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Coordinatore Prof. Carlo Viti

Use of olive oil polyphenols in ruminant feeding as modulators for ruminal fermentations

Studente

Federica Scicutella

Supervisore

Prof.ssa Arianna Buccioni

Coordinatore

Prof. Carlo Viti

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DECLARATION of AUTHORSHIP

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Abstract

The rumen is a large sac located within the first tract of the ruminant digestive apparatus. The main factor affecting the ratio of microbial communities present in the rumen is the diet. Rumen microbiota act on diet ingested by animal that is hydrolysed trough the lipolysis and biohydrogenation processes in which the free fatty acids are formed. In this way rumen FA profile is considered biomarker of rumen ecology. In literature, different feeding strategies have been shown to modulate rumen fermentation and lead to changes in the microbial activities and thus the fatty acid profile of this compartment. Indeed, the presence of specific fatty acids in the rumen can be used as markers of rumen microflora activity. By-products from agro-industrial production rich in polyphenols, such as olive oil pomace, have been employed in ruminant feeding strategies as modulators of rumen fermentation. Polyphenols are functional molecules naturally present in plants, and they exert different antimicrobial activities depending on their chemical structure. Olive oil pomace and olive tree leaves are co-products of the olive milling process, a typical Mediterranean agro-industrial production. The objectives of this thesis were twofold: 1) to evaluate the effects of the dietary inclusion of olive oil pomace on dairy-cow performance by means an *in vivo* trial, and 2) to study how olive oil pomace and olive tree leaves modulate rumen metabolism an *in vitro* 24-h batch fermentation trial.

The dietary inclusion of 8 g/100g DM of olive oil pomace had no effect on dairy-cow performances, but a significant change was found in the rumen fatty acid profile and the microorganism community ratio. Specifically, *Acetobacter, Prevotellaceae_UCG-004, Prevotellaceae_UCG-001, Eubacterium coprostanoligenes, Lachnospira, Acetitomaulatum, and Lachnospiraceae_NK3A20 group* were more abundant in the cows receiving the olive oil pomace (OOP) supplement ($P < 0.05$). The stochiometric relationship between the production of different volatile fatty acids in the rumen suggests that olive oil pomace lowers the methane potential production (control diet = 0.050 mol/L vs experimental diet = 0.024 mol/L, SEM =

0.005, P = 0.0011). Moreover, the milk’s nutritional quality was improved by increasing several important functional fatty acids, specifically linoleic acid, conjugated linoleic acid, oleic acid and vaccenic acid.

The *in vitro* trials showed olive oil pomace and olive tree leaves, to modulate the microbial community in a selective manner. Olive oil pomace increased the content of oleic acid and linolenic acid in the rumen liquor. Concerning the microbial communities, the genera *Butyrivibrio*, *Fibrobacter* and *Pseudobutyrivibrio* were less abundant in rumen liquor fermented with olive oil pomace, whereas *Christensenellaceae_R-7_group*, *Manheimia* and *Uruburuella* were more abundant. Similarly, the diet containing olive tree leaves increased the content of oleic acid and linolenic and decreased the abundance of *Pseudobutyrivibrio* as well as the *Rikenellaceae_RC9_gut_group* in rumen liquor fermented.

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Acronyms

Acid detergent fibre, ADF; Biohydrogenation, BH; Conjugated linoleic acid, CLA; Dimethyl acetal, DMA; Dry matter, DM; Fatty acid, FA; Linoleic acid, LA; Linolenic acid, LNA; Lipolysis, LP; neutral detergent fibre, NDF; Oleic acid, OA; Polyphenol, PP; Polyunsaturated fatty acid, PUFA; Rumen liquor, RL; Solid associated bacteria, SAB; Unsaturated fatty acid, UFA; Vaccenic acid, VA; Volatile fatty acid, VFA.

Introduction

1. Rumen microbiota

The rumen is a large sac located in the first tract of the ruminant digestive apparatus (Figure 1).

Located in the left side of the abdominal cavity, its volume varies according to the species.

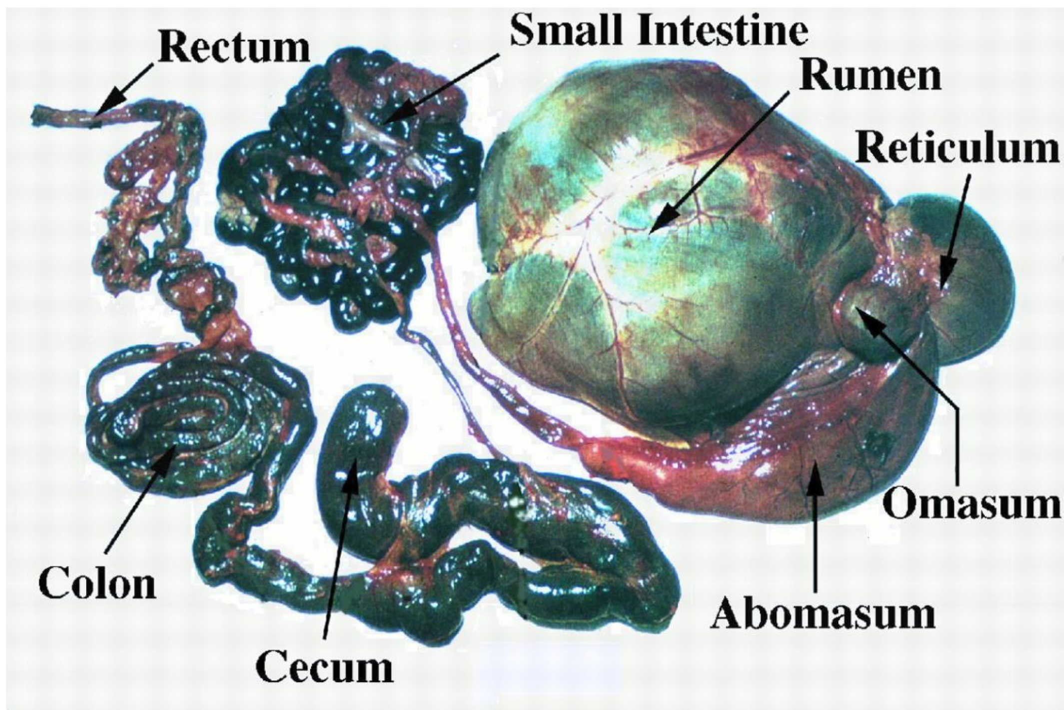


Figure 1. Ruminant digestive tract, from Russell et al. (2001).

The main activity of the rumen is the initial degradation of nutrients. Preceded by oral chewing and the enzymatic activity of the saliva, nutrient degradation occurs thanks to the action of the rumen microbiota, which is responsible for hydrolysing cellulose and hemicellulose, thus releasing glucose for energy purposes.

Several factors affect the ecology of the rumen microflora, including ruminant species and breed, the feeding behaviour and animal management strategy, geographic area, and other environmental inputs. Nevertheless, the predominant bacteria species remain ubiquitous, but at different relative abundances (e.g., *Prevotella*, *Butyrivibrio*, and *Ruminococcus*), while archaea differ only in relation to the methanogen community composition, and protozoa are frequently dissimilar (Henderson et

al., 2015). The diet is the main factor affecting the profile of microbial communities present in the forestomach (Agarwal et al., 2015). A diet with a high forage content increases *Batteroidales* and *Ruminococcaceae* abundances, whereas a low forage content diet enhances *Prevotella* and *Succinivibrionaceae* abundances (Agarwal et al., 2015). The host animal species also influences the rumen microbiota ratio; for example, the *Fibrobacter* genus is more abundant in cattle than in sheep, deer, or camels.

The rumen microflora is mainly anaerobic and made up of Prokaryota (bacteria and archaea) and Eukarya (protozoa and fungi). Specifically, 10^{10} bacteria, 10^{7} protozoa and 10^{-6} fungi and yeasts per mL of rumen liquor (RL). Bacteria are distributed over the feed particle surface (e.g., cellulolytic and hemicellulolytic bacteria) or suspended in the liquid (e.g., amylolytic and proteolytic bacteria), or otherwise attached to the rumen wall on the papillae. Protozoa are mainly present in the liquor and are predominately represented by ciliates, which act as low-efficiency fibre degraders (Choudhury et al., 2015). By contrast, fungi are high-efficient fibre degraders which can also partially degrade lignin tissues (Agarwal et al., 2015). Whilst they born in the rumen, 90% are found in the rumen-reticulum tract, with the remaining 10% distributed equally across the rest of the gut. Both synergic and mutual relationships exist between the different microbial communities, making them difficult to the study by means of pure cultures. One example is the tight relationship between bacteria and protozoa, whereby the former generates the substrate upon which the latter grows (Henderson et al., 2015).

Bacteriophages also inhabit the rumen, and their presence controls bacteria biomass turnover due to their ability to destroy the bacterial cell wall (Choudhury et al., 2015).

The standard environmental conditions in the rumen are a temperature of about 38–39°C, a pH range of 6.0–6.7, and a redox potential of -150-350 mV. When these values change, the activity of the rumen microflora is modulated and, by consequence, the products of fermentation are affected. For example, *Streptococcus bovis* is a starch and sugar degradant producing lactic acid that affects environment pH. Increased *S. bovis* activity reduces the pH and, in turn, the growth of rumen

microorganisms that cannot tolerate acidic conditions (Choudhury et al., 2015). Moreover, archaea and protozoa are less versatile than bacteria (Henderson et al., 2015).

Rumen microbial community ratio depends on the diet and microorganisms exert their activity trough enzymes that are species-specific, even if different microorganisms may be involved in the same pathways across species. Pathways involving microorganisms can be ATP production, glycolysis, lipolysis (LP), or biohydrogenation (BH), such examples.

For instance, methanogens produce methane using H_2 to transform CO_2 into CH_4 and produce ATP, whereas acetogens reduce CO_2 to acetate, and sulphate-reducing bacteria produce H_2S. There are also bacteria which transform fumarate into succinate, i.e., decarboxylate into propionate (Choudhury et al., 2015).

Several bacteria are capable of *de novo* fatty acid (FA) synthesis and use carbohydrates as precursors. Others can release FAs from lipids through LP or transform unsaturated FA (UFA) into saturated FAs via the process of BH. Thus, FAs stored in animal tissues or milk fat offer a source of energy, affecting the quality of the fat profile of end products destined for human consumption (Destaillats et al., 2005; Akraim et al., 2007).

Fifty percent of milk FAs are synthesized in the mammary gland from acetate and beta-hydroxybutyrate obtained from the blood, whereas the remaining 50% is determined by the animal's diet (FAs with long carbon chains > 16), and/or by bacteria metabolism (odd-branched FAs) (Figure 2, Chilliard et al., 2007).

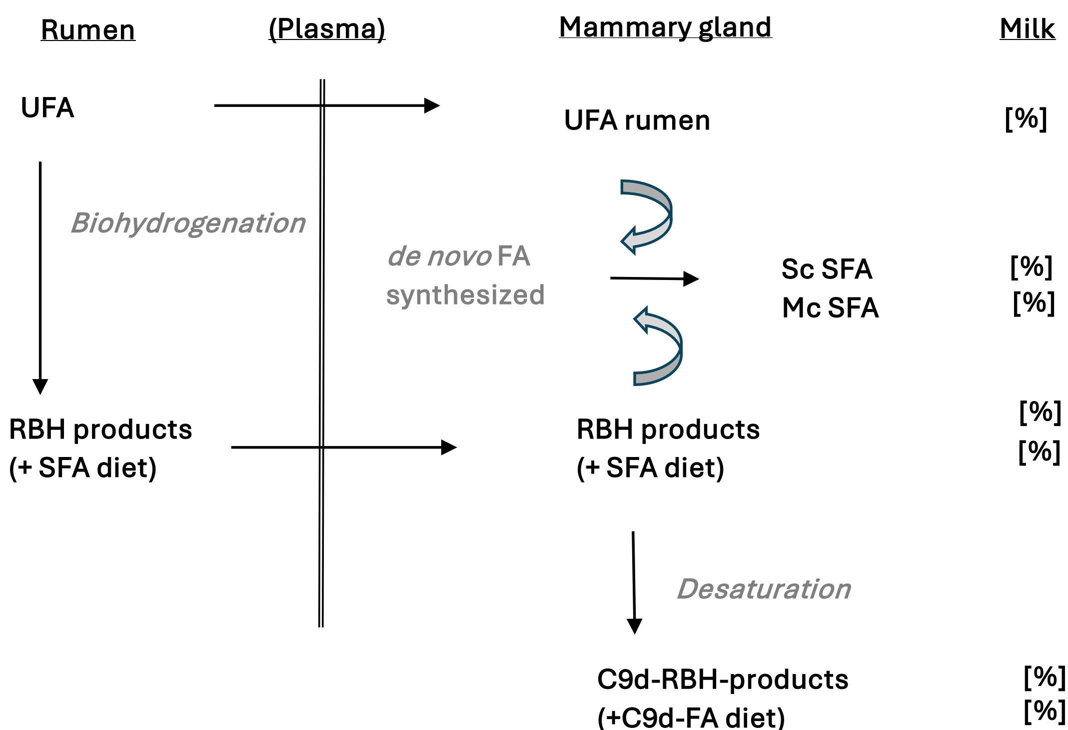


Figure 2. Rumen metabolic pathways producing milk FAs. UFA = unsaturated FA, SFA = saturated FA; Sc = short-chain, Mc = medium-chain; c9D: cis9-desaturase; [%] = changing of FA in milk (g/100 g total FA) (adapted form Chilliard et al., 2007).

Biomarkers of rumen microflora activity have been studied to investigate changes in the microbial community ratio, and thus to assess how rumen fermentation modifies. Odd- and branched-chain FA and dimethyl acetals (DMA) are two such biomarkers. The iso and ante forms of C13/15/17 are lacking in feed ingredients and are solely produced by the rumen microbiota. The iso forms are related to cellulolytic activity, while the ante forms are related to amylolytic activity (Fievez et al., 2012). Instead, the rumen profile of plasmalogens – membrane components of anaerobic bacteria – is strongly affected by environmental conditions and the FA content of the RL (Goldfine, 2010; Alves et al., 2013). Plasmalogens are used as indicators of the bacterial resilience to changes in the rumen ecosystem since their profile changes to ensure the fluidity of the cell membrane (Minato et al., 1988). They form 30–40% of the cell membrane phospholipids in rumen bacteria, and up to 70% of those in protozoa (Saluzzi et al., 1995; Hook et al., 2012).

2. Lipolysis and biohydrogenation

Lipolysis is the process in which glycerol esters are saponified, releasing free FAs and glycerol. Biohydrogenation, on the other hand, is the process through which the double bonds in UFA are reduced, producing the saturated forms of free FAs. Lipolysis is a pre-requisite for BH because hydrogenating bacteria need the carboxylic moiety to be free in order for enzymatic reactions to take place.

The role of microbiota in LP or BH has been identified only for few microorganisms. Several *Anaerovibrio* strains are involved in the hydrolysis of triglycerides, while strains of *Butyrivibrio fibrisolvens* use glycerine as a growth factor that is produced from the hydrolysis of phospholipids (Buccioni et al., 2012). The role of protozoa has yet to be identified, and yeasts seem to participate in the BH of several FAs, for example, the BH of linoleic acid (LA, C18:2 cis9cis12) into vaccenic acid (VA, C18:1 trans11) (Nam and Garnsworthy, 2007).

Diet quality affects LP and BH rates by modulating the ratio between the microbial communities involved in the various steps of these two processes. A low forage diet increases the total amount of bacteria from 0.6 to 1.6 logarithmic units (Latham et al., 1972), and it is associated with a cellulolytic triglyceride hydrolysis rate of about 5.8 %. By contrast, a high forage diet allows 87.6 % of triglycerides to be hydrolysed.

The relative proportions of diet ingredients and their physical form also affect the activity of the rumen microbiota. For instance, oil or fat added to the diet are quickly absorbed from feed particles and the lipolytic activity of microorganisms becomes saturated. Instead, a low fibre diet reduces the abundance of protozoa, even though their relative activity rises by 28 % to 34.9 %. In relation to the physical form of feed particles, small-sized particles transit in a fast manner through the rumen, lowering the exposition time of feed to microbial activity and, consequently, the LP rate (Buccioni et al., 2012).

The first hypothesis formulated to explain the steps of BH of C18 carbon chains (Figure 3) was that by Harfoot and Hazelwood (1997), based on the presence of two main microbial groups involved in

BH steps, nominated group A and group B. These authors affirmed that microorganisms from group A, such as *Butyrivibrio fibrisolvens*, are responsible for the hydrogenation of LA and linolenic acid (LNA, C18:3 cis9cis12cis15) to VA. In the first step, the cis12 position is isomerized to trans11, followed by the saturation of the cis9 position. By contrast, the hypothesis foresees that microorganisms from group B are involved in the last hydrogenation step, that of VA to C18:0. The rate of this reaction is low, and it represents the rate determining step of the whole BH process, which is also what permits VA to pass from the rumen into mammary tissue via the blood (Figure 3). The literature points towards *Butyrivibrio proteoclasticus* as the main bacterial component of group B (Kopečný et al., 2003; Van De Vossenberg and Joblin, 2003; John Wallace et al., 2006; Moon et al., 2008). Recently this theory was overthrown, however, since more bacteria genera were demonstrated to be involved in the same metabolic pathways, such as *Prevotella* and *Lachnospinaceae*, as well as other unclassified bacteria, such as *Bacteroides*, *Clostridiales* and *Ruminococcaceae* (Buccioni et al., 2015).

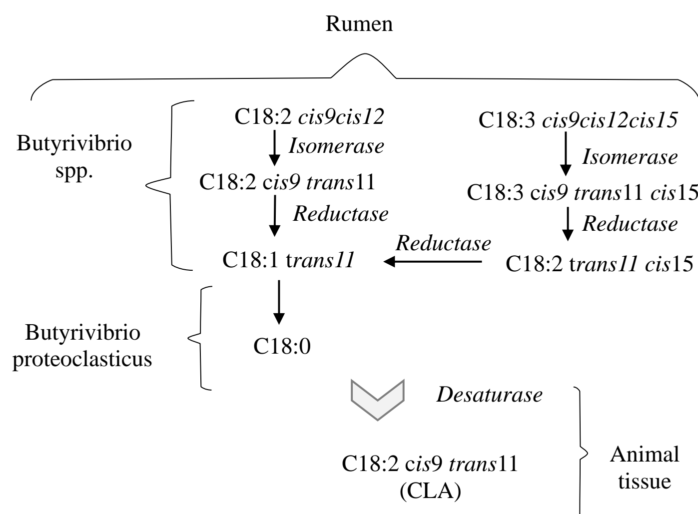


Figure 3. Biohydrogenation of C18:2 cis9cis12 and C18:3 cis9cis12cis15.

3. Strategies for the modulation of rumen fermentation.

As previously mentioned, the rumen microbiota is important for the production of high-quality animal products and it plays a key role in determining the sustainability of production supply. Several feeding strategies have been used to modulate rumen fermentation in order to increase the content of functional molecules in milk and meat, using animals as natural bioreactors.

At first, antibiotics, used as antimicrobials, were applied as modulators of the rumen microbiota. After their prohibition as growth promoters in ruminant species, they were replaced with other natural antimicrobials, such as organic acids. Among these, malate and fumarate are naturally present in animal tissues as intermediates of the tricarboxylic cycle and in the rumen as intermediates of propionic synthesis. However, their dosage needs be controlled carefully since they can lower the pH (Asanuma et al., 1999; Liu et al., 2009). For this reason, malate and fumarate are administered in the salt form so that their release in the rumen is slow, resulting in a reduced variation of the pH; however, this increases the cost of these substances, making them less convenient in economic terms (Carro and Ungerfeld, 2015). Alternatively, ionophores were then considered as antimicrobials, and dietary supplementation was found to be highly efficient at improving performance as well as modulating methane emission and the excretion of nitrogen. However, this practice was also prohibited in Europe because of their antibiotic action.

Feeding strategies have also been employed to improve the quality of animal derived products through their influence on rumen fermentation, such as dietary supplementation with natural components produced by insects or plants as defence molecules. For example, propolis is rich in flavonoids, which can reduce the rate of BH and decrease? nitrogen metabolism through their positive effect on the growth of ammonia-producing bacteria (e.g., *Peptostreptococcus spp. D1* and *Clostridium aminophilum;* de Aguiar et al., 2014). Tannins are other natural compounds which have been studied for their ability to modulate rumen fermentation. They are polyphenols (PPs) and classified as condensed or hydrolysed depending on their chemical-physical properties, such as

water solubility. Tannins are secondary metabolites produced by plants in response to pathogens. When added to ruminant feed, their interaction with microorganisms is related to their chemical structure (Costa et al., 2017). Fish oil and micro-algae have also been evaluated as feed ingredients even if their application is limited since they confer a bad odour to meat and milk and are responsible for "milk fat depressed syndrome" by favouring the synthesis of trans10-FAs responsible for the down-regulation of genes involved in fat synthesis in the mammary gland (Chow et al., 2004; Boeckaert et al., 2007; Or-Rashid et al., 2008). Over the last few years, the employment of agro-food wastes in animal feeds has been considered, which supports the principles of circular economy (educe, reuse and recycle). Most food by-products are edible, and their nutritional composition is characterized by the presence of functional molecules (e.g., PPs and UFAs) that can modulate rumen fermentation, reduce gas emission and improve the quality of animal products (Ianni and Martino, 2020). In fact, in addition to modulating bacteria activities, they were demonstrated to influence the protozoa which compete with archaea for H_2, thus, affecting methane production (Castagnino et al., 2015). In addition, the employment of these kinds of resources in animal diets offers an important element in dealing with the sustainability of this production chain due to feed vs food competition. The increment in the world population expected for 2050 is leading to the breeding of ever greater numbers of animals, the feeding of which competes with the food supply for humans. Thus, using by-products from the agro-food industry limits the land cultivation employed to produce animal feed (van Huis, 2017).

4. Olive oil by-products and the application in animal feeding

The olive tree (*Olea europaea* L., 1753) originated in Asia, where its cultivation commenced in an undefined age (Grigg, 2001). The species is sensitive to low temperatures, but it can also be cultivated in mountainous areas if the climate is favourable. Its fruit production increases after about ten years and can continue for centuries.

The main scope of olive tree cultivation is related to olive production, which are primarily destined for milling to produce oil. Olive oil production in Europe is concentrated in the Mediterranean area. In the last twenty years, two billion tons of olive oil were produced in Europe, with Italy alone generating 520 million tons, being the second largest producer in the world after Spain (data from FAOSTAT, 26 September 2023).

The chemical profile of olive oil is beneficial for human health due to its high content of polyunsaturated FAs (PUFAs), such as oleic acid (C18:1 cis9, OA) that exerts antioxidant activity on low-density lipoprotein (LDL). Other health promoting components are water-soluble PPs, namely hydroxytyrosol and tyrosol that exert antioxidant and anti-inflammatory properties, helping to protect against cardiovascular diseases (Araújo et al., 2015; Medeiros-De-Moraes et al., 2018; Neofytou et al., 2020; Tzamaloukas et al., 2021).

The production chain of olive oil must manage several waste-products, including the olive tree leaves gathered post-harvesting and post-milling and olive pomace. Leaves generate a cost for farms since they need to be disposed of, and their burning is discouraged due to the consequent production of greenhouse gases (GHG; Berbel et al., 2018). Olive pomace is alternatively used as a fertilizer in agronomic practices or as a source of organic matter for biodigesters. In the Mediterranean area, animal grazing is normal practice in areas also dedicated to olive tree cultivation. In fact, goats and sheep, as well as other farmyard animals, are frequently reared in olive groves. Indeed, this practice has become well-suited to modern rearing systems such as Agroforestry management, thus meeting the need to reduce land area dedicated to animal breeding.

From this perspective, abandoned areas could be newly evaluated for such purposes, with the same field being used for different functions contemporaneously. Moreover, considering the potential for the bioactive compounds present in olive tree leaves to modulate rumen fermentation, feeding ruminants olive by-products offers an attractive strategy, and rearing ruminants in olive groves stands to be doubly advantageous.

In accordance with the circular economy and the principles of the 3Rs (reuse, reduce and replace), olive oil pomace and post-milling olive tree leaves have been evaluated for their use in animal feeding in order to exploit their chemical and nutritional properties and reduce both olive oil production costs and environmental pollution, and to improve the animal production chain. These by-products are rich in PPs as well as PUFAs. As reported by Scicutella et al. (2021), PPs exert antimicrobial activity against different microorganisms inhabiting the animal digestion tract. Briefly, phenols interact with sulfhydryl groups or with protein. Flavonoids bind to extracellular and soluble proteins or bacterial cell walls, and may also destroy membranes. Tannins deactivate microbial adhesins, enzymes and cell envelope transport proteins, and they complex with polysaccharides. Hydrolysed tannins are more efficient in penetrating membranes because of their lower molecular weight with respect to condensed tannins.

Several papers have considered the employment of olive oil pomace or olive tree leaves in animal feed as described in the next section; however, the literature lacks information with regard to their effects on rumen metabolism investigated using multifunctional approaches. Moreover, the techniques used to obtain and treat post milling by-products destined for animal feed have recently been updated (Pallara et al., 2014). Thus, investigations into the new feeding strategies integrated with alternative ingredients obtained using these updated technologies are needed, and their effect on rumen metabolism must be subjected to more in depth assessment.

Both *in vivo* and *in vitro* approaches to evaluate the effects of olive pomace and olive tree leaves on the rumen metabolism were applied even if a multifunctional approach is rarely found. The

identification of consist findings by two approaches would strengthen results obtained and the understanding on rumen ecology should be deepen.

4.1. Olive oil milling process and waste description

The olive oil production procedure involves several steps (Angelini et al., 2009). First, olives are mechanically deprived of any attached leaves and washed. Second, mechanical milling is performed, resulting in the "olive cake" which comprises a solid and a liquid phases. Then, the liquid fraction is separated from the olive cake in the press, a slow process lasting 30–40 min and performed within the temperature range of 25–27°C (for cold extraction). This cold extraction procedure is the most common adopted since the quality of the resulting oil is higher. Otherwise, a temperature range of 27–30°C permits a greater quantity of oil to be extracted, but its quality is poorer. At this point, the extracted liquid is usually transferred to a centrifugal decanter to remove the water present, the weight difference of which favours oil extraction. The centrifugal decanter consists of a steel barrel, and the oil's affinity for steel also plays a key role in the separation process.

The waste products generated during the milling process vary depending on the decanter procedure used. The oldest extraction system, referred to as a "three-phase", produces three fractions: pomace, oil must with a low water content, and wastewater with a low oil content. The "two-phase" procedure was then developed, which required the use of less water (important for reasons discussed below) and which produced only two fractions: pomace with wastewater and oil must with a low water content. The most recent upgraded system is the "two-and-a-half-phase" system, in which wet pomace, wastewater and oil must are generated using only a moderate quantity of water.

An important issue of the olive oil milling procedure is linked to the disposal of its waste products. Indeed, olive oil extraction is considered highly polluting due to the high PP content of its waste products. These PPs are water soluble, thus the greater the quantity of water used in the procedure,

the greater the PP content there is to deal with. For this reason, new systems have been developed over recent decades with the scope of reducing the quantity of water used. In addition, to avoid the problems linked with PP disposal, waste products have been applied in other production chains to help make olive oil production more sustainable. Stoner machines are commonly used to deprive pomace of the olive stones, which are then used in pellet production for room heating. Wet pomace or wastewater are often used as fertilizer in agronomic practices. Pomace is also used as a substrate in biogas digesters.

4.2. Polyphenols from olive oil pomace in animal feeding

Thanks to the antimicrobial properties of PPs contained in olive oil pomace, its employment in animal feeding is now being studied in the Mediterranean area. However, olive pomace is mainly used in the dried form since its water content makes it highly perishable, an issue of particular concern in the feeding industry. Whilst drying is the most common preservation method used, thermal treatment causes the oxidation of both PPs and PUFAs, lowering the nutritional and biological value of olive pomace (Neofytou et al., 2021).

4.2.1. Ruminants

The use of destoned, fresh olive oil pomace in the ruminant diet is desirable to increase the animals' dietary intake of PPs and PUFAs, which play an important role in rumen metabolism (Cappucci et al., 2018; Mannelli et al., 2018). Polyphenols can interfere with the enzyme activity of rumen microorganisms involved in LP and BH, processes which influence the characteristics of the metabolites delivered to the tissues through the digestion process, determining the quality of the end-product (milk or meat).

Studies have investigated the effects of feeding destoned olive pomace from two- and three-phase (13 and 11 % on dry matter (DM), respectively) extraction processes to dairy sheep (Mannelli et al., 2018; Cappucci et al., 2018). The dietary inclusion of olive pomace had no effect on milk

production. Olive pomace from three-phase extraction led to an increase in rumen acidity, and two-phase olive pomace enhanced the content of alfa-LNA in the rumen. Indeed, the milk PUFA profile was improved in both experimental diets; however, two-phase olive pomace was more efficient at increasing the UFA content in milk, particularly OA and LNA. The authors emphasized the effect of the different oil extraction methods which had differential effects on the quantity of PPs in the olive pomace, which act as modulators of rumen fermentation.

