA C16:1 that increased during OOPD fermentation. Dimethylacetals are produced from plasmalogens during FA methylation, which is a necessary step for the gas-chromatographic determination of FA. Their structure is characterised by an alkenyl moiety with vinyl ether in Sn1, an acyl group (ester) in the Sn2, and a phosphate moiety in Sn3 position of the glycerol backbone (Alves et al., 2013). Plasmalogens are bacterial membrane components whose profile is strongly affected by environmental conditions and FA content of RL (Goldfine, 2010). Although their role is not well known, they are indicators of the bacterial resilience to changes in the rumen ecosystem, since their profile changes to ensure the fluidity of cell membrane (Minato et al., 1988). The lowering of DMA-C18:1 *trans*11 in RL from animals fed OOPD could be related to the decrease of C18:1 *trans*11 induced by OOP. The increase of DMA C16:1 could be a consequence of the microbial response to maintain the membrane fluidity using another unsaturated FA as a partial replacement of C18:1 *trans*11.

### *Milk fatty acid profile and polyphenols content*

The milk FA profile reflected the effect of OOPD on rumen FA metabolism. In fact, the dietary inclusion of OOP was responsible for the increase of milk C18:1 *cis*9 as recovery from the diet. In addition, milk from cows fed OOPD showed a FA profile characterised by a lower content in medium-chain FAs and a higher content in PUFAs, monounsaturated FAs, and long-chain FAs (carbon chain > 16). In particular, the decrease of medium-chain FAs may be related to the increase of C18:2 *cis*9*cis*12 whose down regulation activity on genes involved in the *ex-novo* syntheses in mammary gland is well known in literature (Zhu et al., 2014). The decrease of C12:0, C14:0, and C16:0 is desirable according to an increase in nutritional value of milk, since these FAs are detrimental for human health (Hadrova et al., 2019). The lowering of biohydrogenation rate at the rumen level in animals fed OOPD reflected the increase of milk C18:2 *cis*9*cis*12 and C18:2 *cis*9*trans*11. In contrast, C18:1 *trans*11, which was found decreased in OOPD RL, increased in milk while C18:3 *cis*9*cis*12*cis*15 increased in OOPD

RL and decreased in milk. All these FAs are considered protective against cardiovascular disease and carcinogenesis and so positive for human health (Minieri et al., 2020).

In contrast, *trans* 10 and *trans* 9 C18:1 isomers are associated with the animal milk fat depression syndrome. In our trial, the concentration of these FAs was doubled in OOPD group with respect to CON. Nevertheless, milk fat depression did not occur, and the milk total fat content was comparable between the two groups. The increase in *trans* 9 C18:1 concentration was due to the higher content of C18:1 *cis*9 in OOPD (Mosley et al., 2002). Considering the *trans*10 *cis*12 C18:2 isomer concentration, one of the main biohydrogenation intermediate involved in milk fat depression syndrome was found in milk only in trace confirming that the condition of rumen environment did not favourite milk fat depression (Ventto et al., 2017).

Alternatively, the high *trans* isomers concentration found in milk of the group fed with OOPD might be linked with the significant decrement of C18:3 *cis*9*cis*12*cis*15. This phenomenon can be linked to an alteration in the biohydrogenation pathways by the PP selective action as reviewed by Vasta et al. (2019).

The higher content of C18:0 in milk from animals fed OOPD with respect to CON was probably due to the highest value of C18:1 *cis*9 in the diet that is biohydrogenated to the correspondent saturated FA form.

No transferring of PP from diet to milk was observed in animals fed OOPD. Despite the study of the possible transfer of PP or their metabolites to the animal tissues, several doubts still exist on the possibility that ruminant physiology allows the transfer of PP from diet to the tissues (Serra et al., 2021). Leparmarai et al. (2019) found a higher total PP content in goat and sheep milk fed a diet with a high PP content. Thus, the presence of PP in cow milk fed OOPD cannot be completely excluded if their concentration was lower than the detection limit of the method used or if the kind of PP used in this study, which are mainly hydrolysable (e.g., hydroxytyrosol and tyrosol), have a less recovery efficiency. Furthermore, the PP metabolism can follow different pathways (Leparmarai et al., 2019). In fact, several PPs may be expelled with the urine or transformed into other derivatives at the rumen level by microorganisms. Since neither the PP concentration in urine nor the presence of PP metabolites have been assessed in this study, further investigations are needed.

## Conclusion

Nowadays, by-products from agro-industry in ruminant feeding are a tool to make more sustainable food chain production. The results of the study suggest that destoned and fresh OOP from a two-phase milling process, with a high PPs and PUFAs content, may be successfully included in the feeding strategy of dairy cows. In fact, OOP dietary supplementation did not lower animal performances, did not compromise the protein and NDF degradability and the biohydrogenation was modulated towards the improvement of C18:1 *cis*9, C18:2 *cis*9*cis*12, C18:1 *trans*11 and C18:2 *cis*9-*trans*11 content increasing the nutritional quality of milk.

## Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100815.

## Ethics approval

Animals were handled according to the guidelines of the Italian law on animal welfare for experimental animals (Ministry of Health, 2014). The body for the protection of well-being has issued the resolution n. 6/2020 approved by the Ethical Committee of the University of Pisa.

## Data and model availability

The nucleotide sequences determined in this study were deposited in the European Nucleotide Archive database under the accession numbers PRJEB51870. The data that support the study findings are available to reviewers, or available from the authors upon request.

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## Declaration of interest

None.

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