Another study assessed increasing levels of olive crude phenolic concentrate (0, 0.6, 0.8 and 1.2 % on DM) in dairy sheep feed, and highlighted the effects of PPs on rumen BH, specifically in levelling down the processing speed and increasing LA and LNA concentrations in rumen liquor (Cappucci et al., 2018), effects that were previously observed in *in vitro* tests by Pallara et al. (2014), and confirmed in the FA profile of the sheep's.

Chiofalo et al. (2020) studied the effects of partially destoned olive cake powder, obtained by means of "two-and-a-half-phase" extraction, added at a dietary inclusion level of 7.5 or 15 % in Limousin young fattening bulls, affecting the meat quality indices (meat tenderness, intramuscular fat and FA profile). Others investigated the effects of adding non-destoned dried olive pomace to dairy cow feeding (10 % on DM) on the nutritional and aromatic properties of milk and cheese (Castellani et al., 2017). Milk protein was affected by the inclusion of the pomace, presumably due to its interactions with the microbial community at the rumen level. The concentration of conjugated LA (CLA) increased in both milk and cheese, bringing about an improvement in their quality. Raw milk differed from pasteurized milk in the volatile aromatic components, and the pomace inclusion improved the volatile profile of both. In the study by Chaves and colleagues, non-destoned olive pomace was added to dairy cow feed at 5, 10, 15 % on DM. The authors observed no differences between control and treated groups in terms of performance parameters, such as milk yield and composition, somatic cell count and milk urea nitrogen, confirming that the level of inclusion of olive pomace in the diet was great to guarantee the nutritional requirements of energy and protein that meet dairy cow post medium milk lactation (Chaves et al., 2020).

4.2.2. Monogastric

Olive pomace has also been added to rabbit feed with the aim of improving meat quality. Rabbit meat is a high-quality protein source, but its high moisture content increases its oxidative status and favours bacterial contamination. Thus, enhancing the level of functional compounds in the meat offers a way of improving its storage and quality. Olive mill vegetation water phenol metabolites were tested at feed inclusion levels of 150 mg/kg and 280 mg/kg (Branciari et al., 2021). The study found no effect on the proximate profile of rabbit meat but a shift of both tyrosol and hydroxytyrosol to their sulphite forms at the higher supplementation level, as well as a reduction in the lag phase process for *Pseudomonas* spp. during 12 days of storage. Furthermore, the antioxidant activity of olive pomace was studied in rabbit (Bakeer et al., 2022). The added value obtained from supplementing rabbit feed with olive oil pomace is related both to its high fibre content that promotes equilibrated digestion and to the presence of polar lipid such as glycerylether-sn-2-acetyl glycolipid, known to inhibit atherosclerosis receptor (Tsantila et al., 2007). However, the effects observed on animal production vary depending on the pomace source, for example, the olive cultivar. One study compared the effects of a diet supplemented with olive pomace obtained from a mixture of olives from the cultivars Coratina, Moraiolo, Frantoio and Ogliarola with two other diets supplemented with pomace obtained from either Coratina and Frantoio cultivars only (Dal Bosco et al., 2012). Pomace obtained from the Coratina cultivar alone, with stones and extracted via the three-phase oil process, had a specific effect on rabbit meat production. In particular, the higher content of hydroxytyrosol and verbascoside improved the oxidative stability of meat, and the higher OA content was associated with a reduction in the atherogenic and thrombogenic meat indexes. In this case, the hemicellulose portion slightly levelled down rabbit performance because feed intake deficiency.

The positive effects of the inclusion of olive pomace in animal feed have also been highlighted in the field of poultry nutrition. For instance, Ross 308 broilers were fed a diet supplemented with two

different levels of olive pomace inclusion (82.5 g/kg and 165 g/kg; Branciari et al., 2016). At the higher inclusion rate, feed intake increased but the feed conversion rate was reduced. Interestingly, the PPs in the feed were not found in the meat, but their metabolites such as the sulphite forms of hydroxytyrosol could be detected in the meat. As observed in other species, the antioxidant activity of meat was improved. The antioxidant activities of PPs do not change in relation to the use of milling processes. In fact, lower protein oxidation and lipid peroxidation and higher total antioxidant capacity in plasma and tissues were also detected in broilers using olive mill wastewater permeate or retentate (Gerasopoulos et al., 2015).

In pigs, greater levels of functional compounds such as UFAs are needed in feeds to improve the meat quality. Olive pomace is a suitable candidate to achieve this, and when destoned olive pomace is used, its lignin content is also limited, improving the digestibility of the feed, as similarly shown in other species. One study analysed the effects of feeding pigs a diet supplemented with 50 or 100 g/kg of olive pomace on DM during the fattening period (Liotta et al., 2019). The 50 g/kg inclusion level showed the best results, resulting in more weight gain and feed intake as well as being associated with the lowest feed conversion rate. The higher inclusion level resulted in less intramuscular fat, but the quality of the fat was improved, with reduced levels of saturated FAs and greater concentrations of OA. Similarly, Iberian pigs fed olive pomace composed of skin, pieces of olive stones and a small fraction of dried (45%) or wet (75%) olive oil, as silage, during the growing period, where no alteration of the growth and slaughter traits studied occurred (García-Casco et al., 2017). In another study, in which 5% (as- fed basis) of olive oil pomace was added to the pig diet during the grower-finishing period, the FA profile of the meat was improved with higher monounsaturated and lower saturated forms and no negative effects on performance (Verge-Mèrida et al., 2021). The same study showed that the acid oils from olive pomace differentially affected meat quality compared with the crude oil form because of the latter's higher content of free FA, moisture, impurities and unsaponifiable matter. The authors of this trial only observed an improvement in FA utilization when the acid form of olive pomace was used in combination with

palm oil (50:50), probably due to a synergic action of saturated and UFAs or due to the higher amounts of diacylglycerols or monoacylglycerols that increase free FA digestibility, since emulsifying improves the inclusion of free FA in mixed micelles.

4.3. Polyphenols from olive tree leaves in animal feeding

Olive leaves have a high content of oleuropein and hydrolysable PP, as well as OA, LA and LNA, but the quality of the fibre in the leaves is much lower compared with that of olive oil pomace (Bahloul et al., 2014; Habeeb et al., 2017; Selin¸sahin and Bilgin, 2017). In addition, olive leaves are rich in bioactive molecules, including the vitamins tocopherols and β-carotene.

Modern mechanical harvesting methods have led to an increase in the quantity of leaves being removed from the tree during olive collection. Annual pruning activity can also generate a waste of up to 25 kg of olive leaves per tree, and a further kg of leaves is collected post-milling (Mattioli et al., 2018).

Olive tree leaves are also likely to be subjected to chemical treatment during their lifetime to improve production, and such treatments might be based on selenium. In animal nutrition, feed additives involving Vitamin E or selenium have been studied because of their antioxidant activities, and a synergic action between the two has also been proposed (Skřivan et al., 2012). Selenium favours Se-dependent glutathione peroxidase activity, which catalyses the reduction of hydrogen peroxide and organic peroxides, and Vitamin E acts on lipid peroxidation.

4.3.1. Ruminants

Olive tree leaves are seasonal by-products and highly perishable, so they need to be conserved. *In vitro* trials conducted to study the chemical composition, rumen degradability and intestinal digestibility of olive tree leaves found air-drying in the shade to be the best conservation method, as it helped the leaves to maintain their nutrient qualities (Martìn-Garcìa et al., 2008).

Olive leaves were fed to male kids at 7.5 or 15 % as an alfa-alfa hay substitute (Hukerdi et al., 2019). Animal performance and carcass traits were not affected by the inclusion of olive leaves. Antioxidant activity in the liver, longissimus muscle and plasma were improved at the 15 % inclusion level, particularly with regard to glutathione peroxidase activity. The effects of including dried olive leaves added to the feed concentrate, replacing 28 % of alfa-alfa hay, was also investigated in dairy sheep (Bolletta et al., 2022). The inclusion led to an increase in cheese yield; in fact, both protein and fat milk were higher than in the control bulk (5.83 vs 5.64, P = 0.001 and 6.94 vs 6.39, P = 0.046, respectively). The cheese was characterized by the high level of PUFAs, and the ingestion of bioactive molecules present in the leaves averted the cheese's deterioration. The FA profile of cheese was improved since saturated forms were less representative, even if their concentrations were higher in both treatments with respects to the control diet. Interestingly, C18:0 was the most abundant saturated FA, which is responsible for atherogenic plaques in the blood vessel, but the human metabolism is able to transform C18:0 into OA. Moreover, the h:H, atherogenic and thrombogenic indices were all lower in cheese from sheep receiving the olive tree leaves in the diet.

Another study supplemented the diet of sheep with olive branches obtained from pruning (sun dried for 3/4 d) and olive cake including peel and stones (oven dried at 40°C for 4 d) at an inclusion level of 30 % on DM (Abbeddou et al., 2011). The authors highlighted a detrimental effect of olive leaf and olive cake on milk yield of young sheep that was greater than that observed in older sheep. The effect may be caused by the high acid detergent fibre (ADF):neutral detergent fibre (NDF) ratio and PP content of olive leaf and by the high fat content of olive cake. Despite the high PP content of olive leaves, the antiradical activity of milk was reduced in the animals receiving the supplemented diet, whilst PUFAs and LNA were increased. Finally, whilst supplementation with olive cake improved the overall fat content of the diet and increased the levels of long chain FAs (mainly OA), it led to a decrease in the milk concentration of short- and medium-chain FAs.

4.3.2. Monogastric

Improving the FA profile of meat is beneficial for humans, but it could have a detrimental effect on meat characteristics and shelf-life due to FA oxidation. The use of selenium-treated olive tree leaves can be useful in this respect. Indeed, not only does selenium treatment improve the resistance of olive trees to water stress, but when rabbit feed was supplemented with these leaves it was found to exert benefits on the animals by increasing the lipid antioxidant activity of the meat (Mattioli et al., 2018, 2020). Selenium doses < 0.10 mg/kg of feed did not bring about any benefits. The inclusion of 10 % olive tree leaves in fattening rabbits can substitute 7 % alfa-alfa hay in the diet without any negative effects on performance, although the values of interscapular and perivisceral fat were reduced, the n-6 series of PUFAs were less abundant and the OA content was higher in meat from rabbits receiving 7 % Se-treated olive leaves in the diet compared with the control group.

Olive tree leaf powder is rich in oleuropein, which decreases its water activity and reduces the enzymatic breakdown of PPs than the fresh leaves. The undigestible fibre content of olive tree leaves reduced the digestibility of feed supplemented with 5 or 10 % olive tree leaves in pigs (Paiva-Martins et al., 2009). However, an effect of oleuropein may also be at work, because although oleuropein activates pepsin, it may also inhibit other digestive enzymes such as trypsin, glycerol dehydrogenase, glycerol-phosphate dehydrogenase, glycerol kinase and lipase. The physical characteristics of the meat were not affected by the dietary inclusion of olive tree leaves, but overall performance and meat quality were diminished. The FA profile of the meat was not altered by diet treatment and only the oxidative stability was improved.

5. The comparison of *in vitro* versus *in vivo* trials: when replacement is possible.

Scientists have long carried out *in vivo* experiments with the aims of advancing knowledge, improving the livestock production chain to increase food quality, and reducing the environmental impact due to the emissions or the exploitation of natural resources. Our understanding of

gastrointestinal physiology has helped to develop *in vitro* techniques for studying certain segments of the digestive tract and the fermentative and digestive characteristics of feeds (Krishnamoorthy et al., 2005). *In vitro* trials are becoming increasingly common, which spares – whenever possible – the use of surgically modified animal models or other forms of experiments with animals (Brooks et al., 1954; Tilley and Terry, 1963; Moore et al., 1962).

Despite the limitations of *in vitro* studies, useful information has been obtained through their use about feed fermentation (Krishnamoorthy et al., 2005). They permit evaluations in standardized conditions that can be conducted over a brief experimental period with lower costs and the need for much less substrate compared with *in vivo* trials (Purba et al., 2020; Tassone et al., 2020). Additionally, *in vitro* experiments permit: the testing of additives in dietary concentrations that would not be tolerated *in vivo* (Deitmers et al., 2022); the carrying out of screening studies before testing *in vivo* (Yáñez-Ruiz et al., 2016); the study of specific physiological mechanisms of a microbial species or the efficacy of a new rumen-protected product (Vinyard and Faciola, 2022); and the acquisition of quantitative data on methanogenesis to insert into models predicting the response and environmental impact of ruminants (Krishnamoorthy et al., 2005). Moreover, since *in vitro* trials do not require the use of surgically-modified animals, is not subordinate to most of the ethical implications linked to animal welfare which characterize *in vivo* trials.

5.1. Rumen liquor sampling: origin and application

The current most common approaches for collecting RL involve a rumen cannula or an oro-esophageal tube (Ramos-Morales et al., 2014). Alternatively, rumen content can be collected at the slaughterhouse.

The oro-esophageal probe is a tube, the size of which varies depending on the ruminant species, which must be inserted down oesophagus, via the oral cavity, until the level of the rumen where a sample of rumen liquor can then be collected (Ramos-Morales et al., 2014). This technique is rapid and relatively un-invasive for the animal, but some considerations should be made before its use.

First, an expert operator is needed to guarantee animal welfare and to avoid salivary contamination (Lodge-Ivey et al., 2009). Second, sample collection must be as heterogeneous as possible such that all microbial communities distributed across the feed particle surfaces in the liquid phase of the liquor or attached to the rumen wall are represented in the sample. Hence, the probe must reach each part of the rumen (de Assis Lage et al., 2020) considering that the pH value and amounts of volatile fatty acids (VFA) may vary across the different parts of the rumen, as reviewed by Yáñez-Ruiz et al. (2016).

An alternative method involves surgical fistulation of the rumen and the insertion of a rumen cannula, the application and maintenance of which are both laborious and expensive (da Cunha et al., 2023).

The comparison of rumen cannulation with stomach tubing techniques reveals several differences to consider. First, rumen liquor collected through the oro-esophageal probe mainly consists of liquid fractions at the expense of negligible solid fractions. Thus, the microbial groups colonizing solid fractions (solid-associated bacteria, SAB) might not be properly sampled with the tube (Ramos-Morales et al., 2014). However, the stomach tube was recently considered a better proxy for evaluating the rumen microbiome and metabolome in a large number of animals compared with the rumen cannula technique (da Cunha et al., 2023). Thus, differences between the two techniques are mostly related to pH, which is frequently higher in samples collected by oro-esophageal tube due to slight saliva contamination (da Cunha et al., 2023), and the total amount of VFA, but not in relation to molar proportions (de Assis Lage et al., 2020).

In *in vivo* trials, rumen content is immediately stored after its collection at -20 or -80°C for preservation until further analysis (e.g. DNA or FA extraction; de Assis Lage et al., 2020; Hinsu et al., 2020). By contrast, in *in vitro* trials, rumen content needs to be transferred very quickly into the lab for incubation at 39°C whilst avoiding oxygen contamination (Yanez-ruiz 2016).

Alternatively, once the rumen bag is collected at the slaughterhouse, it cannot be stored for more than 4 h, after which its environmental conditions will have altered, which will in turn limit the

growth of anaerobic microorganisms that principally inhabit the rumen (Denek et al., 2006). Transfer of the rumen bag is done employing a thermostatic box (39°C), and anaerobiosis within the rumen is guaranteed using CO_2 insufflation.

Prior to incubation, the rumen's content is filtered through layers of cheesecloth, then squeezed into a pre-heated batch and buffered (i.e., rumen liquor:saliva 1:3, V/V as described by McDougall, 1948; rumen liquor:saliva 1:2, V/V as described by Cone et al., 1996; rumen liquor:saliva 1:4 as described by Goering and Van Soest, 1970) in a flask under a flux of CO_2 to maintain anaerobic conditions (Mannelli et al., 2019; Romanzini et al., 2020).

Usually, the source of RL could be collected from mutton, since the rumen microbiota and its activity seem to be mainly affected by the diet rather than the host-donator species (Henderson et al., 2015). Indeed, RL collection intended for *in vitro* trials is generally collected before feeding (Yá˜nez-Ruiz et al., 2016).

For *in vitro* trials, three rumen liquor donors should be used in order to standardize the microbial populations, thus minimizing problems linked to the initial microbial activity that occur during the incubation period (Uden et al., 2012). With regard to the ideal number of "runs", i.e., trial replications carried out on different days, Yá˜nez-Ruiz et al. (2016) recommends three when methane emission is being evaluated *in vitro*. Although, Uden et al. (2012) explained that the run should not form part of the statistical model as the researcher should standardize the experiment and the analysis as much possible as to avoid the need for trial replications.

5.2. Typologies of *in vitro* fermentation.

The choice of the *in vitro* methodology is tightly linked to the aim of the investigation, whether it involves microbiota assays or assessing the nutritive value of ruminant feeds, the FA, volatile compound or plasmalogen profile, methane production or nutrient degradability.

The main typologies of *in vitro* systems are summarized below. It is important to be aware that the parameters under evaluation might favour the use of one methodological approach over another due

to its specific features (e.g. the feed-to-rumen liquor ratio, batch *vs* continuous systems). For example, gas production data for predicting microbial efficiency and VFA production can be used better when the potentials and limitations of batch cultures are taken into consideration (Dijkstra et al., 2005).

5.2.1. Standard in batch incubation

In batch fermentation is commonly conducted using buffered rumen fluid inoculated with the experimental diets. It involves fermenters set to a thermostatic environment (i.e., air circulation chambers or water baths) at 39°C and moderate shaking to simulate the ruminal physiological conditions in an attempt to reproduce *in vivo* conditions (Figures 4a, 4b, 4c). The incubation period and the time(s) at which the data is sampled for evaluation vary in batch incubation experiments depending on the aims of the research (Menke, 1988; Theodorou et al., 1994; Muetzel et al., 2014; Raffrenato et al., 2018). This is especiallycase when the effect of feed additives on microbial activity is the purpose of the trial. For example, Cappucci et al. (2021) used an *in vitro* approach to test the possible effects of adding condensed and hydrolysable tannins to dairy ewe feed. The authors evaluated VFA and FA profiles, methane emissions, ammonia concentration and plasmalogen profile at 6, 12 and 24 h of fermentation. Mannelli et al. (2019) investigated the effects of adding of chestnut tannin, gallic acid and vescalagin to grazing ewe feed, but their study considered just a single sampling time to evaluate NDF diet degradability, rumen microbial community and the plasmalogen profile. Marcos et al. (2019) evaluated the nutritional quality of olive cakes from different oil mill processes by means of two *in vitro* trials. The first was conducted for 120 h with multiple sampling points (3, 6, 9, 12, 15, 22, 26, 31, 36, 48, 58, 72, 96 and 120 h), with the aim of calculating the gas production curve. The second lasted 24 h and samples were collected for evaluating the VFA profile and ammonia production at the end of the fermentation. In the *in vitro* study by Cai et al. (2021), the authors evaluated the role of *Clostridium buthyricum* on

rumen fermentation as a means to investigating the mechanisms underlying improved growth performance in heat-stressed goats; in this case, a 24 h incubation time was used.

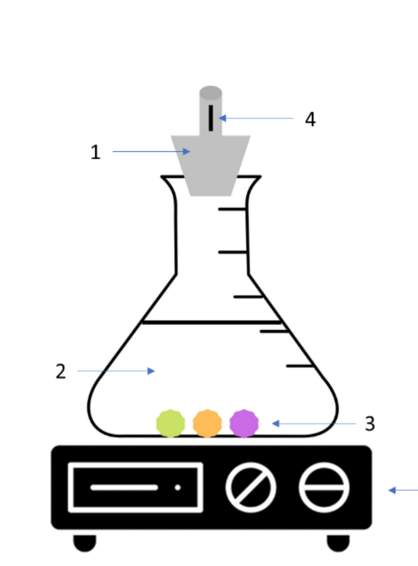


Figure 4 a. Example of standard in batch fermentation; 1: valve to close the batch; 2: inoculum with RL and buffer; 3: Diet; 4: valve for gas release; 5: magnetic stirrer.

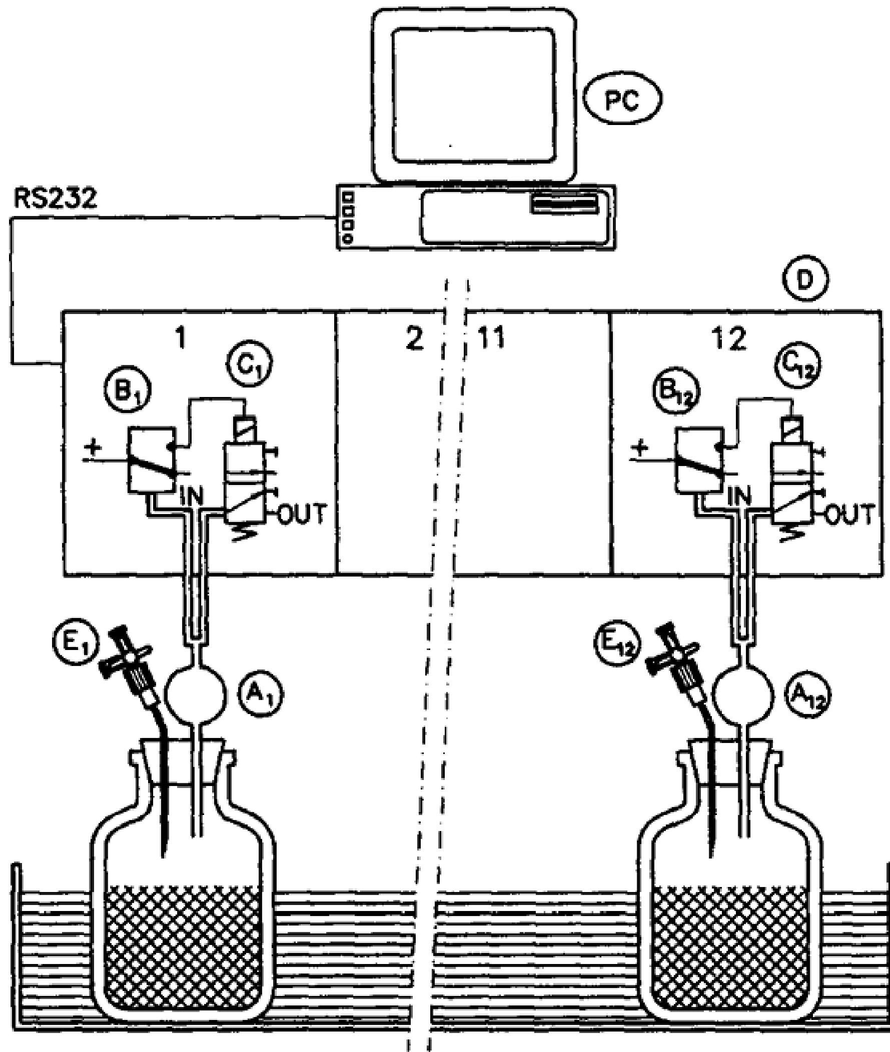
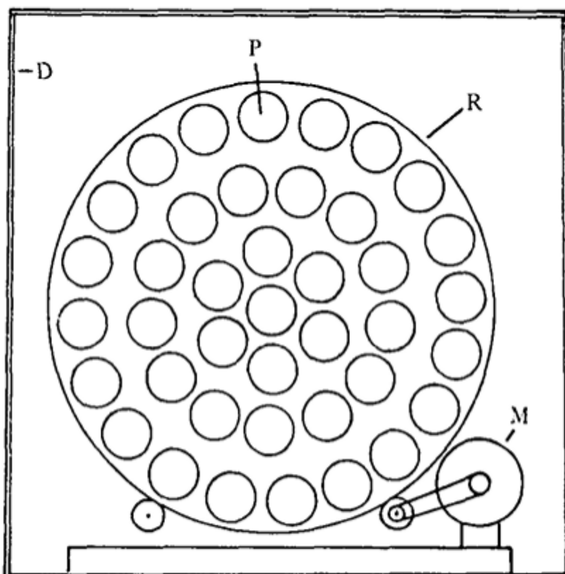


Figure 4 b. Principles of the gas production apparatus from Cone et al. (1996). A: gas produced in 50 mL jar; B: electric valve; C: pressure transducer; D: electronic control unit; E: stirring? Closed 2 min after the start of fermentation.



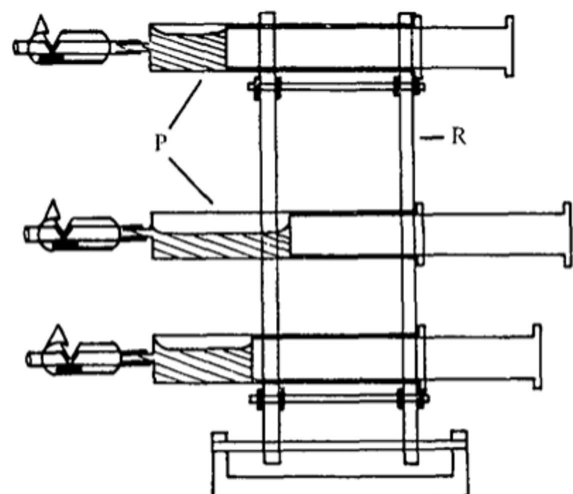


Figure 4 c. Syringe-type closed system from Menke et al., 1979. P: piston-syringes; R: rotor; M: electric motor; D: drying oven.

5.2.2. Continuous batch incubation.

Continuous and semi-continuous batch incubation systems are set up such that the fermented liquor is regularly replaced with new fluid, thus creating a better simulation of rumen conditions. This avoids the recycling of organic matter and enables the established conditions to be maintained for a relatively long period (Soliva and Hess, 2007; Deitmers et al., 2022). Although the inflow and outflow rates can be regulated in continuous systems to mimic the passage rate of living animals – a feature that is absent under batch culture conditions – a full simulation is not possible due to the absence of the rumen wall that guarantees the translocation of VFA into the blood.

Two main types of system exist, distinguishable by their general set-up and the world regions where they have primarily been used: the rumen simulation technique (RUSITEC) is mainly used in Europe, whereas the dual flow system is most popular in North America (Kajikawa et al., 2003).

5.2.2.1. Semicontinuous incubation/long-term rumen simulation technique: RUSITEC fermenters.

The first RUSITEC fermenters were used for short-term incubations lasting 6–8 h to measure methane production (Czerkawski, 1976). However, Czerkawski realized that this length of time was insufficient to allow the growth of all the microorganisms characterizing the rumen microbiota. Thus, the author (1977) proposed a new version of the RUSITEC fermenter which had to meet some basic requirements; for example, the vessels containing the solid and liquid phase had to be gas-tight to enable accurate gas collection and measuring, and the apparatus had to be resistant enough to sustain several weeks of fermentation (Figure 5).

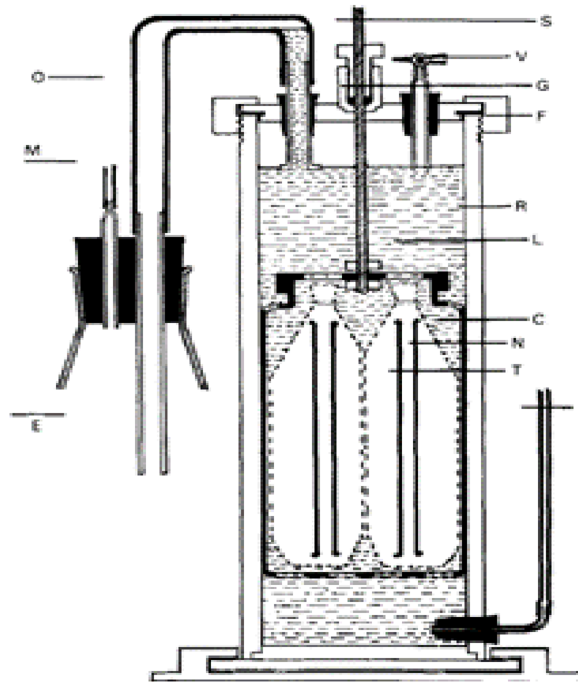


Figure 5. Vessel of the rumen simulation technique described by CZERKAWSK (1977). S: driving shaft; V: sampling valve; G: gland or gas-tight; F: flange; R: main reaction vessel; L: rumen fluid; C: perforated food container; N: nylon gauze bag; T: rigid tube; I: artificial saliva inlet; O: overflow outlet; M: line to gas-collection bag; E: vessel for effluent collection.

In brief, artificial saliva solution (McDougall, 1948) is continuously infused into the fermentation vessels containing rumen liquor, where it is constantly mixed by means of a piston moving up and down and maintained at 39°C using a water bath. Every 48 hours, the nylon bag containing the feed is removed and replaced with a new bag. The resulting effluent is evaluated to assess nutrient degradability. While the rumen fluid is only supplied at the beginning of the fermentation trial, the effluent is removed daily, together with the fermentation gas produced. Hence, analysis of the ruminal fluid and gas produced on a daily basis provides data about fermentation patterns, community composition and the metabolic activity of the rumen microbiota (Brede et al., 2022).

The RUSITEC method has been used to study the effect of diet on the rumen fermentation profile. For example, García-Rodríguez et al. (2020) tested the effects of replacing 16.6 % of the forage in a dairy sheep diet with crude olive cake. Following a 7-day adaptation period, the liquid phase was

evaluated to determine microbial protein synthesis, DM content and ^{15}N enrichment on days 8 and 9. At the same time, the solid phase was also checked for DM content and ^{15}N enrichment, and microbial populations assays were performed. From day 10 to 13 of the trial, methane, VFA and ammonia were checked. Alternatively, Wetzels et al. (2018) used the RUSITEC system to study the effects of a challenge with *Clostridium perfringens* upon the bacterial community and the RL metabolome over a period lasting 13 days. The first seven days comprised the adaptation period, during which a rumen content bag alternated with a feed bag every 24 h for the first four days followed by a steady-stead period lasting three days. During the trial, pH and redox potential were checked daily. *C. perfringens* were added at a concentration of 108 CFU/10mL on days 8, 9, 11, 13 and 14, and rumen bag content samples were collection days 10, 12 and 15 to assess the short chain FA profile, ammonia concentration, bacterial community and metabolome. The same approach was adopted by Kozłowska et al. (2021) to study the partial replacement of grass silage with alfalfa silage cultivars in a trial that lasted 10 days. The first 5 days were dedicated to adaptation to the diet, during which the rumen content bag was alternated with the feed bag.

5.2.2.2. Dual-flow *in vitro* continuous culture (CC) fermenters

The dual-flow CC fermenter system enables the incubation of rumen inoculum for varying lengths of time on the condition that an adaptation period of at least 6 days has been completed (Salfer et al., 2018). It consists of a 1-liter vessel continuously fed with feed, buffer, N_2 and CO_2, and from which solid and liquid effluents can be removed. This approach seems to be well suited for approximating *in vivo* fibre digestion and the VFA profile (Hoover et al. 1976a, 1976b, cited in Salfer et al., 2018). Moreover, dual-flow continuous culture fermenters may be useful for determining the relative abundances of the most abundant groups in the microbial community. Considering the limitation of *in vitro* trials related to the absence of rumen content stratification and papillae absorption, microbial activities are probably stressed making it possible to understanding the rumen ecology. For example, using this system, the addition of camelina sativa to cow feed was

shown to lower the BH rate of C18:1 n-9, resulting in an increase in C18:0, as similarly observed *in vivo* trials where a rise in milk BH intermediates was observed, noting that their presence in this tissue principally arises from their direct flow from rumen to blood to mammary gland (Brandao et al., 2018). Furthermore, flaxseed oil added to dairy cow diet in this *in vitro* set up did not affect the growth of the microbial communities involved in ammonia production since its concentration was below the toxic threshold (8.5 mg/L), as similarly shown in *in vivo* trials (17.3 mg/L vs 16 or 17.6 mg/L). The VFA profile also followed the same trend, with values comparable to those obtained *in vivo* trials (VFA concentration: 120.3 mM vs 122 mM, respectively; Silva et al., 2016).

5.3. Essential differences between *in vitro* and *in vivo* systems

In living animals, contractions of the rumen lead to the stratification of its content, and a part of nutrient absorption is carried out through the rumen wall via the papillae. By contrast, *in vitro* trial mixing can be continuous, and papillae do not exist. To obtain something similar to stratification *in vitro*, the distinct sacs can be subjected to pulsatile peristaltic-like contractions (Salfer et al., 2018). Filtration of rumen liquor is another way to reduce the problems related to the absence of stratification; however, in this case, the evaluation of SAB is limited (Joch et al., 2019).

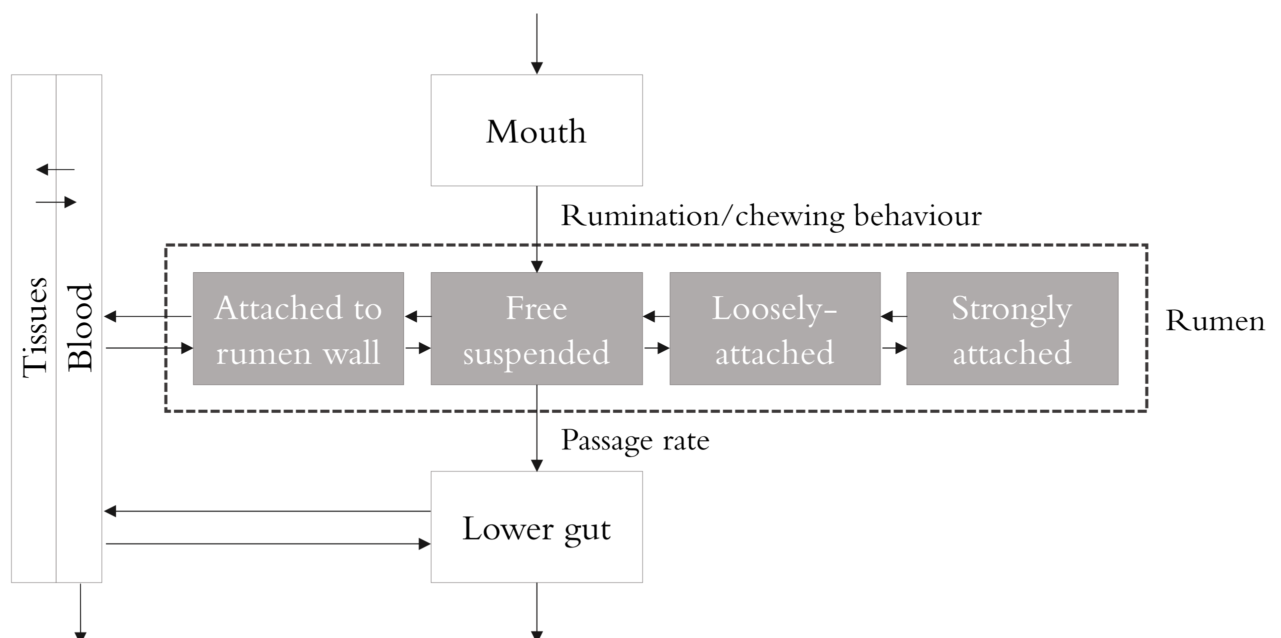
In *in vivo* conditions, certain metabolites derived from fermented feed, such as VFAs, are absorbed by ruminal papillae, whereas others, such as long chain FAs, are absorbed in the intestinal tract (Lock et al., 2006; Aschenbach et al., 2011). The absorbed long chain FAs reach animal tissues through the blood flow, and here they are involved in fat composition. The absorption of VFA in the rumen is bicarbonate dependent, thus, chewing activity and saliva production play a pivotal role (Beauchemin, 2018). Saliva is important for swallowing and regurgitating feed, it allows nitrogen from the urea cycle to be reused, plus it buffers the environment for microbial activity. In particular, the bicarbonate and phosphate components of saliva play a key role in rumen pH buffering. Saliva is constantly produced during chewing and resting, and differences in salvia production on the DM basis (mL/kg of DM) reflect differences in eating rate and the moisture content of the swallowed

bolus. Moisture content depends on the ingredients of the diet. The moisture content of feed concentrate differs to that of forage, which also varies depending on whether the crop is silage or not. A high NDF content requires a longer chewing time and thus more saliva production. For example, dairy cow feed concentrates typically have 1.12–1.19 mL NDF/g of DM, whereas that for forage ranges from 3.40–7.23 mL/g of DM. For a diet based on forage, 50 % of the bicarbonate present in the rumen comes from saliva, whereas in the case of a diet based on feed concentrate it supplies 35 %. The efficiency of saliva may be altered by several components such as the presence of bioactive compounds that bind saliva proteins, which are important for rumen activity. In addition, the type of saliva differ among the animals, particularly in the bicarbonate to phosphate ratio, and being VFA bicarbonate dependent, the estimation of the gas production can strongly be affected (Getachew et al., 2005; Mould et al., 2005). *In vitro* systems have employed different kinds of saliva preparation. Non-filtered saliva, filtered saliva (centrifugated) without microorganisms, double-filtered saliva (large proteins and immunoglobulins removed), and autoclaved saliva (only minerals) have all been used as substitutes for bicarbonate buffer in batch fermentation, and they can differentially affect the fermentation activity in terms of VFA and N-NH_3 (Palma-Hidalgo et al., 2021). The use of artificial saliva *in vitro* can partially simulate the *in vivo* condition and some continuous systems offer the possibility to regulate artificial saliva inflow. However, the removal of end-products (in continuous systems only) can only be controlled by the constant inflow rate of the artificial salvia, but in this case no other factors can be taken into account (Deitmers et al., 2022). Similarly, the passage rate of the fluid and solid phases and the amount of feed per volume of rumen liquor are not comparable between *in vitro* systems and the *in vivo* state (Czerkawski, 1984). Particularly, in an *in vitro* system less than 30 g/L of a substrate (highly variable depending on the *in vitro* methods used) is incubated per unit of buffered rumen fluid on a daily basis, whereas more than 20,000 g of feed can enter the rumen of a dairy cow (considering a rumen liquor volume of 80 L), accounting for about 250 g/L (Hristov et al., 2012). Thus, the absolute number of metabolites and end-products, as well as gases, is never comparable. In addition, this aspect also influences the

in vitro dosage of additives required to obtain similar effects as in *in vivo* conditions (Castro-Montoya et al., 2015).

Regarding the distribution of microbial communities, *in vitro* systems only model three compartments, across which the microorganisms can distribute, rather than the four anatomical compartments of the rumen (Czerkawski, 1984, Figure 6). In the *in vivo* condition, the ruminal microorganisms can be freely suspended in rumen liquor, loosely or strongly attached to the feed, or colonize the rumen wall. Therefore, as recently summarized by Deitmers et al. (2022), *in vitro* systems are ill equipped to evaluate the effects of diet composition (semi-continuous: Hildebrand et al., 2011; batch: Menci et al., 2021), feed additives (semi-continuous: Díaz et al., 2017; batch: Foggi et al., 2022), feed degradability, microbial protein synthesis (semi-continuous: Wischer et al., 2013; batch: Ungerfeld et al., 2020), microbial structure (semi-continuous: Soto et al., 2013; batch: Mannelli et al., 2019) or fermentation patterns (semi-continuous: Carro et al., 2009; batch: Hervás et al., 2022).

However, an advantage of *in vitro* techniques is that several diets can be tested without increasing the number of experimental animals (Yáñez-Ruiz et al., 2016).



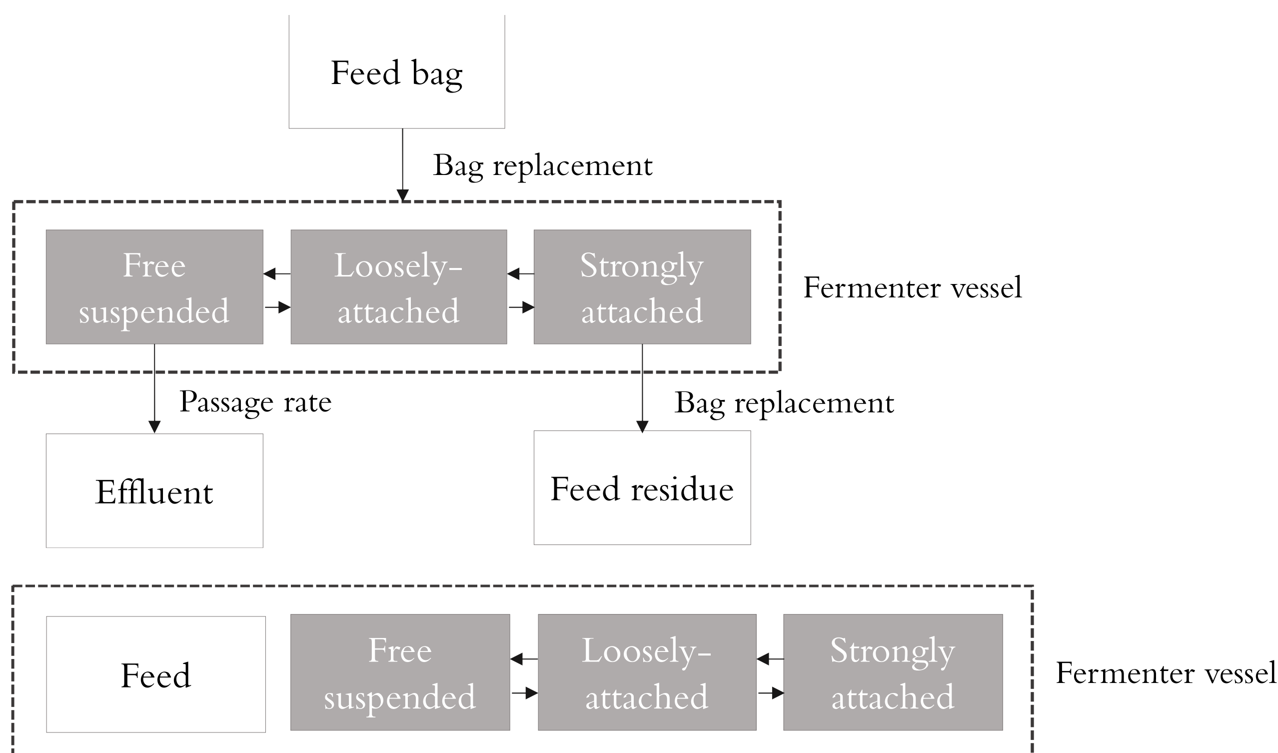


Figure 6. Microbial compartments *in vivo* (a, adapted from; Czerkawski, 1984) and *in vitro* with semi-continuous system (b) or batch culture (c).

Another difference is linked to the adaptation period and to the duration of the trial. In vitro trials are normally carried out over a short period time depending on the system applied (i.e., 24 h, 48 h, 72 h), with one or a more sampling time (i.e., at 0 h, 6 h, 12 h, 24 h). Concerning the adaptation period, animal conditioning in in vivo trials is likely to be planned to coincide with subsequent slaughter. However, several in vitro techniques were evaluated by Castro-Montoya et al. (2015) to overcome the issue of rumen source as the animal origin. Previously, Castro-Montoya et al. (2015) measured the effect on the methane of a blend of essential oils *in vivo* and *in vitro,* with two 24-hour standard incubations, two consecutive batch incubations, and a gas production technique that lasted 72 hours. They did not obtain the same mitigant effect as evaluated *in vivo*, except for a tendency to lower methane formation with the 72-hour fermentation. Together with other effects (e.g., additive dosage *in vivo* vs. *in* vitro), the time interval for adaptation of microorganisms might exert a pivotal role in evaluating an effect, and prolonged incubation time might help in this sense (Castro-Montoya et al., 2015). By contrast, semi-continuous fermentation (i.e., RUSITEC) normally

involves an adaptation period of the rumen liquor both to the fermenter and to the diets fed, which generally lasts 5–7 days (Staerfl et al., 2010; Brede et al., 2022).

5.4. How to apply *in vitro* technique studying rumen fermentation biomarkers.

In vitro trials have long been used to investigate the nutritional aspects of ruminant feeds, their fermentation profile and associated methane production, and a wealth of literature is available on these subjects. More recently, research has focused on the definition of other parameters such as ruminal biomarkers. This final part of the critical analysis addresses the study of animal metabolism through assessment of FA and DMA profiles and the rumen microbiota via *in vitro* methods.

5.4.1. Metabolism of fatty acid and dimethyl acetal from plasmalogens

In vitro systems offer useful tools for studying ruminant metabolism. They are often included in feeding trials, for example, to study the effects of dietary additives. Moreover, *in vitro* approaches that simulate specific aspects of rumen metabolism, such as the passage rate of feeds or the continuous mixing of ingesta, lead to a realistic rumen fermentation and sensible data. For instance, FA biosynthesis has been studied using *in vitro* trials (Buccioni et al., 2006; Pallara et al., 2014; Mannelli et al., 2019; Saeed et al., 2023). However, their concentration depends on two steps: triglyceride LP and FA BH. The second step involves isomerization and saturation reactions. Digestive enzymes are involved in the final BH step, but they are slightly produced in an *in vitro* rumen system. Thus, *in vitro* data pertaining to FA concentrations - are altered respect to the *in vivo* condition. In addition, Devillard et al. (2006) observed that protozoa can incorporate intermediates of BH and thus, when they leave the rumen, they contribute to their transport of FAs to the duodenum of ruminants (400 g/kg of rumenic acid, 300–360 g/kg of trans-10, cis-12 CLA and 400 g/kg of the VA; Yànez-Ruiz et al. 2006; 2007). A long fermentation period may be useful to allow microbiota to fully grow and exert its activity, guaranteeing even the correct animal physiology. In

fact, the other two issues of *in vitro* trials are the lack of rate dilution and a period of treatment adaptation. The first affects the dose needed to observe effects. Instead, both determine the time lasting so that the dose affects the rumen fermentation. Thus, the systems are different, and the dose should be chosen based on the inoculum volume in *in vitro* trials and on the animal's physiology in *in vivo* trials. Nevertheless, using these details, similar effects on the fermentation can be observed because determined principally by the kind of additive. A commercial blend of essential oil active compounds (cresols 50–100 mg/g, thymol 50–105 mg/g, limonene 40–105 mg/g, vanillin 20–60 mg/g, guaiacol 20–45 mg/g, eugenol 10–30 mg/g and salicylates 10–30 mg/g) affected rumen fermentation in dairy cows in the *in vitro* trial at the dose of 600 mg/L, whereas an effect was detected *in vivo* at 20 mg/L (Joch et al., 2019). Meanwhile, tannins influenced CLAs pathways at 0.1-20 g/kg DM in the *in vitro* trial and at 2.1-80 g/kg DM *in vivo* (Purba et al., 2020). These hypotheses are not in complete accordance with the trials carried out by Kim et al. (2020) or by Beigh et al. (2021) that used an *in vitro* trial to assess the dose to apply *in vivo*. However, the authors evaluated different parameters between the *in vitro* and the *in vivo* trials, thus no mean values should be compared.

Plasmalogens are lipid components of anaerobic bacterial membranes. They differ from the more common diacyl polar lipids as they have an alk-10-enyl ether-linked chain at the glycerol sn-1 position. Dimethyl acetals are artifacts that originate from plasmalogens during the FA methylation of microbial membranes. Changes in the plasmalogen profile are expressions of microbial resilience. The feeding strategy used is the main factor affecting the plasmalogen composition of microbial membranes because diet quality, the associative effects of ingredients and their granulometry, and the rate of feed passage through the rumen are all important variables that condition the survival of microorganisms. Indeed, similarly to FAs, the DMA profile is modified by the rumen microbiota as a response to the quality of diet since feedstuff lacks DMA (Alves et al., 2013). Another hypothesis to explain changes in the plasmalogen profile is related to the animal

production attitude. The literature reports that attitude and microbiome composition are strongly related. Recently, several authors demonstrated that in Maremmana and Aubrac breeds, the isoC14:0_DMA concentration was correlated with the presence of Rikenellaceae_RC9_gut_group, which is involved in the growth of amylolytic bacterium associated with high concentrations of iso-form DMAs in RL (Conte et al., 2022). These two hypotheses about the factors influencing the DMA profile (i.e., feed and altitude) are probably correlated because the diet provides the substrate for microbial growth, and the animal production efficiency is strongly related to the rumen's role in valorising feed nutrients for the host animal. Indeed, the host animal affects the rumen microbiota, while the microbial community ratio and its activity principally through the diet quality, as reported by Henderson et al. (2015).

When the objective of an *in vitro* trial is the study of DMAs from plasmalogen metabolism, being ruminal biomarkers, they can be integrated into the considerations reported for FA and for rumen microbiota (see next paragraph).

5.4.2. Rumen microbiota

The differences between trials carried out *in vitro* and *in vivo* are particularly evident when microorganism abundances and activities are the subject of the investigation. Salfer et al. (2018) observed that variability and abundances change more in the short period, with higher values *in vitro*, and changes are only evident for groups that are less representative at every taxonomic level. The authors conclude that this approach is mainly useful for determining the microbial community. Mannelli et al. (2019) sustain a similar point of view. They studied the effects of chestnut tannin extract, vescalagin and gallic acid on the microbial community profile in an *in vitro* study using rumen liquor from slaughtered ewes. Comparing their results of microbiota composition with the core rumen community observed *in vivo* by Henderson et al. (2015) and Baldwin et al. (2012), the authors noticed differences that seemed to be due to the absence of microorganism-host

interactions. Tun et al. (2020) observed different effects *in vitro* compared with *in vivo* when evaluating the effect of fermentation products from Saccharomyces cerevisiae on the microbiota under conditions of subacute ruminal acidosis. They noted that both cellulolytic and amylolytic increased, but when the pH changed, they noted different trends in the two conditions. They supposed this to be associated with the higher abundance of cellulolytic with respect to protozoa *in vitro* that increased with a decrease in pH. Since, since microbiota abundances data might be incomplete (Mannelli et al. 2019; McDermott et al., 2020), studying the rumen microbiota via analysis of RNA extraction instead of DNA extraction may indicate which microorganisms and pathways are actually active (Neves et al., 2020). The authors observed a correlation between bacteria abundances and FA concentrations. Instead, Huws et al. (2016) evaluated which bacteria were correlated with the disappearance of DM over short and long periods of fermentation. At the same time, Li et al. (2016) stated that the Fungi, Protozoa and Archaea domains could also be analysed by means of RNA extraction to further our knowledge about the overall rumen microbiota. From this perspective, *in vitro* studies in which the microbiota is evaluated by RNA extraction could offer a solution for obtaining a more complete overview of rumen metabolism.

Objective

By-products from the agro industry have been successfully studied being edible and rich in functional molecules such as PUFA and PPs, able to modulate rumen fermentation. The reuse of these sources, wherever is possible, meets the principles of 3R (Reuse, Reduce and Recycle). Moreover, the production chain of the by-product source is valorised reducing factory cost and pollution for the disposal. Specifically, in this project the olive oil production chain was considered being a developed sector in the Mediterranean area. The objective of this thesis was to valorise by-products from the olive oil production in ruminant diet that are rich in PUFA, such as OA and LNA, and PPs as oleuropein or hydroxytyrosol and tyrosol. Leaves and pomace were tested in feeding strategy for dairy ruminants.

Reference

Abbeddou, S., B. Rischkowsky, E.K. Richter, H.D. Hess, and M. Kreuzer. 2011. Modification of milk fatty acid composition by feeding forages and agro-industrial byproducts from dry areas to Awassi sheep. J. Dairy Sci. 94:4657–4668. doi:10.3168/jds.2011-4154.

Agarwal, N., D.N. Kamra, and L.C. Chaudhary. 2015. Rumen Microbial Ecosystem of Domesticated Ruminants. Rumen Microbiol. From Evol. to Revolut. 17–30. doi:10.1007/978-81-322-2401-3_2.

de Aguiar, S.C., E.M. de Paula, E.H. Yoshimura, W.B.R. dos Santos, E. Machado, M.V. Valero, G.T. dos Santos, and L.M. Zeoula. 2014. Effects of phenolic compounds in propolis on digestive and ruminal parameters in dairy cows. Rev. Bras. Zootec. 43:197–206. doi:10.1590/S1516-35982014000400006.

Akraim, F., M.C. Nicot, P. Juaneda, and F. Enjalbert. 2007. Conjugated linolenic acid (CLnA), conjugated linoleic acid (CLA) and other biohydrogenation intermediates in plasma and milk fat of cows fed raw or extruded linseed. Animal 1:835–843. doi:10.1017/S175173110700002X.

Alves, S.P., J. Santos-Silva, A.R.J. Cabrita, A.J.M. Fonseca, and R.J.B. Bessa. 2013. Detailed Dimethylacetal and Fatty Acid Composition of Rumen Content from Lambs Fed Lucerne or Concentrate Supplemented with Soybean Oil. PLoS One 8:e58386. doi:10.1371/JOURNAL.PONE.0058386.

Araújo, M., F.B. Pimentel, R.C. Alves, and M.B.P.P. Oliveira. 2015. Phenolic compounds from olive mill wastes: Health effects, analytical approach and application as food antioxidants. Trends Food Sci. Technol. 45:200–211. doi:10.1016/J.TIFS.2015.06.010.

Asanuma, N., M. Iwamoto, and T. Hino. 1999. Effect of the addition of fumarate on methane

production by ruminal microorganisms in vitro. J. Dairy Sci. 82:780–787. doi:10.3168/JDS.S0022-0302(99)75296-3.

Aschenbach, J.R., G.B. Penner, F. Stumpff, and G. Gäbel. 2011. Ruminant nutrition symposium: Role of fermentation acid absorption in the regulation of ruminal pH. J. Anim. Sci. 89:1092–1107. doi:10.2527/jas.2010-3301.

de Assis Lage, C.F., S.E. Räisänen, A. Melgar, K. Nedelkov, X. Chen, J. Oh, M.E. Fetter, N. Indugu, J.S. Bender, B. Vecchiarelli, M.L. Hennessy, D. Pitta, and A.N. Hristov. 2020. Comparison of Two Sampling Techniques for Evaluating Ruminal Fermentation and Microbiota in the Planktonic Phase of Rumen Digesta in Dairy Cows. Front. Microbiol. 11:3330. doi:10.3389/fmicb.2020.618032.

Bahloul, N., N. Kechaou, and N.B. Mihoubi. 2014. Comparative investigation of minerals, chlorophylls contents, fatty acid composition and thermal profiles of olive leaves (Olea europeae L.) as by-product. Grasas y Aceites 65:e035–e035. doi:10.3989/GYA.0102141.

Bakeer, M., H. Abdelrahman, and K. Khalil. 2022. Effects of pomegranate peel and olive pomace supplementation on reproduction and oxidative status of rabbit doe. J. Anim. Physiol. Anim. Nutr. (Berl). 106:655–663. doi:10.1111/jpn.13617.

Baldwin, R., W. Li, C. Li, E. Connor, R.L.- Metagenomics, and U. 2012. 2012. The bacterial community composition of the bovine rumen detected using pyrosequencing of 16S rRNA genes. airitilibrary.com.

Beauchemin, K.A. 2018. Invited review: Current perspectives on eating and rumination activity in dairy cows. J. Dairy Sci. 101:4762–4784. doi:10.3168/JDS.2017-13706.

Beigh, Y.A., A.M. Ganai, and M.I. Wani. 2021. Evaluation of Himalayan Elm (Ulmus wallichiana) leaf meal as a partial substitute for concentrate mixture in total mixed ration of sheep. Small

Rumin. Res. 196:106331. doi:10.1016/j.smallrumres.2021.106331.

Berbel, J., J. Berbel, A. Posadillo, J.B. Biomed, J. Sci, and T. Res. 2018. Opportunities for the Bioeconomy of Olive Oil Byproducts. doi:10.26717/BJSTR.2017.01.000630.

Boeckaert, C., B. Vlaeminck, J. Mestdagh, and V. Fievez. 2007. In vitro examination of DHA-edible micro algae. 1. Effect on rumen lipolysis and biohydrogenation of linoleic and linolenic acids. Anim. Feed Sci. Technol. 136:63–79. doi:10.1016/j.anifeedsci.2006.08.015.

Bolletta, V., M. Pauselli, C. Pomente, A. Natalello, L. Morbidini, G. Veneziani, V. Granese, and B. Valenti. 2022. Dietary olive leaves improve the quality and the consumer preferences of a model sheep cheese. Int. Dairy J. 134:105464. doi:10.1016/J.IDAIRYJ.2022.105464.

Branciari, R., R. Galarini, M. Trabalza-Marinucci, D. Miraglia, R. Roila, G. Acuti, D. Giusepponi, A. Dal Bosco, and D. Ranucci. 2021. Effects of Olive Mill Vegetation Water Phenol Metabolites Transferred to Muscle through Animal Diet on Rabbit Meat Microbial Quality. Sustain. 2021, Vol. 13, Page 4522 13:4522. doi:10.3390/SU13084522.

Branciari, R., D. Ranucci, R. Ortenzi, R. Roila, M. Trabalza-Marinucci, M. Servili, P. Papa, R. Galarini, and A. Valiani. 2016. Dietary administration of olive millwastewater extract reduces campylobacter spp. prevalence in broiler chickens. Sustain. 8. doi:10.3390/su8090837.

Brandao, V.L.N., X. Dai, E.M. Paula, L.G. Silva, M.I. Marcondes, T. Shenkoru, S.R. Poulson, and A.P. Faciola. 2018. Effect of replacing calcium salts of palm oil with camelina seed at 2 dietary ether extract levels on digestion, ruminal fermentation, and nutrient flow in a dual-flow continuous culture system. J. Dairy Sci. 101:5046–5059. doi:10.3168/JDS.2017-13558.

Brede, M., S.B. Haange, S. Riede, B. Engelmann, N. Jehmlich, U. Rolle-Kampzczyk, K. Rohn, D. von Soosten, M. von Bergen, and G. Breves. 2022. Effects of Different Formulations of Glyphosate on Rumen Microbial Metabolism and Bacterial Community Composition in the Rumen Simulation Technique System. Front. Microbiol. 13.

doi:10.3389/FMICB.2022.873101.

Brooks, C.C., G.B. Garner, C.W. Gehrke, M.E. Muhrer, and W.H. Pfander. 1954. The Effect of Added Fat on the Digestion of Cellulose and Protein by Ovine Rumen Microorganisms. J. Anim. Sci. 13:758–764. doi:10.2527/JAS1954.134758X.

Buccioni, A., M. Antongiovanni, F. Petacchi, M. Mele, A. Serra, P. Secchiari, and D. Benvenuti. 2006. Effect of dietary fat quality on C18:1 fatty acids and conjugated linoleic acid production: An in vitro rumen fermentation study. Anim. Feed Sci. Technol. 127:268–282. doi:10.1016/J.ANIFEEDSCI.2005.09.007.

Buccioni, A., M. Decandia, S. Minieri, G. Molle, and A. Cabiddu. 2012. Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. Anim. Feed Sci. Technol. 174:1–25. doi:10.1016/j.anifeedsci.2012.02.009.

Buccioni, A., M. Pauselli, C. Viti, S. Minieri, G. Pallara, V. Roscini, S. Rapaccini, M.T. Marinucci, P. Lupi, G. Conte, and M. Mele. 2015. Milk fatty acid composition, rumen microbial population, and animal performances in response to diets rich in linoleic acid supplemented with chestnut or quebracho tannins in dairy ewes. J. Dairy Sci. 98:1145–1156. doi:10.3168/jds.2014-8651.

Cai, L., R. Hartanto, J. Zhang, and D. Qi. 2021. Clostridium butyricum Improves Rumen Fermentation and Growth Performance of Heat-Stressed Goats In Vitro and In Vivo. doi:10.3390/ani11113261.

Cappucci, A., S.P. Alves, R.J.B. Bessa, A. Buccioni, F. Mannelli, M. Pauselli, C. Viti, R. Pastorelli, V. Roscini, A. Serra, G. Conte, and M. Mele. 2018. Effect of increasing amounts of olive crude phenolic concentrate in the diet of dairy ewes on rumen liquor and milk fatty acid composition. J. Dairy Sci. 101:4992–5005. doi:10.3168/jds.2017-13757.

Cappucci, A., A. Mantino, A. Buccioni, L. Casarosa, G. Conte, A. Serra, F. Mannelli, G. Luciano, G. Foggi, and M. Mele. 2021. Diets supplemented with condensed and hydrolysable tannins affected rumen fatty acid profile and plasmalogen lipids, ammonia and methane production in an in vitro study. Ital. J. Anim. Sci.. doi:10.1080/1828051X.2021.1915189.

Carro, M.D., M.J. Ranilla, A.I. Martín-Garca, and E. Molina-Alcaide. 2009. Comparison of microbial fermentation of high- and low-forage diets in Rusitec, single-flow continuous-culture fermenters and sheep rumen. Animal 3:527–534. doi:10.1017/S1751731108003844.

Carro, M.D., and E.M. Ungerfeld. 2015. Utilization of Organic Acids to Manipulate Ruminal Fermentation and Improve Ruminant Productivity. Rumen Microbiol. From Evol. to Revolut. 177–197. doi:10.1007/978-81-322-2401-3_13.

Castagnino, P.S., J.D. Messana, G. Fiorentini, R.B. de Jesus, E. San Vito, I.P.C. Carvalho, and T.T. Berchielli. 2015. Glycerol combined with oils did not limit biohydrogenation of unsaturated fatty acid but reduced methane production in vitro. Anim. Feed Sci. Technol. 201:14–24. doi:10.1016/j.anifeedsci.2014.12.004.

Castellani, F., A. Vitali, N. Bernardi, E. Marone, F. Palazzo, L. Grotta, and G. Martino. 2017. Dietary supplementation with dried olive pomace in dairy cows modifies the composition of fatty acids and the aromatic profile in milk and related cheese. J. Dairy Sci. 100:8658–8669. doi:10.3168/JDS.2017-12899.

Castro-Montoya, J., N. Peiren, J.W. Cone, B. Zweifel, V. Fievez, and S. De Campeneere. 2015. In vivo and in vitro effects of a blend of essential oils on rumen methane mitigation. Livest. Sci. 180:134–142. doi:10.1016/j.livsci.2015.08.010.

Chaves, B.W., G.A.F. Valles, R.B. Scheibler, J. Schafhäuser Júnior, and J.L. Nörnberg. 2020. Milk yield of cows submitted to different levels of olive pomace in the diet. Acta Sci. Anim. Sci. 43:e51158. doi:10.4025/actascianimsci.v43i1.51158.

Chilliard, Y., F. Glasser, A. Ferlay, L. Bernard, J. Rouel, and M. Doreau. 2007. Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. Eur. J. Lipid Sci. Technol. 109:828–855. doi:10.1002/ejlt.200700080.

Chiofalo, V., L. Liotta, V. Lo Presti, F. Gresta, A.R. Di Rosa, and B. Chiofalo. 2020. Effect of dietary olive cake supplementation on performance, carcass characteristics, and meat quality of beef cattle. Animals 10:1–17. doi:10.3390/ani10071176.

Chow, T.T., V. Fievez, A.P. Moloney, K. Raes, D. Demeyer, and S. De Smet. 2004. Effect of fish oil on in vitro rumen lipolysis, apparent biohydrogenation of linoleic and linolenic acid and accumulation of biohydrogenation intermediates. Anim. Feed Sci. Technol. 117:1–12. doi:10.1016/J.ANIFEEDSCI.2004.08.008.

Cone, J.W., A.H. Van Gelder, G.J.W. Visscher, and L. Oudshoorn. 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Anim. Feed Sci. Technol. 61:113–128. doi:10.1016/0377-8401(96)00950-9.

Conte, G., C. Dimauro, M. Daghio, A. Serra, F. Mannelli, B.M. McAmmond, J.D. Van Hamme, A. Buccioni, C. Viti, A. Mantino, and M. Mele. 2022. Exploring the relationship between bacterial genera and lipid metabolism in bovine rumen. Animal 16:100520. doi:10.1016/j.animal.2022.100520.

da Cunha, L.L., H.F. Monteiro, C.C. Figueiredo, I.F. Canisso, R.C. Bicalho, F.C. Cardoso, B.C. Weimer, and F.S. Lima. 2023. Characterization of rumen microbiome and metabolome from oro-esophageal tubing and rumen cannula in Holstein dairy cows. Sci. Rep. 13:1–14. doi:10.1038/s41598-023-33067-5.

Czerkawski, J.W. 1976. The use of pivalic acid as a reference substance in measurements of production of volatile fatty acids by rumen micro-organisms in vitro. Br. J. Nutr. 36:311–315.

doi:10.1079/BJN19760085.

Czerkawski, J.W. 1984. Microbial fermentation in the rumen. Proc. Nutr. Soc. 43:101–118. doi:10.1079/PNS19840035.

Czerkawski, J.W., and G. Breckenridge. 1977. Design and development of a long-term rumen simulation technique (Rusitec). Br. J. Nutr 38:371. doi:10.1079/BJN19770102.

Dal Bosco, A., E. Mourvaki, R. Cardinali, M. Servili, B. Sebastiani, S. Ruggeri, S. Mattioli, A. Taticchi, S. Esposto, and C. Castellini. 2012. Effect of dietary supplementation with olive pomaces on the performance and meat quality of growing rabbits. Meat Sci. 92:783–788. doi:10.1016/j.meatsci.2012.07.001.

Deitmers, J.H., N. Gresner, and K.H. Südekum. 2022. Opportunities and limitations of a standardisation of the rumen simulation technique (RUSITEC) for analyses of ruminal nutrient degradation and fermentation and on microbial community characteristics. Anim. Feed Sci. Technol. 289:115325. doi:10.1016/J.ANIFEEDSCI.2022.115325.

Denek, N., A. Can, and S. Koncagul. 2006. Use of slaughtered animal rumen fluid for dry matter digestibility of ruminant feeds.

Destaillats, F., J.P. Trottier, J.M.G. Galvez, and P. Angers. 2005. Analysis of α-linolenic acid biohydrogenation intermediates in milk fat with emphasis on conjugated linolenic acids. J. Dairy Sci. 88:3231–3239. doi:10.3168/jds.S0022-0302(05)73006-X.

Devillard, E., F.M. McIntosh, C.J. Newbold, and R.J. Wallace. 2006. Rumen ciliate protozoa contain high concentrations of conjugated linoleic acids and vaccenic acid, yet do not hydrogenate linoleic acid or desaturate stearic acid.. Br. J. Nutr. 96:697–704. doi:10.1079/BJN20061884.

Díaz, A., M.J. Ranilla, C. Saro, M.L. Tejido, M. Pérez-Quintana, and M.D. Carro. 2017. Influence

of increasing doses of a yeast hydrolyzate obtained from sugarcane processing on in vitro rumen fermentation of two different diets and bacterial diversity in batch cultures and Rusitec fermenters. Anim. Feed Sci. Technol. 232:129–138. doi:10.1016/J.ANIFEEDSCI.2017.08.011.

Dijkstra, J., E. Kebreab, A. Bannink, J. France, and S. López. 2005. Application of the gas production technique to feed evaluation systems for ruminants. Anim. Feed Sci. Technol. 123–124:561–578. doi:10.1016/J.ANIFEEDSCI.2005.04.048.

Fievez, V., E. Colman, J.M. Castro-Montoya, I. Stefanov, and B. Vlaeminck. 2012. Milk odd-and branched-chain fatty acids as biomarkers of rumen function-An update. Anim. Feed Sci. Technol. 172:51–65. doi:10.1016/j.anifeedsci.2011.12.008.

Foggi, G., M. Terranova, G. Conte, A. Mantino, S.L. Amelchanka, M. Kreuzer, and M. Mele. 2022. In vitro screening of the ruminal methane and ammonia mitigating potential of mixtures of either chestnut or quebracho tannins with blends of essential oils as feed additives. Ital. J. Anim. Sci. 21:1520–1532. doi:10.1080/1828051X.2022.2130832.

García-Casco, J.M., M. Muñoz, J.M. Martínez-Torres, A. López-García, M.A. Fernández-Barroso, and E. González-Sánchez. 2017. Alternative feeding in Iberian pigs during growth period: Incorporation of olive cake in a dry or wet (silage) form. Agric. Conspec. Sci. 82:147–150.

García-Rodríguez, J., I. Mateos, C. Saro, J.S. González, M.D. Carro, and M.J. Ranilla. 2020. Replacing Forage by Crude Olive Cake in a Dairy Sheep Diet: Effects on Ruminal Fermentation and Microbial Populations in Rusitec Fermenters. Anim. 2020, Vol. 10, Page 2235 10:2235. doi:10.3390/ANI10122235.

Gerasopoulos, K., D. Stagos, S. Kokkas, K. Petrotos, D. Kantas, P. Goulas, and D. Kouretas. 2015. Feed supplemented with byproducts from olive oil mill wastewater processing increases antioxidant capacity in broiler chickens. Food Chem. Toxicol. 82:42–49. doi:10.1016/j.fct.2015.04.021.

Getachew, G., P.H. Robinson, E.J. DePeters, S.J. Taylor, D.D. Gisi, G.E. Higginbotham, and T.J. Riordan. 2005. Methane production from commercial dairy rations estimated using an in vitro gas technique. Anim. Feed Sci. Technol. 123-124 Pa:391–402. doi:10.1016/j.anifeedsci.2005.04.056.

Goldfine, H. 2010. The appearance, disappearance and reappearance of plasmalogens in evolution. Prog. Lipid Res. 49:493–498. doi:10.1016/J.PLIPRES.2010.07.003.

Grigg, D. 2001. Olive oil , the Mediterranean and the world Author (s): David Grigg Published by : Springer Stable URL : https://www.jstor.org/stable/41147597 Olive oil , the Mediterranean and the world 53:163–172.

Habeeb, A.A.M., A.E. Gad, A.A. El-Tarabany, M.M. Mustafa, and M.A.A. Atta. 2017. Using of Olive Oil By-Products In Farm Animals Feeding 6:57–68.

Harfoot, C.G., and G.P. Hazlewood. 1997. Lipid metabolism in the rumen. Rumen Microb. Ecosyst. 382–426. doi:10.1007/978-94-009-1453-7_9.

Henderson, G., F. Cox, S. Ganesh, A. Jonker, W. Young, P.H. Janssen, L. Abecia, E. Angarita, P. Aravena, G.N. Arenas, C. Ariza, G.T. Attwood, J.M. Avila, J. Avila-Stagno, A. Bannink, R. Barahona, M. Batistotti, M.F. Bertelsen, A. Brown-Kav, A.M. Carvajal, L. Cersosimo, A.V. Chaves, J. Church, N. Clipson, M.A. Cobos-Peralta, A.L. Cookson, S. Cravero, O.C. Carballo, K. Crosley, G. Cruz, M.C. Cucchi, R. De La Barra, A.B. De Menezes, E. Detmann, K. Dieho, J. Dijkstra, W.L.S. Dos Reis, M.E.R. Dugan, S.H. Ebrahimi, E. Eythórsdóttir, F.N. Fon, M. Fraga, F. Franco, C. Friedeman, N. Fukuma, D. Gagić, I. Gangnat, D.J. Grilli, L.L. Guan, V.H. Miri, E. Hernandez-Sanabria, A.X.I. Gomez, O.A. Isah, S. Ishaq, E. Jami, J. Jelincic, J. Kantanen, W.J. Kelly, S.H. Kim, A. Klieve, Y. Kobayashi, S. Koike, J. Kopecny, T.N. Kristensen, S.J. Krizsan, H. LaChance, M. Lachman, W.R. Lamberson, S. Lambie, J. Lassen, S.C. Leahy, S.S. Lee, F. Leiber, E. Lewis, B. Lin, R. Lira, P. Lund, E. Macipe, L.L. Mamuad,

H.C. Mantovani, G.A. Marcoppido, C. Márquez, C. Martin, G. Martinez, M.E. Martinez, O.L. Mayorga, T.A. McAllister, C. McSweeney, L. Mestre, E. Minnee, M. Mitsumori, I. Mizrahi, I. Molina, A. Muenger, C. Munoz, B. Murovec, J. Newbold, V. Nsereko, M. O'Donovan, S. Okunade, B. O'Neill, S. Ospina, D. Ouwerkerk, D. Parra, L.G.R. Pereira, C. Pinares-Patino, P.B. Pope, M. Poulsen, M. Rodehutscord, T. Rodriguez, K. Saito, F. Sales, C. Sauer, K. Shingfield, N. Shoji, J. Simunek, Z. Stojanović-Radić, B. Stres, X. Sun, J. Swartz, Z.L. Tan, I. Tapio, T.M. Taxis, N. Tomkins, E. Ungerfeld, R. Valizadeh, P. Van Adrichem, J. Van Hamme, W. Van Hoven, G. Waghorn, R.J. Wallace, M. Wang, S.M. Waters, K. Keogh, M. Witzig, A.D.G. Wright, H. Yamano, T. Yan, D.R. Yanez-Ruiz, C.J. Yeoman, R. Zambrano, J. Zeitz, M. Zhou, H.W. Zhou, C.X. Zou, and P. Zunino. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci. Rep. 5. doi:10.1038/srep14567.

Hervás, G., Y. Boussalia, Y. Labbouz, A. Della Badia, P.G. Toral, and P. Frutos. 2022. Insect oils and chitosan in sheep feeding: Effects on in vitro ruminal biohydrogenation and fermentation. Anim. Feed Sci. Technol. 285. doi:10.1016/j.anifeedsci.2022.115222.

Hildebrand, B., J. Boguhn, and M. Rodehutscord. 2011. Effect of maize silage to grass silage ratio and feed particle size on protein synthesis and amino acid profile in different microbial fractions in a semi-continuous rumen simulation. Animal 5:537–546. doi:10.1017/S1751731110002156.

Hinsu, A.T., A.B. Patel, R.J. Pandit, J.R. Thakkar, R.K. Shah, S.J. Jakhesara, P.G. Koringa, and C.G. Joshi. 2020. MetaRNAseq analysis of surti buffalo rumen content reveals that transcriptionally active microorganisms need not be abundant. Mol. Biol. Rep. 47:5101–5114. doi:10.1007/S11033-020-05581-6/FIGURES/6.

Hook, S.E., J. Dijkstra, A.D.G. Wright, B.W. McBride, and J. France. 2012. Modeling the distribution of ciliate protozoa in the reticulo-rumen using linear programming. J. Dairy Sci.

95:255–265. doi:10.3168/jds.2011-4352.

Hristov, A.N., C. Lee, R. Hristova, P. Huhtanen, and J.L. Firkins. 2012. A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. J. Dairy Sci. 95:5299–5307. doi:10.3168/JDS.2012-5533.

van Huis, A. 2017. New Sources of Animal Proteins: Edible Insects. New Asp. Meat Qual. From Genes to Ethics 443–461. doi:10.1016/B978-0-08-100593-4.00018-7.

Hukerdi, Y.J., M.H.F. Nasri, L. Rashidi, M. Ganjkhanlou, and A. Emami. 2019. E ff ects of dietary olive leaves on performance , carcass traits , meat stability and antioxidant status of fattening Mahabadi male kids. Meat Sci. 153:2–8. doi:10.1016/j.meatsci.2019.03.002.

Huws, S.A., J.E. Edwards, C.J. Creevey, P.R. Stevens, W. Lin, S.E. Girdwood, J.A. Pachebat, and A.H. Kingston-Smith. 2016. Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. FEMS Microbiol. Ecol. 92:137. doi:10.1093/FEMSEC/FIV137.

Ianni, A., and G. Martino. 2020. Dietary grape pomace supplementation in dairy cows: Effect on nutritional quality of milk and its derived dairy products. Foods 9. doi:10.3390/foods9020168.

Joch, M., V. Kudrna, J. Hakl, M. Božik, P. Homolka, J. Illek, Y. Tyrolová, and A. Výborná. 2019. In vitro and in vivo potential of a blend of essential oil compounds to improve rumen fermentation and performance of dairy cows. Anim. Feed Sci. Technol. 251:176–186. doi:10.1016/j.anifeedsci.2019.03.009.

John Wallace, R., L.C. Chaudhary, N. McKain, N.R. McEwan, A.J. Richardson, P.E. Vercoe, N.D. Walker, and D. Paillard. 2006. Clostridium proteoclasticum: a ruminal bacterium that forms stearic acid from linoleic acid. FEMS Microbiol. Lett. 265:195–201. doi:10.1111/J.1574-6968.2006.00487.X.

Kim, T. Bin, J.S. Lee, S.Y. Cho, and H.G. Lee. 2020. In vitro and in vivo studies of rumen-protected microencapsulated supplement comprising linseed oil, vitamin E, rosemary extract, and hydrogenated palm oil on rumen fermentation, physiological profile, milk yield, and milk composition in dairy cows. Animals 10:1–16. doi:10.3390/ani10091631.

Kopečný, J., M. Zorec, J. Mrázek, Y. Kobayashi, and R. Marinšek-Logar. 2003. Butyrivibrio hungatei sp. nov. and Pseudobutyrivibrio xylanivorans sp. nov., butyrate-producing bacteria from the rumen. Int. J. Syst. Evol. Microbiol. 53:201–209. doi:10.1099/IJS.0.02345-0.

Kozłowska, M., A. Cieślak, A. Jóźwik, M. El-Sherbiny, M. Gogulski, D. Lechniak, M. Gao, Y.R. Yanza, M. Vazirigohar, and M. Szumacher-Strabel. 2021. Effects of partially replacing grass silage by lucerne silage cultivars in a high-forage diet on ruminal fermentation, methane production, and fatty acid composition in the rumen and milk of dairy cows. Anim. Feed Sci. Technol. 277. doi:10.1016/j.anifeedsci.2021.114959.

Krishnamoorthy, U., C. Rymer, and P.H. Robinson. 2005. The in vitro gas production technique: Limitations and opportunities. Anim. Feed Sci. Technol. 123–124:1–7. doi:10.1016/J.ANIFEEDSCI.2005.04.015.

Latham, M.J., J.E. Storry, and M.E. Sharpe. 1972. Effect of Low-Roughage Diets on the Microflora and Lipid Metabolism in the Rumen.

Li, F., G. Henderson, X. Sun, F. Cox, P.H. Janssen, and L.L. Guan. 2016. Taxonomic assessment of rumen microbiota using total rna and targeted amplicon sequencing approaches. Front. Microbiol. 7:987. doi:10.3389/FMICB.2016.00987/BIBTEX.

Liotta, L., V. Chiofalo, V. Lo Presti, and B. Chiofalo. 2019. In vivo performances, carcass traits, and meat quality of pigs fed olive cake processing waste. Animals 9:1–13. doi:10.3390/ani9121155.

Liu, K., J. Wang, D. Bu, S. Zhao, C. McSweeney, P. Yu, and D. Li. 2009. Isolation and

biochemical characterization of two lipases from a metagenomic library of China Holstein cow rumen. Biochem. Biophys. Res. Commun. 385:605–611. doi:10.1016/j.bbrc.2009.05.110.

Lock, A.L., K.J. Harvatine, J.K. Drackley, and D.E. Bauman. 2006. Concepts in fat and fatty acid digestion in ruminants. Proc Intermt. Nutr Conf 85–100.

Lodge-Ivey, S.L., J. Browne-Silva, and M.B. Horvath. 2009. Technical note: Bacterial diversity and fermentation end products in rumen fluid samples collected via oral lavage or rumen cannula. J. Anim. Sci. 87:2333–2337. doi:10.2527/jas.2008-1472.

Mannelli, F., A. Cappucci, F. Pini, R. Pastorelli, F. Decorosi, L. Giovannetti, M. Mele, S. Minieri, G. Conte, M. Pauselli, S. Rapaccini, C. Viti, and A. Buccioni. 2018. Effect of different types of olive oil pomace dietary supplementation on the rumen microbial community profile in Comisana ewes. Sci. Rep. 8. doi:10.1038/S41598-018-26713-W.

Mannelli, F., M. Daghio, S.P. Alves, R.J.B. Bessa, S. Minieri, L. Giovannetti, G. Conte, M. Mele, A. Messini, S. Rapaccini, C. Viti, and A. Buccioni. 2019. Effects of chestnut tannin extract, vescalagin and gallic acid on the dimethyl acetals profile and microbial community composition in rumen liquor: An in vitro study. Microorganisms 7. doi:10.3390/microorganisms7070202.

Marcos, C.N., T. de Evan, P. García-Rebollar, C. de Blas, and M.D. Carro. 2019. Influence of storage time and processing on chemical composition and in vitro ruminal fermentation of olive cake. J. Anim. Physiol. Anim. Nutr. (Berl). 103:1303–1312. doi:10.1111/jpn.13149.

Martín-García, A.I., and E. Molina-Alcaide. 2008. Effect of different drying procedures on the nutritive value of olive (Olea europaea var. europaea) leaves for ruminants. Anim. Feed Sci. Technol. 142:317–329. doi:10.1016/j.anifeedsci.2007.09.005.

Mattioli, S., J.M. Machado Duarte, C. Castellini, R. D’Amato, L. Regni, P. Proietti, D. Businelli, E. Cotozzolo, M. Rodrigues, and A. Dal Bosco. 2018. Use of olive leaves (whether or not

fortified with sodium selenate) in rabbit feeding: Effect on performance, carcass and meat characteristics, and estimated indexes of fatty acid metabolism. Meat Sci. 143:230–236. doi:10.1016/J.MEATSCI.2018.05.010.

Mattioli, S., P. Rosignoli, R. D'amato, M.C. Fontanella, L. Regni, C. Castellini, P. Proietti, A.C. Elia, R. Fabiani, G.M. Beone, D. Businelli, and A. Dal Bosco. 2020. Effect of Feed Supplemented with Selenium-Enriched Olive Leaves on Plasma Oxidative Status, Mineral Profile, and Leukocyte DNA Damage in Growing Rabbits. Anim. 2020, Vol. 10, Page 274 10:274. doi:10.3390/ANI10020274.

McDermott, K., M.R.F. Lee, K.J. McDowall, and H.M.R. Greathead. 2020. Cross Inoculation of Rumen Fluid to Improve Dry Matter Disappearance and Its Effect on Bacterial Composition Using an in vitro Batch Culture Model. Front. Microbiol. 11. doi:10.3389/fmicb.2020.531404.

McDougall. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J. 43:99. doi:10.1042/bj0430099.

Medeiros-De-Moraes, I.M., C.F. Gonçalves-De-Albuquerque, A.R.M. Kurz, F.M. De Jesus Oliveira, V.H. Pereira de Abreu, R.C. Torres, V.F. Carvalho, V. Estato, P.T. Bozza, M. Sperandio, H.C. De Castro-Faria-Neto, and A.R. Silva. 2018. Omega-9 oleic acid, the main compound of olive oil, mitigates inflammation during experimental sepsis. Oxid. Med. Cell. Longev. 2018. doi:10.1155/2018/6053492.

Menci, R., M. Coppa, A. Torrent, A. Natalello, B. Valenti, G. Luciano, A. Priolo, and V. Niderkorn. 2021. Effects of two tannin extracts at different doses in interaction with a green or dry forage substrate on in vitro rumen fermentation and biohydrogenation. Anim. Feed Sci. Technol. 278:114977. doi:10.1016/j.anifeedsci.2021.114977.

Menke, K. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid.

Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. J. Agric. Sci. 93:217–222. doi:10.1017/S0021859600086305.

Minato, H., S. Ishibashi, and T. Hamaoka. 1988. Cellular fatty acid and sugar compositionof representativestrains of rumen bacteria. J. Gen. Appl. Microbiol. 34:303–319. doi:10.2323/JGAM.34.303.

Moon, C.D., D.M. Pacheco, W.J. Kelly, S.C. Leahy, D. Li, J. Kopečný, and G.T. Attwood. 2008. Reclassification of Clostridium proteoclasticum as Butyrivibrio proteoclasticus comb. nov., a butyrate-producing ruminal bacterium.. Int. J. Syst. Evol. Microbiol. 58:2041–2045. doi:10.1099/IJS.0.65845-0.

Mould, F.L., K.E. Kliem, R. Morgan, and R.M. Mauricio. 2005. In vitro microbial inoculum: A review of its function and properties. Anim. Feed Sci. Technol. 123-124 Pa:31–50. doi:10.1016/j.anifeedsci.2005.04.028.

Muetzel, S., C. Hunt, and M.H. Tavendale. 2014. A fully automated incubation system for the measurement of gas production and gas composition. Anim. Feed Sci. Technol. 196:1–11. doi:10.1016/J.ANIFEEDSCI.2014.05.016.

Nam, I.S., and P.C. Garnsworthy. 2007. Biohydrogenation of linoleic acid by rumen fungi compared with rumen bacteria. J. Appl. Microbiol. 103:551–556. doi:10.1111/j.1365-2672.2007.03317.x.

Neofytou, M.C., D. Miltiadou, E. Sfakianaki, C. Constantinou, S. Symeou, D. Sparaggis, A.L. Hager-Theodorides, and O. Tzamaloukas. 2020. The use of ensiled olive cake in the diets of Friesian cows increases beneficial fatty acids in milk and Halloumi cheese and alters the expression of SREBF1 in adipose tissue. J. Dairy Sci. 103:8998–9011. doi:10.3168/JDS.2020-

18235.

Neofytou, M.C., D. Miltiadou, S. Symeou, D. Sparaggis, and O. Tzamaloukas. 2021. Short-term forage substitution with ensiled olive cake increases beneficial milk fatty acids in lactating cows. Trop. Anim. Health Prod. 53:1–6. doi:10.1007/S11250-021-02706-2/TABLES/3.

Neves, A.L.A., Y. Chen, K.A. Lê Cao, S. Mandal, T.J. Sharpton, T. McAllister, and L.L. Guan. 2020. Taxonomic and functional assessment using metatranscriptomics reveals the effect of Angus cattle on rumen microbial signatures. animal 14:731–744. doi:10.1017/S1751731119002453.

Or-Rashid, M.M., J.K.G. Kramer, M.A. Wood, and B.W. McBride. 2008. Supplemental algal meal alters the ruminal trans-18:1 fatty acid and conjugated linoleic acid composition in cattle. J. Anim. Sci. 86:187–196. doi:10.2527/jas.2007-0085.

Paiva-Martins, F., S. Barbosa, V. Pinheiro, J.L. Mourão, and D. Outor-Monteiro. 2009. The effect of olive leaves supplementation on the feed digestibility, growth performances of pigs and quality of pork meat. Meat Sci. 82:438–443. doi:10.1016/J.MEATSCI.2009.02.014.

Pallara, G., A. Buccioni, R. Pastorelli, S. Minieri, M. Mele, S. Rapaccini, A. Messini, M. Pauselli, M. Servili, L. Giovannetti, and C. Viti. 2014. Effect of stoned olive pomace on rumen microbial communities and polyunsaturated fatty acid biohydrogenation: An in vitro study. BMC Vet. Res. 10. doi:10.1186/s12917-014-0271-y.

Palma-Hidalgo, J.M., A. Belanche, E. Jiménez, A.I. Martín-García, C.J. Newbold, and D.R. Yáñez-Ruiz. 2021. Short communication: Saliva and salivary components affect goat rumen fermentation in short-term batch incubations. Animal 15:100267. doi:10.1016/j.animal.2021.100267.

Purba, R.A.P., P. Paengkoum, and S. Paengkoum. 2020. The links between supplementary tannin levels and conjugated linoleic acid (CLA) formation in ruminants: A systematic review and

meta-analysis. PLoS One 15:1–23. doi:10.1371/journal.pone.0216187.

Raffrenato, E., D.A. Ross, and M.E. Van Amburgh. 2018. Development of an in vitro method to determine rumen undigested aNDFom for use in feed evaluation. J. Dairy Sci. 101:9888–9900. doi:10.3168/JDS.2018-15101.

Ramos-Morales, E., A. Arco-Pérez, A.I. Martín-García, D.R. Yáñez-Ruiz, P. Frutos, and G. Hervás. 2014. Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. Anim. Feed Sci. Technol. 198:57–66. doi:10.1016/J.ANIFEEDSCI.2014.09.016.

Romanzini, E.P., A.G. Da Silva Sobrinho, R. De Lima Valença, T.H. Borghi, F. De Almeida Merlim, N. De Andrade, N.M.B.L. Zeola, P. De Souza Castagnino, and P.A. Bernardes. 2020. Biodiesel co-products modified the rumen parameters of feedlot lambs but did not change methane production in vitro. Acta Sci. Vet. 48:1–10. doi:10.22456/1679-9216.100003.

Russell, J.B., and J.L. Rychlik. 2001. Factors that alter rumen microbial ecology. Science (80-.). 292:1119–1122. doi:10.1126/SCIENCE.1058830.

Saeed, O.A., U.M. Sani, A.Q. Sazili, H. Akit, A.R. Alimon, and A.A. Samsudin. 2023. Profiling of Fatty Acids and Rumen Ecosystem of Sheep Fed on a Palm Kernel Cake-Based Diet Substituted with Corn. Agric. 2023, Vol. 13, Page 643 13:643. doi:10.3390/AGRICULTURE13030643.

Salfer, I.J., C. Staley, H.E. Johnson, M.J. Sadowsky, and M.D. Stern. 2018. Comparisons of bacterial and archaeal communities in the rumen and a dual-flow continuous culture fermentation system using amplicon sequencing. J. Anim. Sci. 96:1059–1072. doi:10.1093/jas/skx056.

Saluzzi, L., C.S. Stewart, H.J. Flint, and A. Smith. 1995. Plasmalogens of microbial communities associated with barley straw and clover in the rumen. FEMS Microbiol. Ecol. 17:47–56.

doi:10.1016/0168-6496(95)00010-8.

Scicutella, F., F. Mannelli, M. Daghio, C. Viti, and A. Buccioni. 2021. Polyphenols and organic acids as alternatives to antimicrobials in poultry rearing: A review. Antibiotics 10. doi:10.3390/antibiotics10081010.

Selin¸sahin, S.S., and M. Bilgin. 2017. Olive tree (Olea europaea L.) leaf as a waste by-product of table olive and olive oil industry: a review. doi:10.1002/jsfa.8619.

Silva, L.G., J. Bunkers, E.M. Paula, T. Shenkoru, Y. Yeh, B. Amorati, D. Holcombe, and A.P. Faciola. 2016. Effects of flaxseed and chia seed on ruminal fermentation, nutrient digestibility, and long-chain fatty acid flow in a dual-flow continuous culture system. J. Anim. Sci. 94:1600–1609. doi:10.2527/JAS.2015-9750.

Skřivan, M., M. Marounek, M. Englmaierová, and E. Skřivanová. 2012. Influence of dietary vitamin C and selenium, alone and in combination, on the composition and oxidative stability of meat of broilers. Food Chem. 130:660–664. doi:10.1016/J.FOODCHEM.2011.07.103.

Soliva, C.R., and H.D. Hess. 2007. Measuring methane emission of ruminants by in vitro and in vivo techniques. Meas. Methane Prod. from Ruminants 15–31. doi:10.1007/978-1-4020-6133-2_2/COVER.

Soto, E.C., E. Molina-Alcaide, H. Khelil, and D.R. Yáñez-Ruiz. 2013. Ruminal microbiota developing in different in vitro simulation systems inoculated with goats' rumen liquor. Anim. Feed Sci. Technol. 185:9–18. doi:10.1016/J.ANIFEEDSCI.2013.06.003.

Staerfl, S.M., M. Kreuzer, and C.R. Soliva. 2010. In vitro screening of unconventional feeds and various natural supplements for their ruminai methane mitigation potential when included in a maize-silage based diet. J. Anim. Feed Sci. 19:651–664. doi:10.22358/JAFS/66338/2010.

Tassone, S., R. Fortina, and P.G. Peiretti. 2020. In Vitro Techniques Using the DaisyII Incubator

for the Assessment of Digestibility: A Review. Anim. an open access J. from MDPI 10. doi:10.3390/ANI10050775.

Theodorou, M.K., B.A. Williams, M.S. Dhanoa, A.B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48:185–197. doi:10.1016/0377-8401(94)90171-6.

Tilley, J.M.A., and R.A. Terry. 1963. A two-stage technique for the in vitro digestion of forage crops. Grass Forage Sci. 18:104–111. doi:10.1111/J.1365-2494.1963.TB00335.X.

Tsantila, N., H.C. Karantonis, D.N. Perrea, S.E. Theocharis, D.G. Iliopoulos, S. Antonopoulou, and C.A. Demopoulos. 2007. Antithrombotic and antiatherosclerotic properties of olive oil and olive pomace polar extracts in rabbits. hindawi.com 36204:11. doi:10.1155/2007/36204.

Tun, H.M., S. Li, I. Yoon, S.J. Meale, P.A. Azevedo, E. Khafipour, and J.C. Plaizier. 2020. Saccharomyces cerevisiae fermentation products (SCFP) stabilize the ruminal microbiota of lactating dairy cows during periods of a depressed rumen pH. BMC Vet. Res. 16:1–17. doi:10.1186/s12917-020-02437-w.

Tzamaloukas, O., M.C. Neofytou, and P.E. Simitzis. 2021. Application of Olive By-Products in Livestock with Emphasis on Small Ruminants: Implications on Rumen Function, Growth Performance, Milk and Meat Quality. Anim. 2021, Vol. 11, Page 531 11:531. doi:10.3390/ANI11020531.

Ungerfeld, E.M., M.F. Aedo, C. Muñoz, N.L. Urrutia, E.D. Martínez, and M. Saldivia. 2020. Inhibiting methanogenesis stimulated de novo synthesis of microbial amino acids in mixed rumen batch cultures growing on starch but not on cellulose. Microorganisms 8. doi:10.3390/microorganisms8060799.

Udén, P., P.H. Robinson, G.G. Mateos, and R. Blank. 2012. Use of replicates in statistical analyses in papers submitted for publication in Animal Feed Science and Technology. Anim. Feed Sci.

Technol. 171:1–5. doi:10.1016/J.ANIFEEDSCI.2011.10.008.

Verge-Mèrida, G., A.C. Barroeta, F. Guardiola, M. Verdú, M. Balart, M. Font-i-Furnols, and D. Solà-Oriol. 2021. Crude and acid oils from olive pomace as alternative fat sources in growing-finishing pigs. Animal 15. doi:10.1016/J.ANIMAL.2021.100389.

Vinyard, J.R., and A.P. Faciola. 2022. Unraveling the pros and cons of various in vitro methodologies for ruminant nutrition: a review. Transl. Anim. Sci. 6. doi:10.1093/TAS/TXAC130.

Van De Vossenberg, J.L.C.M., and K.N. Joblin. 2003. Biohydrogenation of C18 unsaturated fatty acids to stearic acid by a strain of Butyrivibrio hungatei from the bovine rumen. Lett. Appl. Microbiol. 37:424–428. doi:10.1046/j.1472-765X.2003.01421.x.

Wetzels, S.U., M. Eger, M. Burmester, L. Kreienbrock, A. Abdulmawjood, B. Pinior, M. Wagner, G. Breves, and E. Mann. 2018. The application of rumen simulation technique (RUSITEC) for studying dynamics of the bacterial community and metabolome in rumen fluid and the effects of a challenge with Clostridium perfringens. doi:10.1371/journal.pone.0192256.

Wischer, G., J. Boguhn, H. Steingaß, M. Schollenberger, and M. Rodehutscord. 2013. Effects of different tannin-rich extracts and rapeseed tannin monomers on methane formation and microbial protein synthesis in vitro. Animal 7:1796–1805. doi:10.1017/S1751731113001481.

Yáñez-Ruiz, D.R., A. Bannink, J. Dijkstra, E. Kebreab, D.P. Morgavi, P. O'Kiely, C.K. Reynolds, A. Schwarm, K.J. Shingfield, Z. Yu, and A.N. Hristov. 2016. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants-a review. Anim. Feed Sci. Technol. 216:1–18. doi:10.1016/j.anifeedsci.2016.03.016.

Yáñez-Ruiz, D.R., S. Williams, and C.J. Newbold. 2007. The effect of absence of protozoa on rumen biohydrogenation and the fatty acid composition of lamb muscle. Br. J. Nutr. 97:938–

948. doi:10.1017/S0007114507675187.

Yáñez-Ruiz, D.R., N.D. Scollan, R.J. Merry, and C.J. Newbold. 2006. Contribution of rumen protozoa to duodenal flow of nitrogen, conjugated linoleic acid and vaccenic acid in steers fed silages differing in their water-soluble carbohydrate content. Br. J. Nutr. 96:861–869. doi:10.1017/BJN20061927.

Trial 1

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

Abstract

The use of alternative feed ingredients from Agro industry could be an efficient tool to improve the sustainability of dairy cow production. Since the richness in polyphenols (**PP**s), olive oil pomace (**OOP**), produced during olive-oil milling, seems a promising by-product to ameliorate milk 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 multidisciplinary approach. Forty multiparous Italian-Friesian dairy cows, at middle lactation, were randomly allotted into 2 homogenous groups and fed respectively a commercial diet (**CON**) and the experimental diet (**OOPD**) obtained 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 dry matter intake, rumen % DEG and milk production. The milk nutritional quality was improved 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 of rumen liquor (**RL**) biohydrogenation rate of unsaturated FAs. The stochiometric relation between volatile FA production in the rumen and methanogenesis, suggested that OOP lower 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 rumen degradability of the diet or causing strong perturbation of rumen ecosystem and maintaining animal performances.

Key words: By-product, Functional fatty acids, Polyphenols, Biohydrogenation, Rumen ecosystem.

Implications

The use of alternative feed ingredients from 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 is one of the fields in which the possibility to reuse them has been evaluated (Wilkinson and Lee, 2018). Most of food by-products are edible and their nutritional composition is characterized 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 nutritional value because is rich in soluble polyphenols (**PP**; 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 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 stones 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 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 Center “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 body weight (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 2 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 difference between offering and orts. Cows were milked twice daily

(07:00 am; 07:00 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 in five aliquots for i) microbiological analysis, ii) proximate assays, iii) rheological characteristic determination, iv) FA profile characterization, v) PP content and profile determination.

At the end of the trial (29th day), the rumen liquor (**RL**) was individually collected with an oro-esophageal 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 characterization and microbial assays.

Moreover, 2 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 isoproteic and isoenergetic to meet the nutritional requirements of dairy cows at middle lactation according to 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 characterized 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 fiber, ADF, and ADL were determined according to Van Soest et al. (1985) using heat-stable amylase and sodium sulfite. Results were inclusive of residual ash. Metabolizable energy and metabolizable 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 characterization

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 Folin-Ciocalteu method (S Makkar et al., 1996) while the PP profile was determined according to Cecchi et al. (2017). 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 method (1963). Each diet was fermented in triplicates. Crude protein 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:

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

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 24h of fermentation.

In vivo rumen liquor analysis

Rumen liquor fatty acid and dimethylacetals profile, methane production potential

Total rumen lipids were extracted with chloroform/methanol (2:1, vol/vol) as described previously by Folch et al (1957) as follows: In a tube equipped with a teflon-lined screw cap, 2.5 ml of rumen fluid was added with 0.5 ml of 6N HCl to acidify the sample and to permit the free fatty acid extraction and the stabilization of volatile fatty acids. The solution was homogenized with a 2: 1 chloroform-methanol mixture (2.5 ml) and maintained for 1 h in a water bath at 50°C. After, the sample was cooled at room temperature overnight. Then, it was centrifugated at 3000 g for 15 min at 4°C to separate the water and organic phases. The organic phase was collected and transferred in another tube equipped with a Teflon-lined screw cap. The extraction was repeated twice. After, the organic fraction containing the lipid extract was extracted with 2 ml of toluene. The methylation procedure was carried out according to Kramer et al. (1997) as follows: In a culture tube equipped with teflon-lined screw cap, 2 ml of lipid extract in toluene was added with a solution HCl/methanol (10%) and acetyl/Chloride/methanol and heated for 1 h at 80°C. After NaOCH3/Methanol 6M was added and the mixture was newly heated for 15 min at 50°C. The esters were extracted with 2 mL of hexane containing C9 and C19 methyl ester (Sigma Chemical Co., St. Louis, MO) as internal standards and analyzed directly by gas-chromatography as described in Mannelli et al. (2018):

the FAMEs were separated on GC equipped with a capillary column CP-Select CB for FAMEs Varian, Middelburg, the Netherlands: 100 m 0.25 mm i.d., film thickness 0.20 mm. The injector and flame ionization detector temperatures were 270 °C and 300 °C, respectively. The programmed temperature was 40 °C for 4 min, increased to 120 °C at a rate of 10 °C min^{-1}, maintained at 120 °C for 1 min, increased to 180 °C at a rate of 5 °C min^{-1}, maintained at 180 °C for 18 min, increased to 200 °C at a rate of 2 °C min^{-1}, maintained at 200 °C for 1 min, increased to 230 °C at a rate of 2 °C min^{-1} and maintained at this last temperature for 19 min. The split ratio was 1:100 and helium was the carrier gas with a flux of 1 mL/ min. Individual FAMEs were identified by comparison of the relative retention times of FAME peaks from samples, with those of the standard mixture 37 Component FAME Mix (Supelco, Bellefonte, PA, USA). Individual trans9 C18:1, trans11 C18:1, trans12 C18:1, trans13 C18:1 (Supelco), individual cis9, trans11 and trans10, cis12 C18:2

(Matreya Inc.), CLA mix standard (Sigma Chemical Co.) and published isomeric profile (Griinari et al., 1998; Kramer et al., 2004) were used to identify trans C18:1 and CLA isomers of interest.

The dimethylacetals (**DMA**) profile was determined according to the procedure described by Mannelli et al. (2018). Fatty acid contents are expressed as g/100g*10^{-3} of DM while DMA as g/100g of DMA.

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

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

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 Bionformatics

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’- CCTACGGGNBGCASCAG -3’/ Pro805R 5’- GACTACNVGGGTATCTAATCC -3’ (Takashi et al., 2014) for universal bacterial and

archaeal 16S rRNA, and AF-LSU-F 5'- GCTCAAAYTTGAAATCTTMAAG -3'/AF-LSU-R 5'-CTTGTTAAMYRAAAAGTGCATT -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 then 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 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 (qPCR) 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 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. Amplification reactions were carried out in a 10 µL mixture containing 1X Blas Taq qPCR MasterMix (Applied Biological Materials Inc., Richmond, BC, Canada), 400 nM of each primer (Table 2), distilled water (RNase/DNase free, Promega), and 2 µL of ten-fold diluted DNA extracts or standard

DNA, using the ABI StepOneTM real-time PCR system (Applied Biosystems, Foster City, CA, USA). The cycling conditions for each primer set are reported in Table 2. All samples were run in triplicate in optical 96-well plates together with negative control and standard curve. Fluorescent light outputs were collected during each elongation step and analyzed with ABI Step One Plus Real-Time System SDS software (Version 2.3). Optimization assay conditions were performed for each template DNA concentration and primer set. Melting curves were generated after amplification to verify the absence of primer dimers or artifacts. In addition, the amplification products were also checked on 1% agarose gel (w/v).

Standard curves for absolute quantification were created using plasmids containing an amplified fragment of the target gene. The PCR reactions were carried out in a T100 Thermal Cycler (Bio-rad Laboratories, Hertfordshire, UK) in a 25 µL volume containing 1X Xpert Taq reaction buffer (GRiSP Research Solutions, Porto, Portugal), 1.5 mM $MgCl_2$, 250 µM deoxynucleotide triphosphates (dNTPs), 400 nM each primer, and 1U Xpert Taq DNA polymerase (GRiSP Research Solutions). The amplicons were purified by using the PureLinkTMQuick PCR Purification kit (Invitrogen-Life Technologies, Carlsbad, CA, USA) and cloned using the pGEM-T Easy Vector System (Promega, Madison, WI) according to the manufacturer instructions. Transformation of the recombinant plasmid was performed using *Escherichia coli* JM109 high efficiency competent cells (Promega) and transformants were selected on LB/ampicillin plates. Two positive clones were chosen for each gene and subjected to DNA sequencing by BMR Genomics (Padova, IT). The web-based BLAST tool available at the NCBI website (http://www.ncbi.nlm.nih.gov) was used to check specificity. Plasmid DNA was isolated from standard clones using the QIAprep®Spin Miniprep kit (Qiagen, Hilden, Germany) and quantified using NanoDrop™1000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA).

Milk composition and analysis

Proximate profile, microbiological assay, and rheological assay

Crude protein 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 et al. (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).

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

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

The PP content in milk samples (from animals fed CON and OOPD) collected at the beginning and at the end of 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 characterization of their profile.

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

m = 0.04928 ± 4.26439E-4; b = 0.02228 ± 0.00485.

The milk extracts were analyzed to determined PP profile by means 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. The polyphenols content in milk samples from animals fed control diet and treated diet collected at the beginning and at the end of trial (1 and 4 weeks) was determined following the method described by Vázquez et al. (2014). Briefly, 0.5 mL of milk was diluted with 625 μL of methanol solution (methanol water 1:1) and vortexed for 1 minute. Then, the samples were diluted with 31.25 μL of Carrez 1 and 31.25 μL di Carrez 2 solution; after each addition, samples were vortexed for 1 min. Milk solutions were diluted with 312.5 μL of acetonitrile and vortexed for 1 min and, finally, diluted with 62.5 μL di CH_3OH/H_2O (1:1) solution. The samples were stored at room temperature for 30 min to favor proteins precipitation. After, samples were centrifuged at 7800 x g for 15 min at 4°C. Supernatant was withdrawn, transferred in an Eppendorf tube, and stored at -20°C. The extracts were used for the determination of total content of polyphenols and for the characterization of their profile.

Firstly, the analysis of total polyphenols content was carried out using the Folin–Ciocalteu method (Makkar et al., 1996). An aliquot (30 L) of each milk extract was transferred in a 96 multiwall and diluted with 70 μL of bidistilled water. Then, each sample was mixed with 100 μL of a water solution containing 1:10 Folin–Ciocalteu reagent. The samples were stored at room temperature in the dark for 10 min before the addition of 50 μL of 15% sodium carbonate solution. Samples were mixed and stored for further 60 min in the dark at room temperature. After this time, the quantification of samples was carried out measuring the absorbance at 750 nm using a microplate reader (iMark Microplate Absorbance Reader |

Bio-Rad Laboratories, Hercules, California, USA). The reference curve was prepared using increasing amount (2-20 nmoles) of gallic acid (Merck Life Science S.r.l., Italy). The calculated standard-curve parameters were:

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

Secondly, the milk extracts were analyzed to determine polyphenols profile firstly by means HPLC and then by Chromatography–Mass Spectrometry (GC–MS) (Genovese et al., 2022). The HPLC apparatus (Thermo Scientific UltiMate 3000 UHPLC) was equipped with RP-HPLC column (Kinetex Column C18; Phenomenex) with dimensions of 250 x 4.6 mm. The silica packing particles were 5 µm diameter and 100 Å pores. The mobile phase was 10 mM solution of trifluoroacetic acid in water (Solvent A) and 10 mM solution of trifluoroacetic acid in acetonitrile (solvent B). The injection volume was 10 µL and the elution gradient was: 0-10 min, 0% B; 10-25 min, 0-15% B; 25-35 min, 15-40% B; 35-45 min, 40-100% B. Flow rate was 0.8 mL/min. Chromatograms were obtained recording the signal at 330 nm wavelength.

Then, to prepare sugar-free extracts, milk extracts newly were injected in the HPLC apparatus equilibrated at 0% organic solvent and fluxed for 20 min to ensure the elution of sugars and other polar compounds. After this interval, 0% of the solvent A for 10 min, 0-15% of the solvent A for 15 min, 15–40% of the solvent A for 10 min, 40–100% of the solvent A for 10 min were applied to favor the elution of less-polar compounds such as polyphenols (flow = 0.8 mL/min).

For SCAN mode, HPLC sugar free fractions were dried under vacuum, dissolved in 60 µL of 2% methoxyamine hydrochloride in pyridine (Thermo Fisher Scientific, Waltham, MA,

USA), and then incubated at 30 °C for 2 h. After dissolution and reaction, 90 μL N-trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) were added and samples were further incubated at 37 °C for 60 min. Gas chromatographic runs were performed with helium as carrier gas at 0.6 mL/min. The split inject temperature was set to 250 °C and the injection volume of 1 μL. A split ratio of 1:10 was used. The GC oven temperature ramp was from 60 to 325 °C at 10 °C/min. The data acquisition rate was 10 Hz. For the Quadrupole, an Electron Ionization (EI) source (70 eV) was used, and full-scan spectra (mass range from 50 to 600) was recorded in the positive ion mode. The ion source and transfer line temperatures were set, respectively, to 250 and 290 °C. 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.

Statistical analysis

Milk

Milk data (e.g., performances or qualitative profile) recorded throughout the experiment were processed as completely randomized 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; μ is the overall mean; D_i the fixed effect of diet (i = 1 to 2); T_j the fixed effect of sampling time (j = 1 to 4); I_k is the random effect of the cow within the diet (k = 1 to 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 were selected based on Akaike's information criterion of the mixed model of SAS. Statistical significance of the diet effect was tested against 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; μ is the overall mean; D_i the diet (i = 1 to 2) and e_{ij} the residual error. Multiple comparisons among means were performed using the Tukey's test.

Rumen liquor microbial communities

The data on microbial community structure were analyzed 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 nonmetric multidimensional scaling (NMDS) and a permutational multivariate analysis of variance (PERMANOVA) performed with the Bray–Curtis dissimilarity index at 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 analyzed 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 dry matter intake (CON = 24.10 ± 0.15 kg of DM day per head; OOPD = 24.90 ± 0.13kg 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 ± 1.95 kg of DM day per head). Moreover, 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 dimethylacetals 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, 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 characterized by high-throughput sequencing 16S and 28S rRNA gene amplicons. The α-diversity indices (Taxa_S, Shannon, Evenness and Chao1 indices; Figure 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 by the NMDS (Figures 2-3).

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 (Figure 4). 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 % (Figure 5). *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 (Figure 6), *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 specifity, Class *Neocallimastigomycetes*, Family *Neocallimastigaceae* in both CON and OOPD conditions. A total of 6 genera was observed (Figure 7) 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 %).

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 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 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 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 (Figures 8, 9, 10). The post-run 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 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, dry matter 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 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 was 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 respect to those fed CON was observed. At the same time, a comparable C2:0 concentration was found. In rumen, the fiber 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 microrganisms and used by archea in the methanogenesis. The lower 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 of 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, methanogens competitors in using H_2 as 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 utilization as consequence of a less favorable 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 use H_2 to produce ammonia during amino acid fermentation while *Acetobacter* uses H_2 as substrate during the acetogenesis (Wallace et al., 2003; Kersters et al., 2006). Several authors hypothesized 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., 2015). 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 characterization 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.

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 to a C18:1 *trans*11 decrement. Specifically, C18:2 *cis*9*cis*12 biohydrogenation seems to be slowed down either during the *cis*12 isomerization 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 of 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 PP act in 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, the present study, the isomerization in the 12 position and the saturation in the 9 and 15 positions of carbon chain seem 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 biohydrogeantion 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 the process need free carboxyl group (Jenkins et al., 2008).

Considering DMA profile, no great variation was observed confirming that OOP did not strongly perturb 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 characterized 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 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 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 characterized 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 of nutritional value of milk, since these FA 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 respect to CON. Nevertheless, milk fat depression did not occur, and the milk total fat content was comparable between the two groups. The increase of *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 favorite 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 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 of 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 PPs 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 PP may be expelled with the urine or transformed in 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 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 compromised the protein and NDF degradability and the biohydrogenation was modulated toward 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.

Ethics approval

Animal 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 (ENA) database under the accession numbers PRJEB51870. The data that support the study findings are available to reviewers, or available from the authors upon request.

References

Alves SP, Santos-Silva J, Cabrita ARJ, Fonseca AJM and Bessa RJB 2013. Detailed Dimethylacetal and Fatty Acid Composition of Rumen Content from Lambs Fed Lucerne or Concentrate Supplemented with Soybean Oil. PLOS ONE 8, e58386.

AOAC International 1995. Official methods of analysis. Association of Official Analytical Chemists, Washington DC, USA.

Araújo M, Pimentel FB, Alves RC and Oliveira MBPP 2015. Phenolic compounds from olive mill wastes: Health effects, analytical approach and application as food antioxidants. Trends in Food Science & Technology 45, 200–211.

Benincasa C, Pellegrino M, Veltri L, Claps S, Fallara C and Perri E 2021. Dried destoned virgin olive pomace: a promising new by-product from pomace extraction process. Molecules 26, 1–12.

Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA and Gregory Caporaso J 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 1–17.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang K Bin, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik A V., Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R and Caporaso JG 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology 37, 852–857.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA and Holmes SP 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nature methods 13, 581–583.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N and Knight R 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences of the United States of America 108, 4516–4522.

Cappucci A, Alves SP, Bessa RJB, Buccioni A, Mannelli F, Pauselli M, Viti C, Pastorelli R, Roscini V, Serra A, Conte G and Mele M 2018. Effect of increasing amounts of olive crude phenolic concentrate in the diet of dairy ewes on rumen liquor and milk fatty acid composition. Journal of Dairy Science 101, 4992–5005.

Carreño D, Toral G, Belenguer A, Yáñez-Ruiz DR, Hervás G, Mcewan R and Newbold J 2019. Rumen bacterial community responses to DPA, EPA and DHA in cattle and sheep: A comparative in vitro study. Scientific Reports, 9,11857.

Cecchi L, Bellumori M, Cipriani C, Mocali A, Innocenti M, Mulinacci N and Giovannelli L 2018. A two-phase olive mill by-product (pâté) as a convenient source of phenolic compounds: Content, stability, and antiaging properties in cultured human fibroblasts. Journal of functional food, 40, 751-759.

Chen Y, Cheng JJ and Creamer KS 2008. Inhibition of anaerobic digestion process: a review. Bioresource technology 99, 4044–4064.

Clarke KR and Gorley RN, 2015. Getting started with PRIMER v7. Plymouth Routines In Multivariate Ecological Research, 1st edition. Auckland, New Zealand.

Cucu MA, Marhan S, Said-Pullicino D, Celi L, Kandeler E and Rasche F 2017. Resource driven community dynamics of NH4+ assimilating and N2O reducing archaea in a temperate paddy soil. Pedobiologia 62, 16–27.

Dehority B, 2003. Rumen microbiology. Nottingham University Press, Nottingham, NG11 OAX, UK, 2003, Hardcover, ISBN 1-897676-99-9, £ 40, 178-210; 229-329; 193-229.

Dollhofer V, Callaghan TM, Dorn-In S, Bauer J and Lebuhn M 2016. Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi. Journal of microbiological methods 127, 28–40.

Enjalbert F, Combes S, Zened A and Meynadier A 2017. Rumen microbiota and dietary fat: a mutual shaping. Journal of Applied Microbiology 123, 782–797.

Faith DP 1992. Conservation evaluation and phylogenetic diversity. Biological Conservation 61, 1–10.

Fievez V, Colman E, Castro-Montoya JM, Stefanov I and Vlaeminck B 2012. Milk odd-and branched-chain fatty acids as biomarkers of rumen function-An update. Animal Feed Science and Technology 172, 51–65.

Genovese M, Luti S, Pardella E, Vivoli-Vega M, Pazzagli L, Parri M, Caselli A, Cirri P and Paoli P 2022. Differential impact of cold and hot tea extracts on tyrosine phosphatases regulating insulin receptor activity: a focus on PTP1B and LMW-PTP. European Journal of Nutrition 61(4), 1905–1918.

Goldfine H 2010. The appearance, disappearance and reappearance of plasmalogens in evolution. Progress in Lipid Research 49, 493–498.

Hadrova S, Vesely A and Krizova L 2019. Assesment of bovine milk fat quality from the view of human health. Proceedings of the ICAR Conference 2019 , Prague, Czech Republic, Technical Series 24 pp. 217–221.

Hammer DAT, Ryan PD, Hammer Ø and Harper DAT 2001. Past: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4, 178.

Ianni A and Martino G 2020. Dietary grape pomace supplementation in dairy cows: Effect on nutritional quality of milk and its derived dairy products. Foods 9(2), 168, 1–20.

Ishida K, Kishi Y, Oishi K, Hirooka H and Kumagai H 2015. Effects of feeding polyphenol-rich winery wastes on digestibility, nitrogen utilization, ruminal fermentation, antioxidant status and oxidative stress in wethers. Animal Science Journal 86, 260–269.

Jenkins TC, Wallace RJ, Moate PJ and Mosley EE 2008. Board-Invited Review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. Journal of Animal Science 86, 397–412.

Kersters K, Lisdiyanti P, Komagata K and Swings J 2006. The Family Acetobacteraceae: The

Genera Acetobacter, Acidomonas, Asaia, Gluconacetobacter, Gluconobacter, and Kozakia. In The Prokaryotes: a Handbook on the Biology of Bacteria (Editor-in-Chief Dworkin,M., ed. Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E.). Springer, New York, USA, pp. 163–200.

Klieve A V., Hennessy D, Ouwerkerk D, Forster RJ, Mackie RI and Attwood GT 2003. Establishing populations of Megasphaera elsdenii YE 34 and Butyrivibrio fibrisolvens YE 44 in the rumen of cattle fed high grain diets. Journal of applied microbiology 95, 621–630.

Leparmarai PT, Sinz S, Kunz C, Liesegang A, Ortmann S, Kreuzer M and Marquardt S 2019. Transfer of total phenols from a grapeseed-supplemented diet to dairy sheep and goat milk, and effects on performance and milk quality. Journal of Animal Science 97, 1840–1851.

Littell RC, Henry PR and Ammerman CB 1998. Statistical analysis of repeated measures data using SAS procedures. Journal of Animal Science 76, 1216–1231.

Mannelli F, Cappucci A, Pini F, Pastorelli R, Decorosi F, Giovannetti L, Mele M, Minieri S, Conte G, Pauselli M, Rapaccini S, Viti C and Buccioni A 2018. Effect of different types of olive oil pomace dietary supplementation on the rumen microbial community profile in Comisana ewes. Scientific Reports 8(1), 1-11.

Medeiros-De-Moraes IM, Gonçalves-De-Albuquerque CF, Kurz ARM, De Jesus Oliveira FM, Pereira de Abreu VH, Torres RC, Carvalho VF, Estato V, Bozza PT, Sperandio M, De Castro-Faria-Neto HC and Silva AR 2018. Omega-9 oleic acid, the main compound of olive oil, mitigates inflammation during experimental sepsis. Oxidative Medicine and Cellular Longevity 2018, 6053492.

Minato H, Ishibashi S and Hamaoka T 1988. Cellular fatty acid and sugar compositionof representativestrains of rumen bacteria. The Journal of General and Applied Microbiology 34, 303–319.

Minieri S, Sofi F, Mannelli F, Messini A, Piras S and Buccioni A 2020. Milk and conjugated linoleic acid a review of the effects on human health. Topics in Clinical Nutrition 35, 320–328.

Mosley EE, Powell GL, Riley MB and Jenkins TC 2002. Microbial biohydrogenation of oleic acid to trans isomers in vitro. Journal of Lipid Research 43, 290–296.

Moss AR, Jouany JP and Newbold J 2000. Methane production by ruminants: its contribution to global warming. Annales de Zootechnie 49, 231–253.

Muyzer G, De Waal EC and Uitterlinden AG 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695.

Neofytou MC, Miltiadou D, Sfakianaki E, Constantinou C, Symeou S, Sparaggis D, Hager-Theodorides AL and Tzamaloukas O 2020. The use of ensiled olive cake in the diets of Friesian cows increases beneficial fatty acids in milk and Halloumi cheese and alters the expression of SREBF1 in adipose tissue. Journal of Dairy Science 103, 8998–9011.

Neofytou MC, Miltiadou D, Symeou S, Sparaggis D and Tzamaloukas O 2021. Short-term forage substitution with ensiled olive cake increases beneficial milk fatty acids in lactating cows. Tropical Animal Health and Production 53, 1–6.

Papadopoulou A and Frazier RA 2004. Characterization of protein–polyphenol interactions. Trends in Food Science & Technology 15, 186–190.

Raskin L, Stromley JM, Rittmann BE and Stahl DA 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Applied and Environmental Microbiology 60, 1232.

Rico DE, Chouinard PY, Hassanat F, Benchaar C and Gervais R 2016. Prediction of enteric methane emissions from Holstein dairy cows fed various forage sources. Animal 10, 203–211.

S Makkar HP, Goodchild A V, Abd El-Moneim AM and Becker K 1996. Cell-Constituents, Tannin Levels by Chemical and Biological Assays and Nutritional Value of Some Legume Foliage and Straws. Journal of the Science of Food Agriculture 71, 129–136.

Serra V, Salvatori G, Pastorelli G and Romero C 2021. Dietary Polyphenol Supplementation in

Food Producing Animals: Effects on the Quality of Derived Products. Animals 11(401), 1–44.

Servili M, Esposto S, Taticchi A, Urbani S, Selvaggini R, Di Maio I and Veneziani G 2011. Innovation in extraction technology for improved virgin olive oil quality and by-product valorisation. Acta Horticulturae 888, 303–315.

Shook GE 1993. Genetic improvement of mastitis through selection on somatic cell count. The Veterinary clinics of North America. Food animal practice 9, 563–577.

Van Soest PJ and Robertson JB 1985. Polysaccharides in Relation to Animal Nutrition. Journal of Dairy Science 74, 3583–3597.

Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M and Benno Y 2001. Diet-Dependent Shifts in the Bacterial Population of the Rumen Revealed with Real-Time PCR. Applied and Environmental Microbiology 67, 2766.

Takai K and Horikoshi K 2000. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Applied and environmental microbiology 66, 5066–5072.

Takahashi S, Tomita J, Nishioka K, Hisada T and Nishijima M 2014. Development of a Prokaryotic Universal Primer for Simultaneous Analysis of Bacteria and Archaea Using Next-Generation Sequencing. PLOS ONE 9, e105592.

Tilley JMA and Terry RA 1963. A two-stage technique for the in vitro digestion of forage crops. Grass and Forage Science 18, 104–111.

Toral PG, Hervás G, Leskinen H, Shingfield KJ and Frutos P 2018. In vitro ruminal biohydrogenation of eicosapentaenoic (EPA), docosapentaenoic (DPA), and docosahexaenoic acid (DHA) in cows and ewes: Intermediate metabolites and pathways. Journal of Dairy Science 101, 6109–6121.

Tzamaloukas O, Neofytou MC and Simitzis PE 2021. Application of Olive By-Products in Livestock with Emphasis on Small Ruminants: Implications on Rumen Function, Growth Performance, Milk

and Meat Quality. Animals 11, 531.

Vasta V, Daghio M, Cappucci A, Buccioni A, Serra A, Viti C and Mele M 2019. Invited review: Plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: Experimental evidence and methodological approaches. Journal of Dairy Science 102, 3781–3804.

Vázquez CV, Rojas MGV, Ramírez CA, Chávez-Servín JL, García-Gasca T, Ferriz Martínez RA, García OP, Rosado JL, López-Sabater CM, Castellote AI, Montemayor HMA and De La Torre Carbot K 2014. Total phenolic compounds in milk from different species. Design of an extraction technique for quantification using the Folin-Ciocalteu method. Food Chemistry 176, 480–486.

Ventto L, Leskinen H, Kairenius P, Stefański T, Bayat AR, Vilkki J and Shingfield KJ 2017. Diet-induced milk fat depression is associated with alterations in ruminal biohydrogenation pathways and formation of novel fatty acid intermediates in lactating cows. British Journal of Nutrition 117, 364–376.

Wallace RJ, McKain N, McEwan NR, Miyagawa E, Chaudhary LC, King TP, Walker ND, Apajalahti JHA and Newbold CJ 2003. Eubacterium pyruvativorans sp. nov., a novel non-saccharolytic anaerobe from the rumen that ferments pyruvate and amino acids, forms caproate and utilizes acetate and propionate. International Journal of Systematic and Evolutionary Microbiology 53, 965–970.

Wilkinson JM and Lee MRF 2018. Review: Use of human-edible animal feeds by ruminant livestock. Animal 12, 1735–1743.

Zhou M, Hernandez-Sanabria E and Le LG 2009. Assessment of the Microbial Ecology of Ruminal Methanogens in Cattle with Different Feed Efficiencies. Applied and Environmental Microbiology 75, 6524.

Zannoni M and Annibaldi S 1981. Standardization of the rennet ability of milk by Formagraph-I. AGRIS 32, 79–94.

Zhu JJ, Luo J, Wang W, Yu K, Wang HB, Shi HB, Sun YT, Lin XZ and Li J 2014. Inhibition of FASN reduces the synthesis of medium-chain fatty acids in goat mammary gland. animal 8, 1469–1478.

Tables

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/100g 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/100g 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
Others	1.7	1.57	
TFA[10]	2.71	3.94	
Polyphenols profile (g/kg of DM)			
Hydroxytyrosol			11.091
Tyrosol			3.166

[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 fiber.

[7]PS = soluble protein.

[8]ME = metabolisable energy.

[9]MP = metabolisable proteins.

[10]TFA = trans fatty acids.

*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/100g DM;C16:0 13.49 g/100g DM;C18:0 2.27 g/100g DM;C18:1 cis9 71.11 g/100g DM;C18:2 cis9cis12 12.21 g/100g DM;C18:3 cis9cis12cis15 0.99 g/100g DM.

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 15s, 60°C 45s, 72°C 30s 82°C 10s
	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 15s, 61°C 35s, 72°C 45s 83°C 10s
	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 15s, 60°C 45s, 72°C 30s 83°C 10s
	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 15s, 60°C 30s, 72°C 30s, 82°C 10s
	uniMet1-R (Zhou et al., 2009)		
	5’ CGGTCTTGCCCAGCTCTTATTC 3’		
Anaerovibrio lipolytica	ALF (Tajima et al., 2001)		45cycles
	5′ TGGGTGTTAGAAATGGATTC 3′		95°C 15s, 60°C 45s, 72°C 30s 82°C 10s
	ALR (Tajima et al., 2001)		
	5′ CTCTCCTGCACTCAAGAATT 3′		

Butyrivibrio fibrisolvens	BfibF (Klieve et al., 2003)		40 cycles
	5′ ACACACCGCCCGTCACA 3′		95°C 15s, 60°C 30s, 72°C 30s, 81°C 10s
	BfibR (Klieve et al., 2003)		
	5′ TCCTTACGGTTGGGTCACAGA 3′		
Prevotella ruminicola	PRF (Tajima et al., 2001)		40 cycles
	5′ GGTTATCTTGAGTGAGTT 3′		95°C 15s, 60°C 45s, 72°C 30s 80°C 10s
	PRR (Tajima et al., 2001)		
	5′ CTGATGGCAACTAAAGAA 3′		
PCR			
All bacteria		464	35 cycles
(16S rRNA gene)			95°C 30s, 60°C 45s 72°C 45s/72°C 10min
All archaea		461	35 cycles
(16S rRNA gene)			95°C 30s, 60°C 45s 72°C 45s/72°C 10min
Anaerobic fungi		441	35 cycles
(28S rRNA gene)			95°C 30s, 55°C 45s, 72°C 45s/72°C 5min
Methanogens		163	35 cycles
			95°C 30s, 55°C 45s, 72°C 30s/72°C 10min
Anaerovibrio lipolytica		597	35 cycles
			95°C 30s, 61°C 35s, 72°C 60s/72°C 5min
Butyrivibrio fibrisolvens		90	35 cycles
			95°C 30s, 55°C 45s, 72°C 30s/72°C 10min
Prevotella ruminicola		485	35 cycles
			95°C 30s, 57,7°C 35s, 72°C 60s/72°C 5min

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/100g 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	10.428a	4.032b	1.739	0.014
C4:0	132.368a	61.221b	14.402	0.0014
C5:0 iso	18.435a	4.31b	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
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
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
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 = umen odd and branched chain fatty acid profile.

[5]MCFA = rumen medium chain fatty acid profile.

[6]LCFA = umen long chain fatty acid profile.

[7]FA = fatty acids.

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.

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/100g of fat)	Diet		SEM	P		
	CON[1]	OOPD[2]		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
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 x 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) / (anteiso 15:0 + anteiso 17:0).

[10]DI = desaturation index (cis9 14:1 / 14:0 + cis9 14:1).

[11]<16:0, de novo fatty acid (according to Fiviez et al., 2012).

[12]>16:0, preformed fatty acids (according to Chillard et al., 2000 and to Fiviez et al., 2012).

Figures

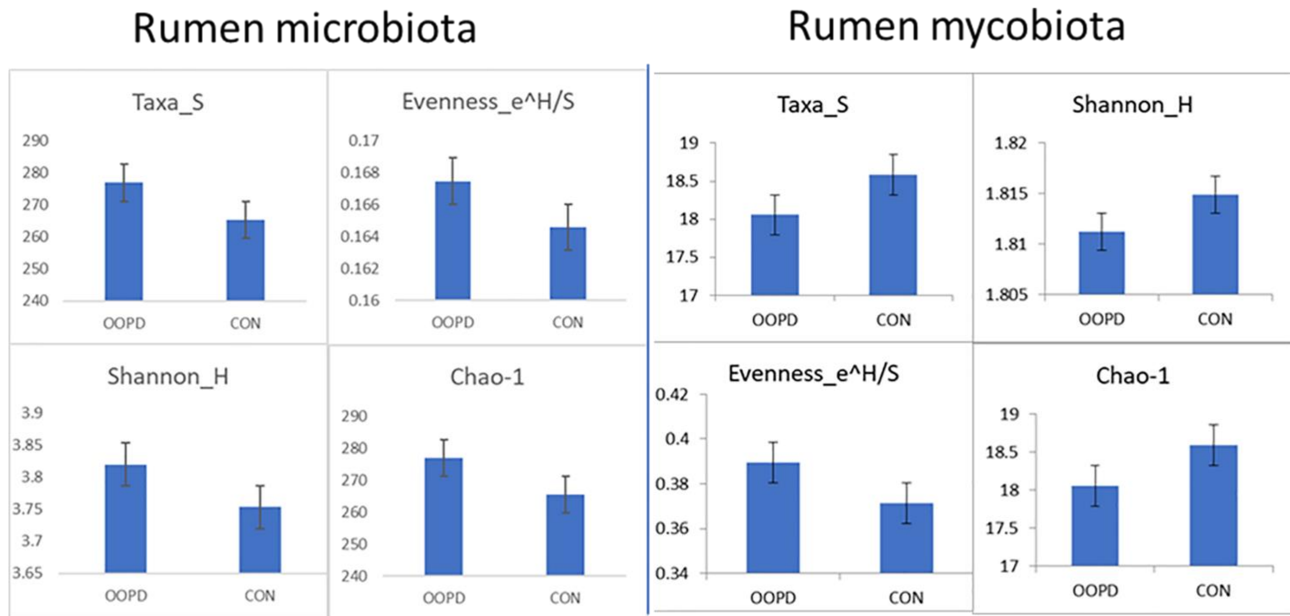


Figure 1. The α-diversity indices (e.g., Taxa_S, Evenness, Shannon and Chao1) of the rumen microbiota A) and mycobiota B) from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

Abbreviations: CON = control diet; OOPD = treated diet, with olive oil pomace.

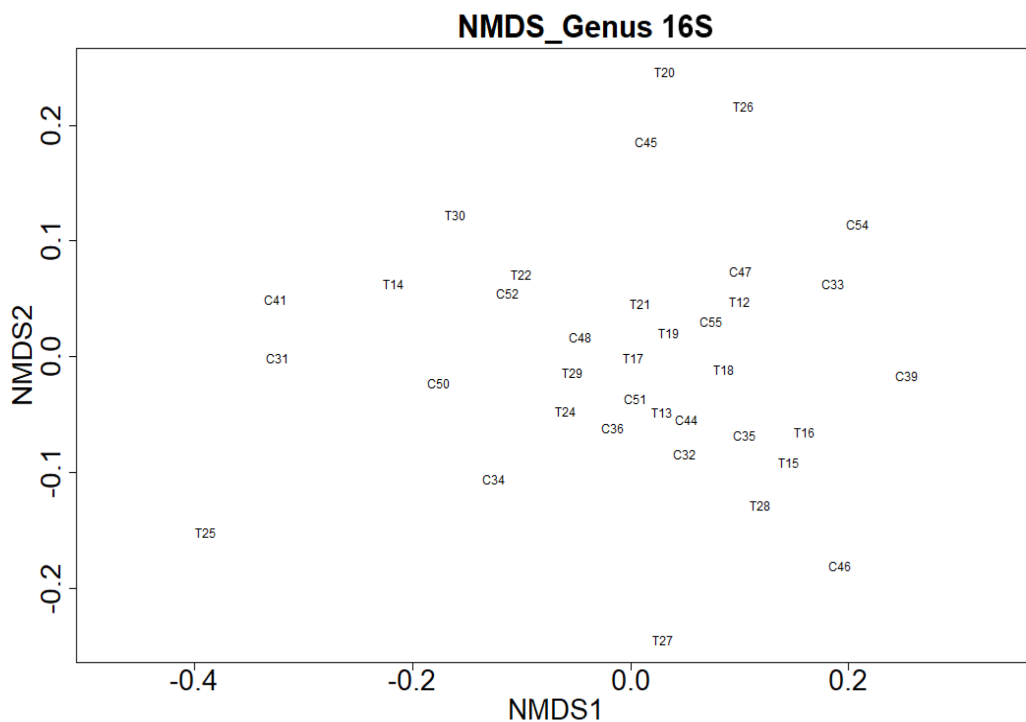


Figure 2. Nonmetric multidimensional scaling visualizations of β-diversity analysis using the Bray–Curtis metric of bacterial and archaeal 16S microbial communities of rumen liquor from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace

Abbreviations: NMDS = Nonmetric multidimensional scaling; C = acronym for control diet; T = acronym for treated diet with olive oil pomace.

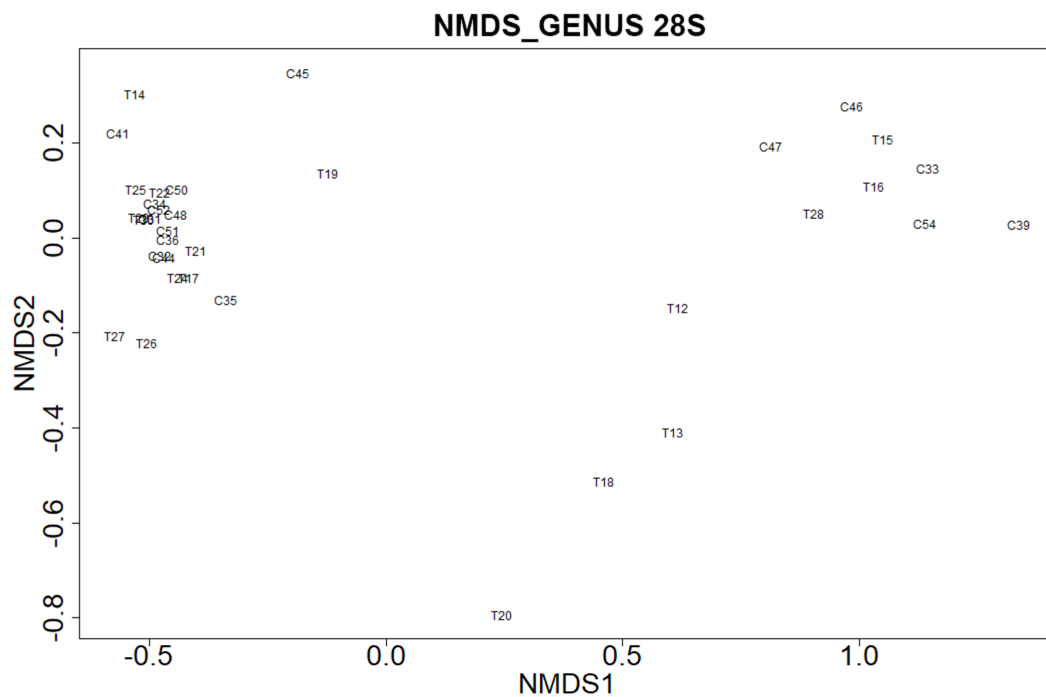


Figure 3. Nonmetric multidimensional scaling visualizations of β-diversity analysis using the Bray–Curtis metric of anaerobic fungal 28S microbial communities of rumen liquor from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace

Abbreviations: NMDS = Nonmetric multidimensional scaling; C = acronym for control diet; T = acronym for treated diet, with olive oil pomace.

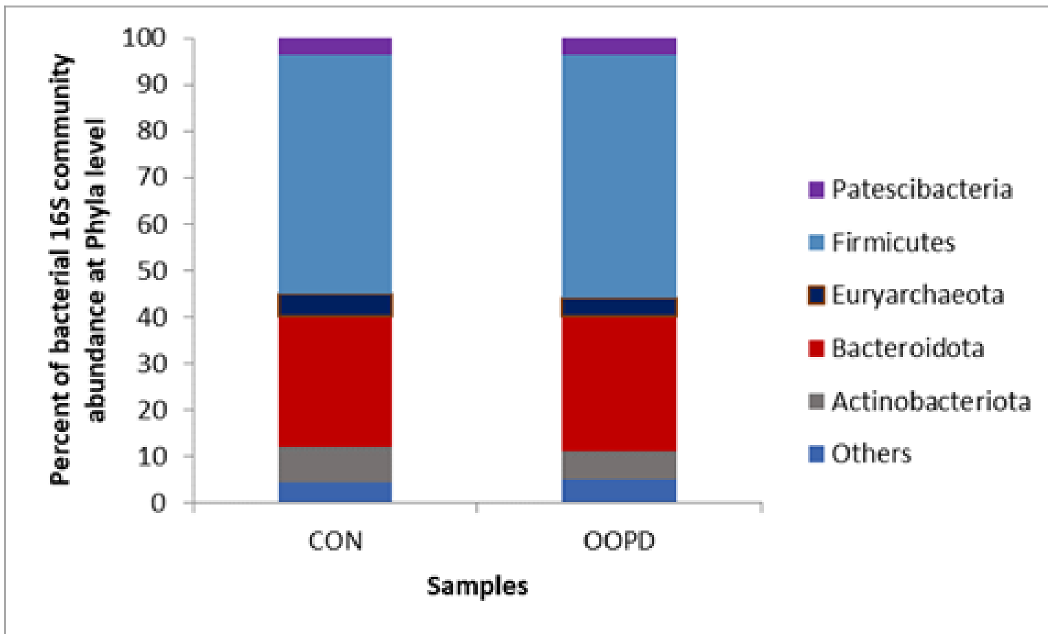


Figure 4. Percent composition of predominant bacterial phyla of rumen liquor from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

Abbreviations: CON = control diet; OOPD = treated diet, with olive oil pomace.

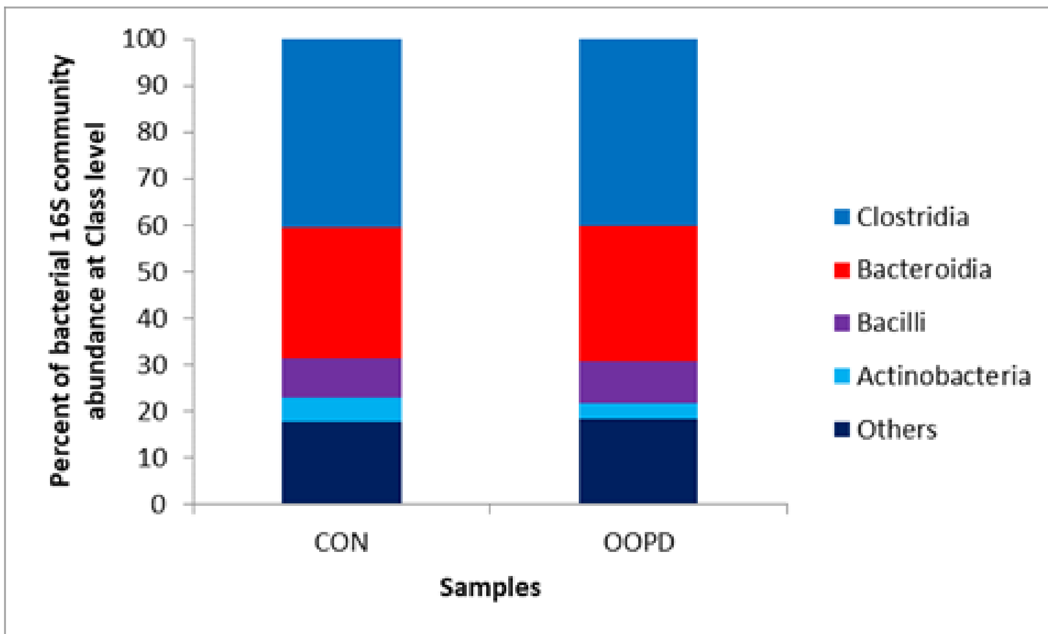


Figure 5. Percent composition of predominant bacterial classes of rumen liquor from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

Abbreviations: CON = control diet; OOPD = treated diet with olive oil pomace.

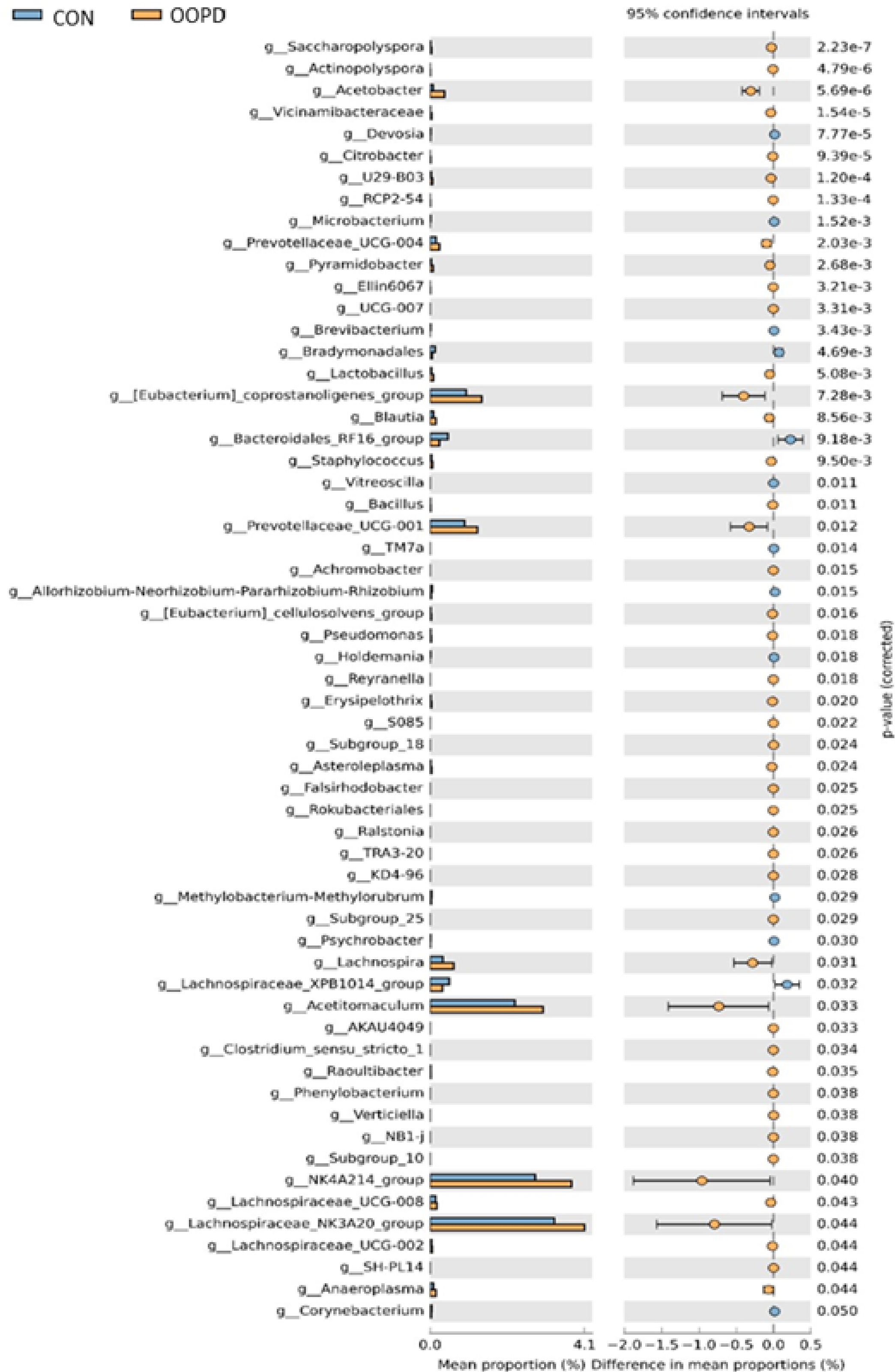


Figure 6. Percent of the genera detected in rumen liquor microbiota from Holstein cows fed with the control diet or the diet supplemented with olive oil pomace and the significant differences.

Abbreviations: CON = control diet; OOPD = treated diet, with olive oil pomace.

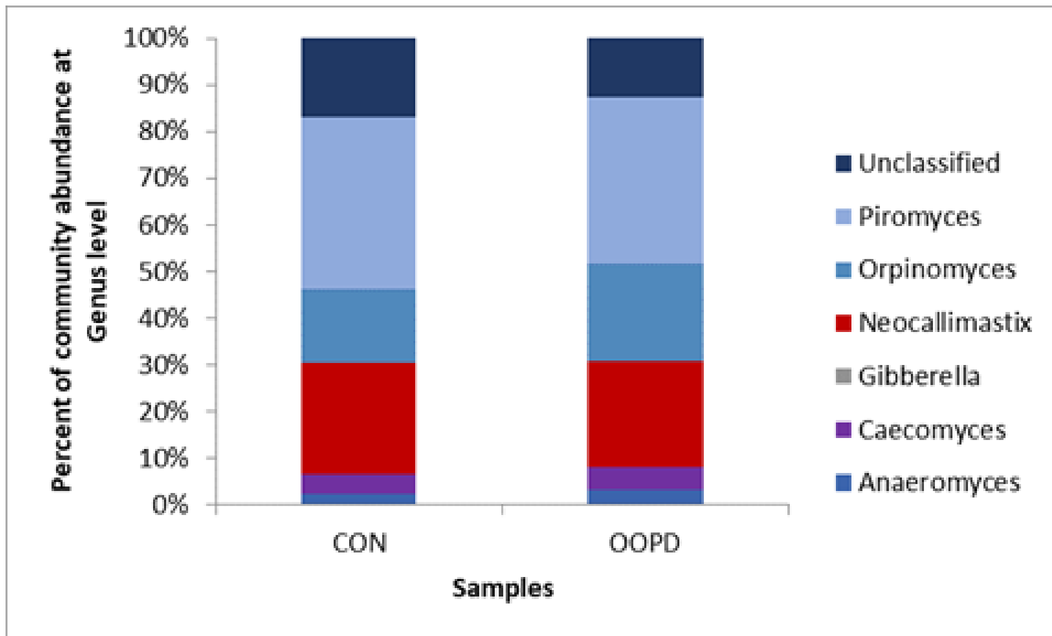


Figure 7. Percent of community abundance at the genus level detected in rumen liquor mycobiota from lactating Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

Abbreviations: CON = control diet; OOPD = treated diet, with olive oil pomace.

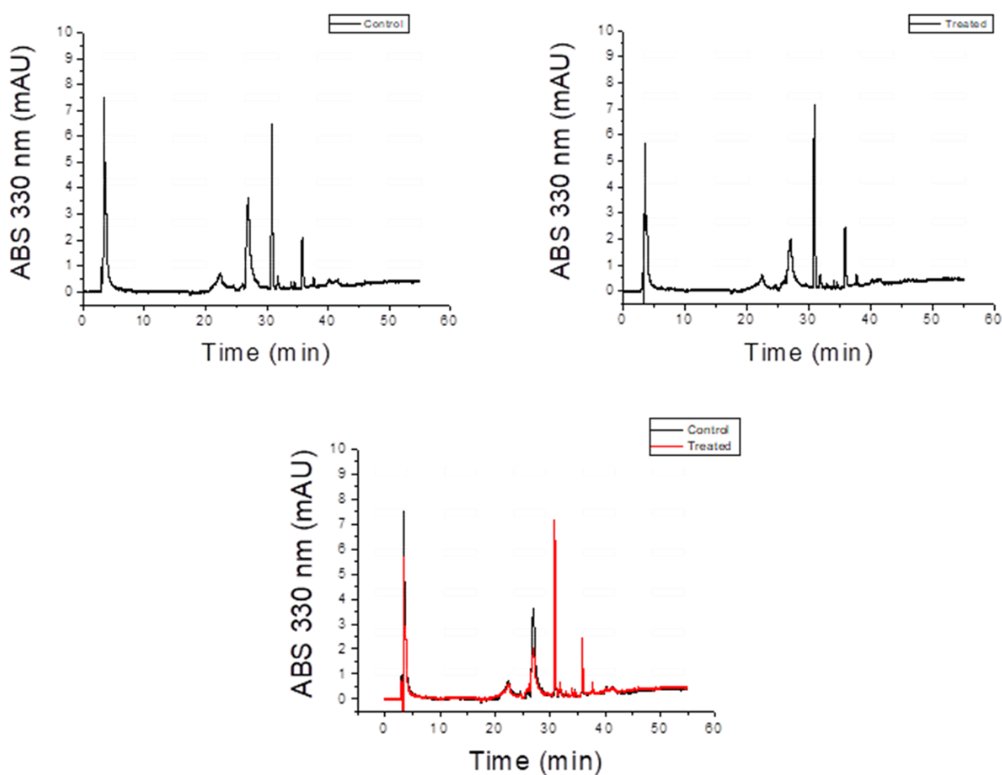


Figure 8. Chromatograms, obtained with HPLC analysis, about polyphenols of milk samples collected after 1 week of treatment from Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

Abbreviations: ABS = absorbance

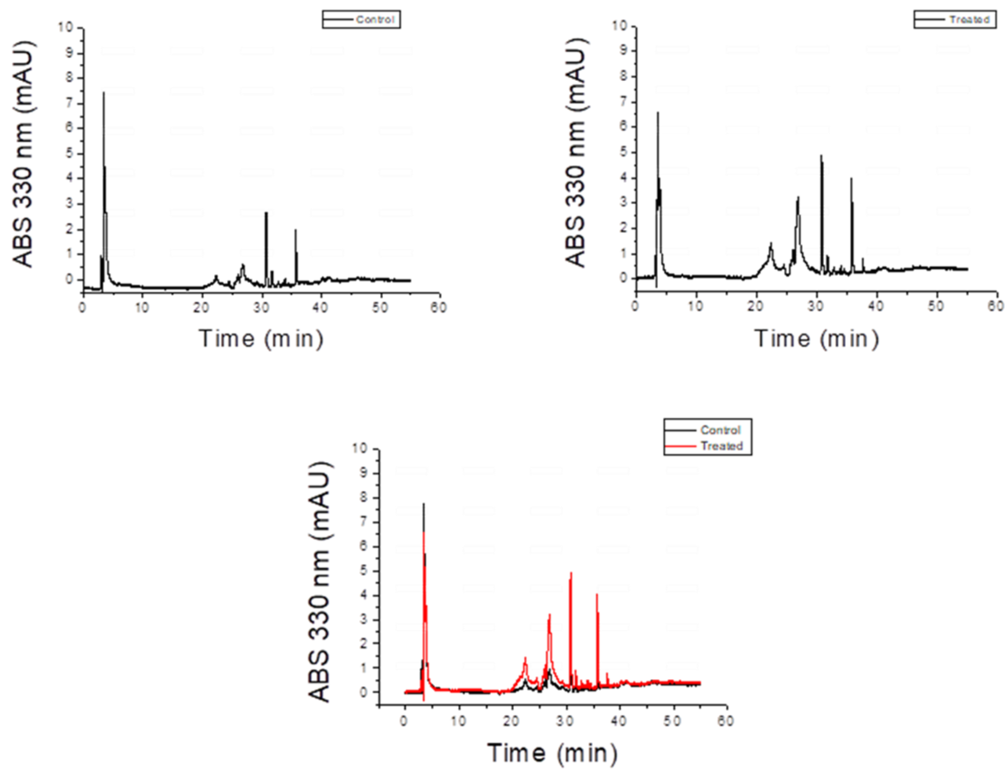


Figure 9. Chromatograms, obtained with HPLC analysis, about polyphenols of milk samples collected after 4 weeks of treatment from Holstein cows fed with the control diet or the diet supplemented with olive oil pomace.

Abbreviations: ABS = absorbance

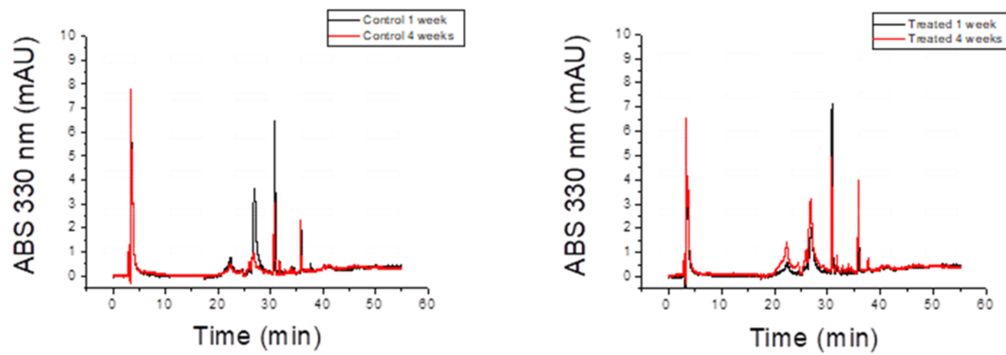


Figure 10. Superimposition of chromatograms, obtained with HPLC analysis, about polyphenols of milk samples collected after 1 and 4 weeks of treatment from Holstein cows fed with the control diet or the diet supplemented with olive oil pomace. The first is related to the control (1st week and 4th week) and the second is related to the treated (1st week and 4th week).

Abbreviations: ABS = absorbance

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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

F. Scicutella[a], M.A. Cucu[b], F. Mannelli[a], R. Pastorelli[b], M. Daghio[a], P. Paoli[c], L. Pazzagli[c], L. Turini[d,e], A. Mantino[d,e], S. Luti[c], M. Genovese[c], C. Viti[a], A. Buccioni[a,f,*]

[a] Dipartimento di Scienze e Tecnologie Agrarie, Alimentari, Ambientali e Forestali. University of Florence, Piazzale delle Cascine 18, 50144 Firenze, Italy
[b] Centro di ricerca Agricoltura e Ambiente, Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA-AA), via di Lanciola 12/A, 50125 Firenze, Italy
[c] Dipartimento di Scienze Biomediche, Sperimentali e Cliniche Mario Serio, Università degli studi di Firenze, 50100 Florence, Italy
[d] Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, Università di Pisa, via del borghetto, 80, 56124 Pisa, Italy
[e] Centro di Ricerche Agro-ambientali "E. Avanzi", Università di Pisa, via Vecchia di Marina, 6, 56122, Pisa, Italy
[f] Centro Interdipartimentale di Ricerca e la Valorizzazione degli Alimenti, University of Florence, viale Pieraccini 6, 50139 Firenze, Italy

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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 (**FAs**; 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*, *Acetitomaculatum*, *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.

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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

* Corresponding author.
E-mail address: arianna.buccioni@unifi.it (A. Buccioni).

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Trial 2

Effect of tree leaves and pomace from olive-oil production chain on rumen microbial communities: an *in vitro* study.

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 characterized 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 c9 c12 c15. 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 c9 c12 c15 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.

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 (Ianni and Martino 2020; Correddu et al. 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 co-products (Molina-Alcaide and Yanez-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 (Khadir et al. (2018). Both are already employed as fertilizers 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 c9 c12c15; LNA), but the quality of the fiber is usually worse than that of OOP (Bahloul et al. 2014; Selin¸sahin 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 hypothesized to replace conventional such as roughage (Bolletta et al., 2022; Innosa et al., 2020), or concentrate ingredients (Chiofalo et al., 2020; Shdaifat et al. 2013). 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, 20A, 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 et al. 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 (1995), respectively. Neutral detergent fiber (NDF), acid detergent fiber (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 and Robertson (1991). Metabolizable energy was calculated according to CNCPS system ver. 6.55.

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 according to Yá˜nez-Ruiz et al. (2016). 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 artifacts. 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 characterization 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 0.01g/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 randomized 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 value < 0.1 and were considered significant for P value < 0.05. A trend towards significance was considered for 0.05 ≤ P value < 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 (Table 4), C10:1 showed a significant feed · time effect and was higher in fermenters with OTLF than in ones with CONL (P value =0.0389) 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 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 5), 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 6). Only DMA 18:1 c12 showed a feed · time effect (P value = 0.0464). 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 value = 0.0500). Instead, the concentration of DMA 15:0 and DMA 16:1 showed a sampling time effect (P value ≤ 0.05). In particular, DMA 15:0 was higher (SEM = 0.876; P value = 0.0009) and DMA 16:1 (SEM = 0.659; P value = 0.0252) was lower at 24h.

Experiment 2

The presence of OOP in the feed lowered C5:0 iso concentration regardless of the sampling time (Table 7). 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 7).

Considering OLCFAs and OBCFAs (Table 7), 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 value =0.0371) 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 8).

The profile of FA involved in the BH pathway of LA and LNA is reported in Table 9. The RL with the inclusion of OOPF showed a higher concentration of t5, t6-8, t9, t10, and c12 C18:1 (P value ≤ 0.05), LA (P value =0.0472), and LNA (P value < 0.0001). 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 9). 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 value = 0.0268), since its concentration was lower in fermenters with OOPF than in ones with CONP. The complete report of DMA data is shown in Table 10.

Characterization of the microbial communities.

Experiment 1

The microbial community was characterized 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 11). Whilst the PERMANOVA evidenced no significant differences of the beta-diversity (R2 = 0.26912; P value = 0.1). Six phyla were identified (Figure 1). 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 2). Twenty-five genera were identified (Figure 3). 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 value = 0.0450) and *Rikenellaceae_RC9_gut_group* (P value = 0.0374) 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 4). The genera *Ruminococcus* and *Treponema* correlated positively with C13:0 iso and C15:0 iso (Figure 4). The genus *Manheimia* correlated negatively with C15:0 iso and C17:0 iso (Figure 4). The genus *Butyrivibrio* correlated negatively with LNA, LA, OA, C18:1 t11, C18:1 t9, SA (Figure 5). The genus *Ruminobacter* correlated positively with LNA, OA, C18:1 t11, C18:1 t9, SA (Figure 5).

Experiment 2

The microbial community was characterized 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 12). Whilst PERMANOVA evidenced no significant differences of the beta-diversity (R^2 = 0.31494; P value = 0.1). Six phyla were identified (Figure 6). The most abundant phyla were *Proteobacteria* (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 7). At the genus level, 21 taxa were identified (Figure 8). The most abundant genera were *Ruminobacter* (CONP ~39 % and OOPF ~42 %) and *Prevotella* (CONP ~12 % and OOPF ~11 %). A lower relative abundance of the genera *Butyrivibrio* (P value = 0.0161), *Fibrobacter* (P value = 0.0104), *Pseudobutyrivibrio* (P value = 0.0103) was observed in OOPF, while the relative abundance of *Christensenellaceae_R-7_group* (P value = 0.0550), *Manheimia* (P value = 0.0450),

Uruburuella (P value =0.0040) 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 9). *Christensenellaceae_R-7_group* and *Manheimia* correlated negatively with C17:0 ante (Figure 9). The genera *Butyrivibrio*, *Fibrobacter*, and *Pseudobutyrivibrio* correlated positively with OA (Figure 10). *Christensenellaceae_R-7_group* correlated negatively with LNA, LA, OA, C18:1 t10, and C18:1 t9 (Figure 10). 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 toward 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-cellulolytic bacteria in the fiber 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 fiber digestion (Suen et al. 2011).

The involvement of *Prevotella* in fiber 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 fiber 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 fiber 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 isomerization 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 isomerization 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 isomerization 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 isomerize or reduce the double bonds of unsaturated FA. Thus, these authors hypothesized 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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References

Anter J, Tasset I, Demyda-Peyrás S, Ranchal I, Moreno-Millán M, Romero-Jimenez M, Muntané J, Luque de Castro MD, Muñoz-Serrano A, Alonso-Moraga Á. 2014. Evaluation of potential antigenotoxic, cytotoxic and proapoptotic effects of the olive oil by-product “alperujo”, hydroxytyrosol, tyrosol and verbascoside. MRGTEM. 772: 25–33. doi:10.1016/J.MRGENTOX.2014.07.002

AOAC International 1995. Official methods of analysis. Association of Official Analytical Chemists, Washington DC.

Bahloul N, Kechaou N, Mihoubi NB. 2014. Comparative investigation of minerals, chlorophylls contents, fatty acid composition and thermal profiles of olive leaves (Olea europeae L.) as by-product. Grasas Aceites 65 (3). doi: http://dx.doi.org/10.3989/gya.0102141.

Bellucci M, Borruso L, Piergiacomo F, Brusetti L, Beneduce L. 2022. The effect of substituting energy crop with agricultural waste on the dynamics of bacterial communities in a two-stage anaerobic digester. Chemosphere. 294. doi: 10.1016/J.CHEMOSPHERE.2022.133776

Benincasa C, Pellegrino M, Veltri L, Claps S, Fallara C, Perri E. 2021. Dried destoned virgin olive pomace: a promising new by-product from pomace extraction process. Molecules. 26: 1–12. doi: 10.3390/molecules26144337.

Bennato F, Martino C, Domenico M Di, Ianni A, Chai B, Marcantonio L Di, Camm C, Martino G. 2022. Metagenomic characterization and volatile compounds determination in rumen from Saanen goat kids fed olive leaves. Vet.Sci. 9(9): 452. https://doi.org/10.3390/vetsci9090452

Berbel J, Posadillo A. 2018. Opportunities for the Bioeconomy of Olive Oil Byproducts. Biomed J Sci & Tech Res. 2(1). DOI : 10.26717/BJSTR.2018.02.000630-

Bolletta V, Pauselli M, Pomente C, Natalello A, Morbidini L, Veneziani G, Granese V, Valenti B.

2022. Dietary olive leaves improve the quality and the consumer preferences of a model sheep cheese. IdairyJ. 134. 105464. https://doi.org/10.1016/j.idairyj.2022.105464

Buccioni A, Antongiovanni M, Petacchi F, Mele M, Serra A, Secchiari P, Benvenuti D. 2006. Effect of dietary fat quality on C18:1 fatty acids and conjugated linoleic acid production: An in vitro rumen fermentation study. Animal Feed Science and Technology. 127:268–282. doi: 10.1016/J.ANIFEEDSCI.2005.09.007

Buccioni A, Minieri S, Rapaccini S, Antongiovanni M, Mele M. 2011. Effect of chestnut and quebracho tannins on fatty acid profile in rumen liquid- and solid-associated bacteria: An in vitro study. Animal 5(10):1521–1530. doi:10.1017/S1751731111000759

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nature methods. 13(7):581–583. doi:10.1038/nMeth.3869

Castellani F, Vitali A, Bernardi N, Marone E, Palazzo F, Grotta L, Martino G. 2017. Dietary supplementation with dried olive pomace in dairy cows modifies the composition of fatty acids and the aromatic profile in milk and related cheese. J. Dairy Sci. 100:8658–8669. https://doi.org/10.3168/jds.2017-12899

Chiofalo B, Di Rosa AR, Lo Presti V, Chiofalo V, Liotta L. 2020. Effect of Supplementation of Herd Diet with Olive Cake on the Composition Profile of Milk and on the Composition, Quality and Sensory Profile of Cheeses Made Therefrom. Animals. 10. 977. https://doi.org/10.3390/ani10060977

Correddu F, Lunesu MF, Buffa G, Atzori AS, Nudda A, Battacone G, Pulina G. 2020. Can agro-industrial by-products rich in polyphenols be advantageously used in the feeding and nutrition of dairy small ruminants? Animals. 10. 131. doi:10.3390/ani10010131

Dehority B. 2003. Rumen microbiology.

Denek N, Can A, Koncagul S. 2006. Use of slaughtered animal rumen fluid for dry matter digestibility of ruminant feeds. J. Anim. Vet. Adv. 5(6): 459-461.

Dherbécourt J, Maillard MB, Catheline D, Thierry A. 2008. Production of branched-chain aroma compounds by Propionibacterium freudenreichii: links with the biosynthesis of membrane fatty acids. Journal of Applied Microbiology. 105:977–985. doi:10.1111/j.1365-2672.2008.03830.x

Difonzo G, Troilo M, Squeo G, Pasqualone A, Caponio F. 2021. Functional compounds from olive pomace to obtain high-added value foods – a review. J Sci Food Agric. 101:15–26. DOI 10.1002/jsfa.10478.

FAO. 2021. Emissions from agriculture and forest land. Global, regional and country trends 1990–2019. ISSN 2709-0078.

Fievez V, Colman E, Castro-Montoya JM, Stefanov I, Vlaeminck B. 2012. Milk odd-and branched-chain fatty acids as biomarkers of rumen function-An update. Animal Feed Science and Technology 172:51–65. doi: 10.1016/j.anifeedsci.2011.12.008

Van Gylswyk NO, Hippe H, Rainey FA. 1996. Pseudobutyrivibrio ruminis gen. nov., sp. nov., a butyrate-producing bacterium from the rumen that closely resembles Butyrivibrio fibrisolvens in phenotype. Int. J. Syst. Bacteriol. 46(2):559–563.

Ianni A, Martino G. 2020. Dietary grape pomace supplementation in dairy cows: Effect on nutritional quality of milk and its derived dairy products. Foods. 9. 168. doi:10.3390/foods9020168

Jenkins TC, Wallace RJ, Moate PJ, Mosley EE. 2008. Board-Invited Review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J Anim Sci. 86:397-412. doi: 10.2527/jas.2007-0588.

Khdair AI, Abu-Rumman G, Khdair SI. 2018. Evaluation the mechanical harvesting efficiency of olive with the application of fruit loosening spray. Agricultural Engineering International: CIGR

Journal. 20:69-75.

Kholif AE, Olafadehan OA. 2021. Essential oils and phytogenic feed additives in ruminant diet: chemistry, ruminal microbiota and fermentation, feed utilization and productive performance. Phytochem Rev. 20:1087–1108. https://doi.org/10.1007/s11101-021-09739-3

Kramer JK, Fellnera V, Dugana MER, Sauera FD, Mossobab MM, Yurawecz MP. 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasison conjugated dienes and total trans fatty acids. Lipids. 32(11):1219–1228.

Littell RC, Henry PR, Ammerman CB. 1998. Statistical analysis of repeated measures data using SAS procedures. J. Anim. Sci. 76:1216–1231.

Lutakome P, Kabi F, Tibayungwa F, Laswai GH, Kimambo A, Ebong C. 2017. Rumen liquor from slaughtered cattle as inoculum for feed evaluation. Animal nutrition. 3:300–308. https://doi.org/10.1016/j.aninu.2017.06.010

Mannelli F, Cappucci A, Pini F, Pastorelli R, Decorosi F, Giovannetti L, Mele M, Minieri S, Conte G, Pauselli M et al. 2018. Effect of different types of olive oil pomace dietary supplementation on the rumen microbial community profile in Comisana ewes. SCiEntiFiC RepoRts. 8:8455. doi:10.1038/s41598-018-26713-w

Marcel M. 2011. Cutadapt removes adapter sequences from high-throughput sequncing reads. EMBnet.journal. 17:10–12. doi:https://doi.org/10.14806/ej.17.1.200

McDougall EI. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochemical Journal. 43(1):100-109.

Molina-Alcaide E, Yanez-Ruiz D. 2008. Potential use of olive by-products in ruminant feeding: A review. Animal Feed Science and Technology. 147: 247-264.

Mosley EE, Powell GL, Riley MB, Jenkins TC. 2002. Microbial biohydrogenation of oleic acid to

trans isomers in vitro. J. Lipid Res. 43: 290–296.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, et al. 2020. vegan: Community Ecology Package. R package version 2.5-7. https://CRAN.R-project.org/package=vegan

Patra AK, Saxena J. 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry. 71:1198–1222. doi:10.1016/j.phytochem.2010.05.010

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic acids research. 35(21):7188–7196. doi:10.1093/nar/gkm864

R Core Team. 2020. European Environment Agency. Retrieved on 11 February 2022, from *https://www.eea.europa.eu/data-and-maps/indicators/oxygen-consuming-substances-in-rivers/r-development-core-team-2006*.

Redondo LM, Dominguez JE, Rabinovitz BC, Redondo EA, Fernández Miyakawa ME. 2015. Hydrolyzable and condensed tannins resistance in Clostridium perfringens. Anaerobe. 34:139–145. https://doi.org/10.1016/j.anaerobe.2015.05.010

Romani A, Ieri F, Urciuoli S, Noce A, Marrone G, Nediani C, Bernini R. 2019. Health effects of phenolic compounds found in extra-virgin olive oil, by-products, and leaf of Olea europaea L. Nutrients. 11. 1776. doi:10.3390/nu11081776

Salami SA, Luciano G, O'Grady MN, Biondi L, Newbold CJ, Kerry JP, Priolo A. 2019 Sustainability of feeding plant by-products: A review of the implications for ruminant meat production. Animal Feed Science and Technology. 251:37-55

Scicutella F, Cucu MA, Mannelli F, Pastorelli R, Daghio M, Paoli P, Pazzagli L, Turini L, Mantino

A, Luti S et al. 2023. Rumen microbial community and milk quality in Holstein lactating cows fed olive oil pomace as part in a sustainable feeding strategy. Animal. 17. 100815. https://doi.org/10.1016/j.animal.2023.100815

Selin¸sahin SS, Bilgin M. 2017. Olive tree (Olea europaea L.) leaf as a waste by-product of table olive and olive oil industry: a review. J. Sci. Food Agric. 98:1271-1279. doi:10.1002/jsfa.8619

Shdaifat M, Al-Barakah F, Kanan A, Obeidat B. 2013. The effect of feeding agricultural by-products on performance of lactating Awassi ewes. Small Ruminant Researc. 113: 11–14

Van Soest PJ, Robertson JB. 1991. Polysaccharides in Relation to Animal Nutrition. J. Dairy Sci. 74(10): 3583-3597.

Suen G, Weimer PJ, Stevenson DM, Aylward FO, Boyum J, Deneke J, Drinkwater C, Ivanova NN, Mikhailova N, Chertkov O et al. 2011. The complete genome sequence of *Fibrobacter succinogenes* S85 reveals a cellulolytic and metabolic Specialist. PLoS ONE 6(4): e18814. doi:10.1371/journal.pone.0018814

Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. 2014. Development of a prokaryotic universal primer for simultaneous analysis of Bacteria and Archaea using next-generation sequencing. PLoSONE 9(8):e105592. doi:10.1371/journal.pone.0105592

Tavella T, Rampelli S, Guidarelli G, Bazzocchi A, Gasperini C, Pujos-Guillot E, Comte B, Barone M, Biagi E, Candela M, Nicoletti C et al. 2021. Elevated gut microbiome abundance of Christensenellaceae, Porphyromonadaceae and Rikenellaceae is associated with reduced visceral adipose tissue and healthier metabolic profile in Italian elderly. Gut Microbes. 13:1. 1880221. doi: 10.1080/19490976.2021.1880221

Tsiplakou E, Zervas G. 2008. The effect of dietary inclusion of olive tree leaves and grape marc on the content of conjugated linoleic acid and vaccenic acid in the milk of dairy sheep and goats. Journal of Dairy Research. 75:270–278. doi:10.1017/S0022029908003270

Vasta V, Daghio M, Cappucci A, Buccioni A, Serra A, Viti C, Mele M. 2019. Invited review: Plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: Experimental evidence and methodological approaches. J. Dairy Sci. 102:3781–3804. https://doi.org/10.3168/jds.2018-14985

Zilio DM, Bartocci S, Giovanni S Di, Servili M, Chiariotti A, Terramoccia S, Zilio DM, Bartocci S, Giovanni S Di, Servili M, Chiariotti A, Terramoccia S. 2014. Evaluation of dried stoned olive pomace as supplementation for lactating Holstein cattle: effect on milk production and quality. Animal Production Science. 55:185–188. doi:10.1071/AN14254

Tables

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 DM)		
Crude protein	15.9	16.2
Ether extract	2.19	2.51
Neutral detergent fiber	42.2	40.5
Acid detergent fiber	30.9	30.0
Lignin	6.52	7.34
Metabolizable energy (Mcal/Kg DM)	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).

Ingredients (% of DM)	**Feed[1]**	
	CONP	OOPF
OOP[2]	0	8.02
Corn 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 profile (g/kg of DM)		
Crude protein	13.97	14.04
Ether extract	3.45	4.82
Neutral detergent fiber	38.67	38.92
Acid detergent fiber	23	24.17
Lignin	4.63	6.44
Metabolizable energy (Mcal/Kg DM)	9.89	9.38
Fatty acid (g/100g fatty acids)		

C14:0	0.19	0.19
C16:0	20.33	19.73
C18:0	4.87	4.7
C18:1 c9	19.19	23.34
C18:2 c9c12	32.31	30.7
C18:3 c9c12c15	17.85	16.49

[1]Feed, CONP control feed from Experiment 2, OOPF treated feed with olive oil pomace inclusion from Experiment 2.

[2]OOP, Olive oil pomace.

*Ferrero Mangimi spa, Via Fornace, 15 12060 Farigliano (CN) Italia.

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

Fatty acid	CONL[1]	OTLF[1]	CONL[1]	OTLF[1]	SEM[2]	P value[3]		
mg/100g of DM	6h		24h			F	T	FxT
C4:0 iso	4.82	4.88	11.11	11.74	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	0.0350	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	0.067
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 (FxT), (P value ≤ 0.05).

Table 4. Rumen fatty acid profile excluded odd-linear chain fatty acids and odd-branched chain fatty acids and C18-fatty acid involved in biohydrogenation pathways of rumen liquor fermented in experiment 1.

Fatty acid	**CONL**[1]	**OTLF**[1]	**CONL**[1]	**OTLF**[1]	**SEM**[2]	**P value**[3]		
mg/100g	**6h**		**24h**			**F**	**T**	**F×T**
C5:0	0.64	2.57	0.64	2.57	0.52	1.00	0.003	1.00
C8:0	10.66	13.36	14.21	13.56	1.68	0.57	0.30	0.36
C10:0	12.28	14.63	14.17	12.35	1.95	0.90	0.93	0.32
C10:1	0.18	0.18	0.134b	0.243a	0.02	0.05	0.70	0.039
C12:0	1.70	1.76	1.64	1.74	0.26	0.77	0.89	0.95
C12:1	2.81	3.35	2.02	1.68	0.49	0.85	0.029	0.40
C14:0	3.04	3.64	3.98	4.62	0.60	0.34	0.14	0.98
C14:1	2.39	2.49	2.84	2.67	0.45	0.94	0.51	0.78
C16:0	52.45	56.72	50.71	40.24	7.32	0.69	0.25	0.35
C16:1	1.13	1.12	1.24	1.08	0.18	0.65	0.83	0.69
C18:4	0.11	0.29	0.30	0.51	0.15	0.22	0.20	0.91
C20:0	1.86	2.04	2.79	3.12	0.42	0.57	0.035	0.87
C20:1	1.09	0.85	0.95	1.17	0.28	0.98	0.77	0.45
C20:2	0.12	0.23	0.21	0.19	0.07	0.53	0.69	0.37
C20:3 n3	0.18	0.14	0.20	0.14	0.08	0.58	0.93	0.94

C20:3 n6	5.57	6.93	5.86	5.00	0.65	0.72	0.25	0.12
C20:4 n6	1.85	1.91	2.02	2.23	0.36	0.73	0.51	0.84
C22:0	0.58	0.54	0.69	0.87	1.49	0.68	0.23	0.53
C22:1	0.71	0.76	0.85	0.56	0.17	0.29	0.77	0.15
C22:6 n3	0.24	0.19	0.46	0.27	0.11	0.21	0.11	0.49
C23:0	2.04	2.26	2.29	2.55	0.09	0.61	0.56	0.96
C24:0 + C22:3	0.19	0.35	0.19	0.25	0.44	0.055	0.42	0.39
C24:1	0.34	0.29	0.21	0.18	0.05	0.32	0.004	0.76

[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 value ≤ 0.05).

Table 5. Fatty acid profile involved in C18-fatty acid biohydrogenation-pathway of the rumen liquor fermented in experiment 1.

Fatty acid mg/100g of DM	CONL[1]	OTLF[1]	CONL[1]	OTLF[1]	SEM[2]	P value[3]		
	6h		24h			F	T	FxT
C18:0	22.00	20.24	52.87	53.68	11.01	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	12.34	19.13	9.73	13.18	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	42.24	47.50	20.33	25.81	4.88	0.27	0.000	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	0.000	0.28
C18:3 c9c12c15	29.42	39.76	13.37	17.72	3.54	0.044	<0.0001	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 (FxT), (P value ≤ 0.05).

Table 6. Dimethylacetals from rumen liquor fermented in experiment 1.

Dimethylacetal g/100g DMA	**CONL[1]**	**OTLF[1]**	**CONL[1]**	**OTLF[1]**	**SEM[2]**	**P value[3]**		
	6h		**24h**			**F**	**T**	**FxT**
DMA 14	8.06	8.71	6.21	5.42	1.39	0.96	0.09	0.62
DMA 15 ante	8.36	7.77	8.92	9.27	1.06	0.91	0.36	0.68
DMA 15	10.96	9.77	13.62	14.42	0.88	0.87	0.001	0.29
DMA 16	30.60	25.99	33.53	33.74	2.46	0.43	0.053	0.36
DMA 16:1	3.67	5.15	3.01	2.44	0.66	0.56	0.025	0.16
DMA 18	22.76	25.80	19.54	21.27	2.95	0.45	0.23	0.83
DMA 18:1 t11	1.64	0.23	1.52	1.27	0.37	0.050	0.25	0.15
DMA 18:1 c9	5.78	4.86	5.88	5.69	0.57	0.35	0.45	0.55
DMA 18:1 c11	4.68	5.17	3.78	3.50	1.02	0.92	0.24	0.72
DMA 18:1 c12	3.51b	6.55aα	3.99	2.99β	0.90	0.34	0.12	0.046

[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 value ≤ 0.05).

Table 7. Odd-branched chain fatty acid profile of the rumen liquor fermented in experiment 2.

Fatty acid mg/100g of DM	CONP[1]	OOPF[1]	CONP[1]	OOPF[1]	SEM[2]	P value[3]		
	6h		24h			F	T	FxT
C4:0 iso	12.98	12.90	43.55	30.02	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	0.000	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	0.00	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 (FxT), (P value ≤ 0.05).

Table 8. Rumen fatty acid profile excluded odd-linear chain fatty acids and odd-branched chain fatty acids and C18-fatty acid involved in biohydrogenation pathways of rumen liquor fermented in experiment 2.

Fatty acid mg/100g	CONP[1]	OOPF[1]	CONP[1]	OOPF[1]	SEM[2]	P value[3]		
	6h		24h			F	T	F×T
C5:0	1.64	1.60	8.64	5.70	1.24	0.24	0.00	0.27
C8:0	11.50	12.50	12.06	11.11	1.79	0.99	0.82	0.60
C10:0	10.65	10.75	10.84	10.30	1.81	0.91	0.95	0.86
C10:1	0.31	0.17	0.04	0.17	0.07	0.93	0.08	0.07
C12:0	1.31	1.39	2.59	3.03	0.30	0.41	0.000	0.56
C12:1	0.83	0.85	1.09	1.05	0.16	0.95	0.17	0.85
C14:0	2.70	3.47	4.81	5.89	0.41	0.040	<0.0001	0.71
C14:1	2.06	1.83	2.08	2.25	0.37	0.94	0.57	0.59
C16:0	38.92	54.72	50.89	66.42	6.34	0.026	0.08	0.98
C16:1	0.97	1.23	1.04	0.94	0.21	0.71	0.62	0.40
C18:4	0.20	0.57	0.28	0.04	0.17	0.70	0.21	0.09
C20:0	1.59	2.72	1.97	1.85	0.57	0.40	0.68	0.30
C20:1	1.64	2.45	1.63	2.28	0.31	0.035	0.79	0.79
C20:2	0.37	0.20	0.34	0.20	0.11	0.17	0.92	0.87
C20:3 n3	0.11	0.38	0.40	0.23	0.15	0.88	0.65	0.15

C20:3 n6	4.62	5.01	4.92	4.29	0.45	0.80	0.66	0.28
C20:4 n6	1.57	2.07	1.90	1.96	0.29	0.36	0.71	0.46
C22:0	0.59	0.59	0.68	0.24	1.50	0.23	0.46	0.23
C22:1	0.63	0.44	0.74	0.56	0.17	0.20	0.43	0.98
C23:0	1.61	1.95	1.93	1.84	0.14	0.71	0.76	0.53
C24:0 + **C22:3**	0.53	0.33	0.48	0.22	0.32	0.07	0.53	0.81
C24:1	0.95	0.40	0.29	0.36	0.12	0.43	0.26	0.30
C22:6 n3	0.88	1.53	0.67	0.35	0.29	0.70	0.12	0.28

[1]CONP, control feed; OOPF, experimental feed formulated 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 value ≤ 0.05).

Table 9. Fatty acid profile involved in C18-fatty acid biohydrogenation-pathway of the rumen liquor fermented in experiment 2.

Fatty acid mg/100g of DM	CONP[1]	OOPF[1]	CONP[1]	OOPF[1]	SEM[2]	P value[3]		
	6h		24h			F	T	FxT
C18:0	21.80	24.52	54.25	73.56	11.65	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	18.81b	67.08aα	15.29b	38.99aβ	4.47	<0.0001	0.003	0.015
C18:1 c11	0.42	0.46	0.80	1.15	0.11	0.11	0.000	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	40.93	63.78	25.77	35.70	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	13.17bα	25.36aα	8.07β	11.49β	1.54	<0.0001	<0.0001	0.01
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, probability of significant effect due to feed (F), sampling time (T), or to the interaction between feed and sampling time (FxT), (P value ≤ 0.05); Within the same feed, means with different Latin superscripts are significantly different; between the two feed, means with different Greek superscripts are significantly different.

Table 10. Dimethylacetals from rumen liquor fermented in experiment 2.

Dimethylacetal g/100g DMA	CONP[1] 6h	OOPF[1] 6h	CONP[1] 24h	OOPF[1] 24h	SEM[2]	P value[3] F	P value[3] T	P value[3] F×T
DMA 14	9.32	13.34	7.61	12.02	2.56	0.10	0.56	0.94
DMA 15 ante	9.90	8.32	6.82	8.85	1.82	0.90	0.49	0.33
DMA 15	9.57	10.13	9.08	9.02	1.70	0.88	0.64	0.86
DMA 16	25.67	25.56	21.71	27.65	2.51	0.25	0.71	0.24
DMA 16:1	3.75	3.25	3.05	4.54	1.05	0.63	0.78	0.36
DMA 18	23.95	26.01	29.44	26.19	2.23	0.80	0.22	0.25
DMA 18:1 t11	0.20	0.37	0.80	0.61	0.27	0.97	0.14	0.52
DMA 18:1 c9	2.13	3.14	4.04	3.46	1.01	0.83	0.28	0.44
DMA 18:1 c11	6.55	5.02	5.73	3.70	1.83	0.32	0.56	0.89
DMA 18:1 c12	8.98	4.88	11.72	3.97	2.58	0.027	0.73	0.49

[1]CONP, control feed; OOPF, experimental feed formulated 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 value ≤ 0.05).

Table 11. 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	0.2322
Shannon Index	4.95	4.66	0.25	0.4364
Chao1 Index	428	431	48	0.9663
Number of ASVs	419	418	46	0.9880

[1]SEM, Standard error mean

[2]P value, probability of significant effect due to feed (P value ≤ 0.05)

Table 12. 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	0.8778
Shannon Index	3.99	3.98	0.22	0.9904
Chao1 Index	346	368	24	0.5491
Number of ASVs	341	363	24	0.5349

[1]SEM, Standard error mean

[2]P value, probability of significant effect due to feed (P value ≤ 0.05)

Figures

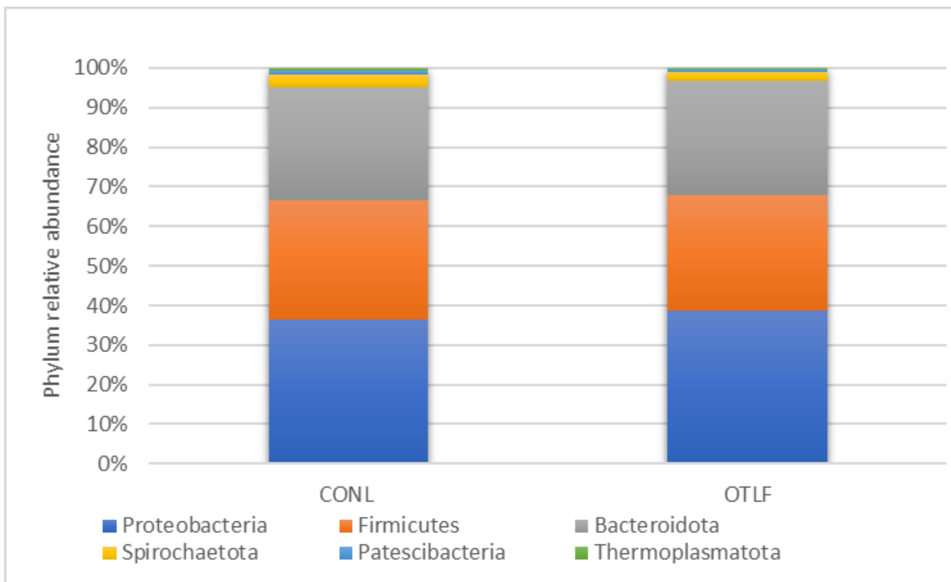

Figure 1. Phyla detected in microbiota from rumen liquor fermented with the control feed without olive tree leaves (CONL) or the treated feed with olive tree leaves (OTLF).

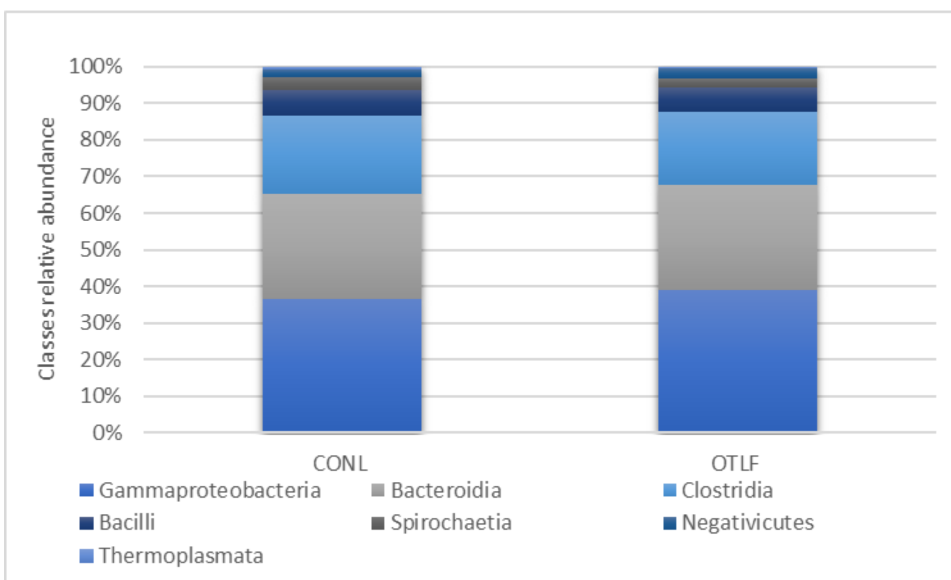

Figure 2. Classes detected in microbiota from rumen liquor fermented with the control feed without olive tree leaves (CONL) or the treated feed with olive tree leaves (OTLF).

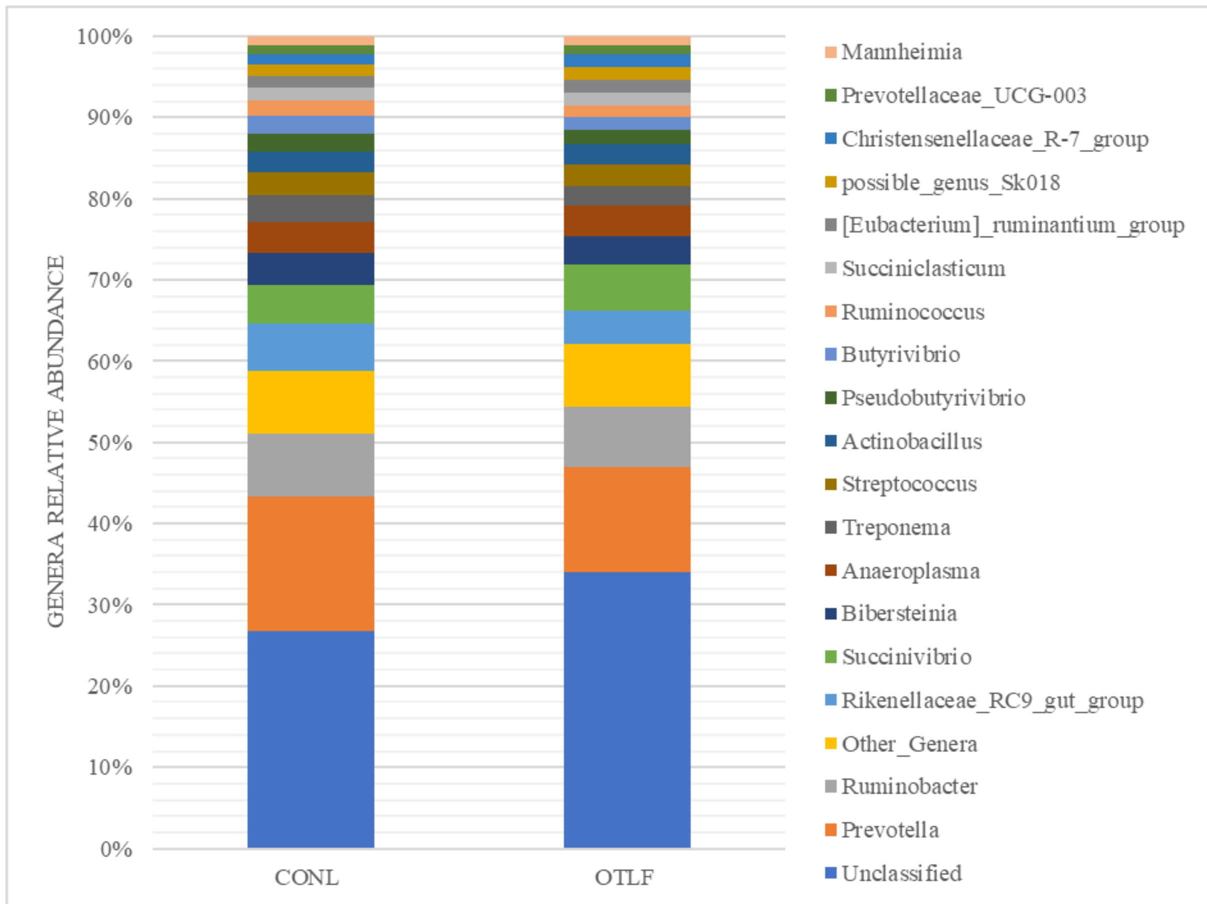

Figure 3. Genera detected in microbiota from rumen liquor fermented with the control feed without olive tree leaves (CONL) or the treated feed with olive tree leaves (OTLF). Only Genera with a relative abundance > 1 % are reported.

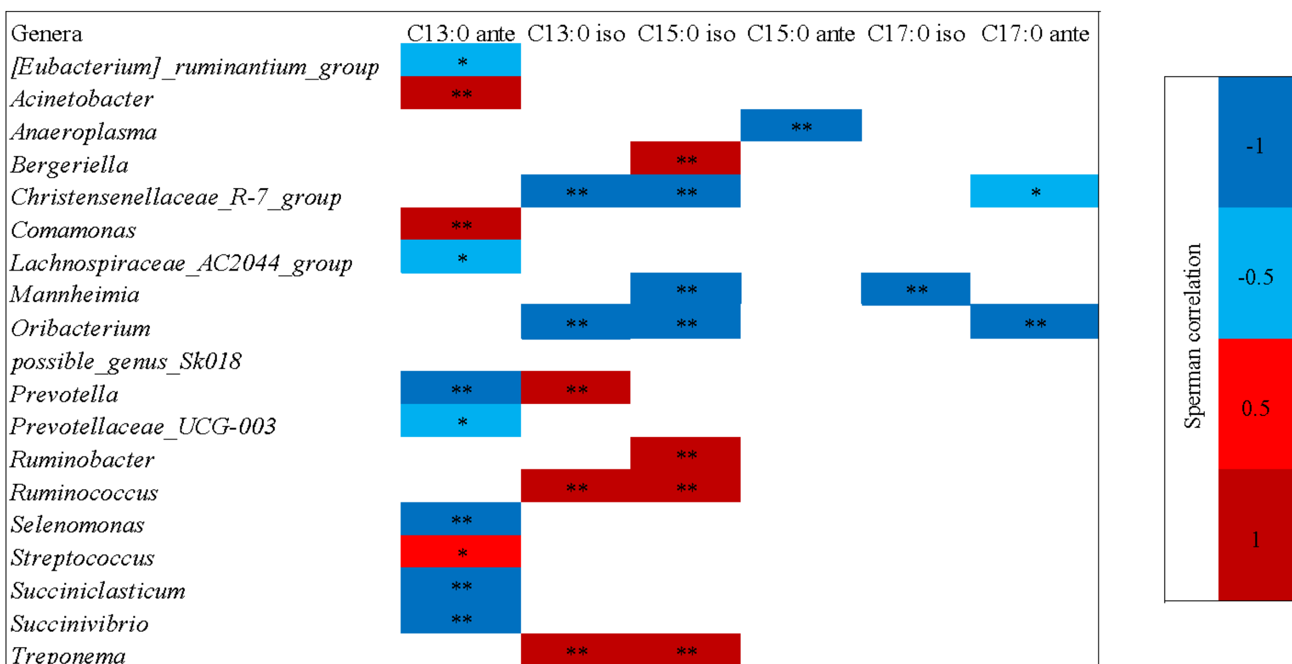

Figure 4. Correlations between odd-branched chain fatty acids and Genera in microbiota from rumen liquor fermented with the control feed without olive tree leaves (CONL) or the treated feed with olive tree leaves (OTLF). Positive correlation is indicated in red and negative correlation is indicated in blue (* for -0.5 < P value < 0.5; ** for P value < -0.5 and P value > 0.5). Only Genera with a relative abundance > 0.1 % were correlated.

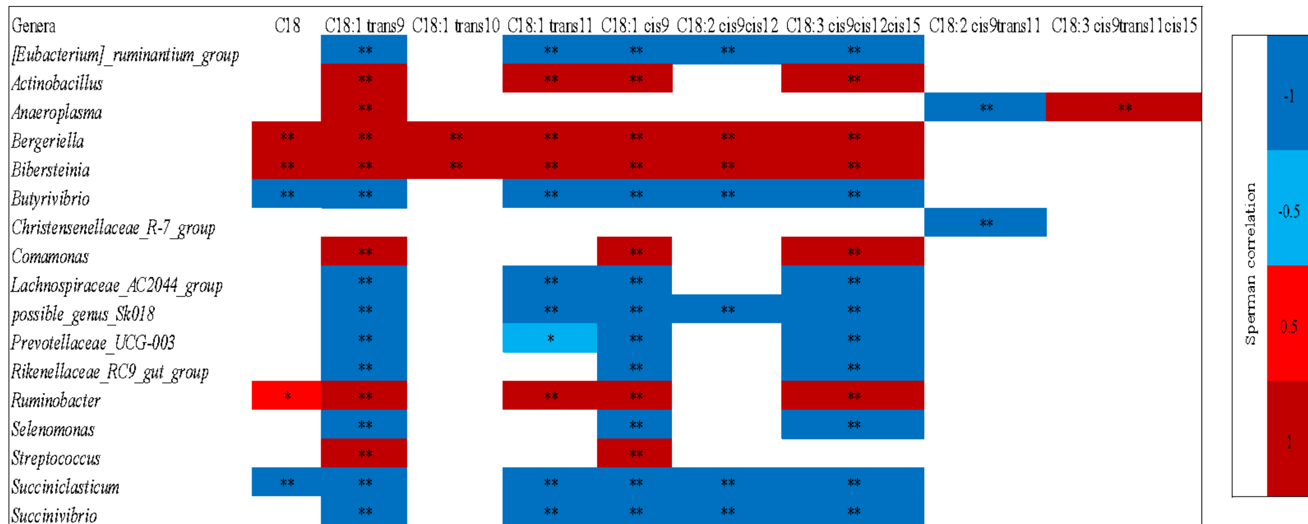

Figure 5. Correlations between C18 biohydrogenation chain and Genera detected in microbiota from rumen liquor fermented with the control feed without olive tree leaves (CONL) or the treated feed with olive tree leaves (OTLF). Positive correlation is indicated in red and negative correlation is indicated in blue (* for -0.5 < P value < 0.5; ** for P value < -0.5 and P value > 0.5). Only Genera with a relative abundance > 0.1 % were correlated.

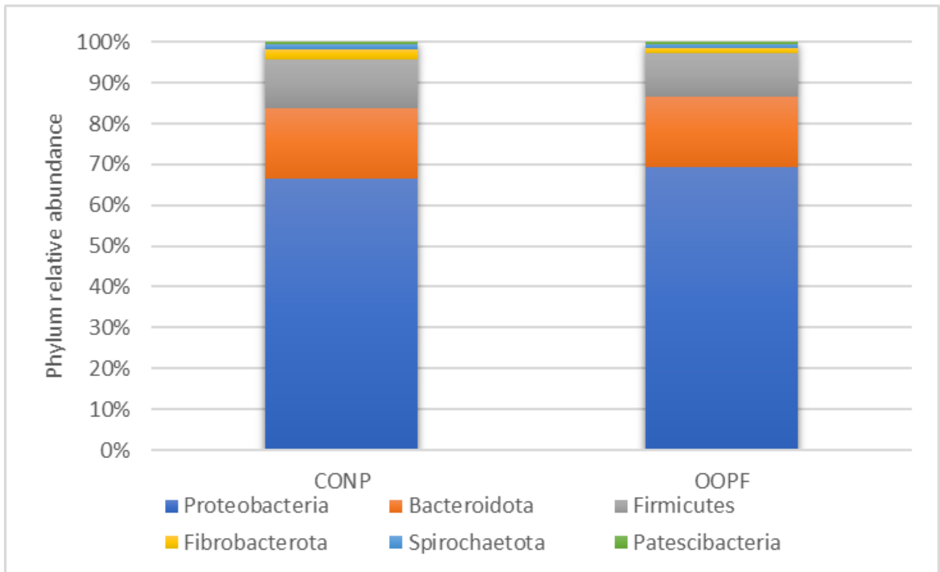

Figure 6. Phyla detected in microbiota from rumen liquor fermented with the control feed without olive oil pomace (CONP) or the treated feed with olive oil pomace (OOPF).

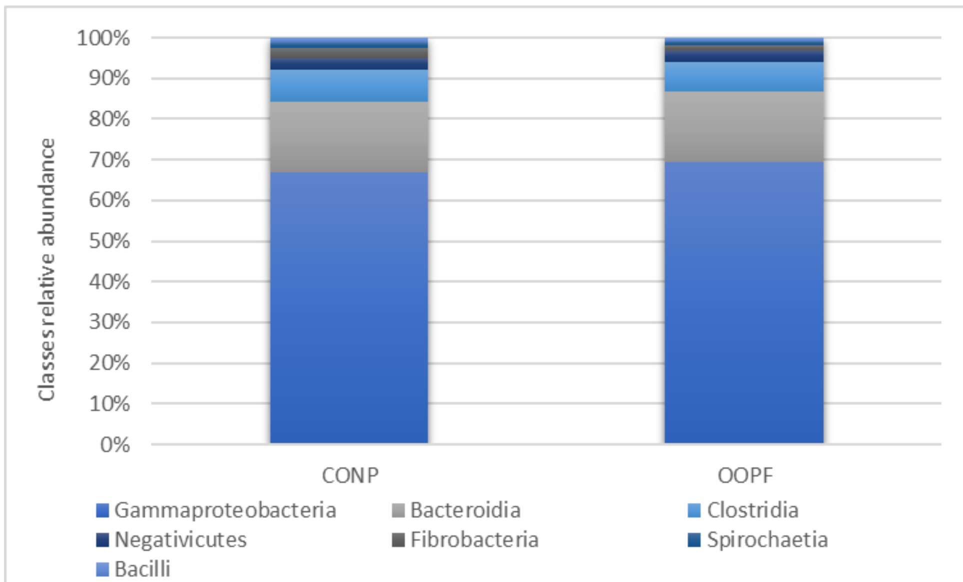

Figure 7. Classes detected in in microbiota from rumen liquor fermented with the control feed without olive oil pomace (CONP) or the treated feed with olive oil pomace (OOPF).

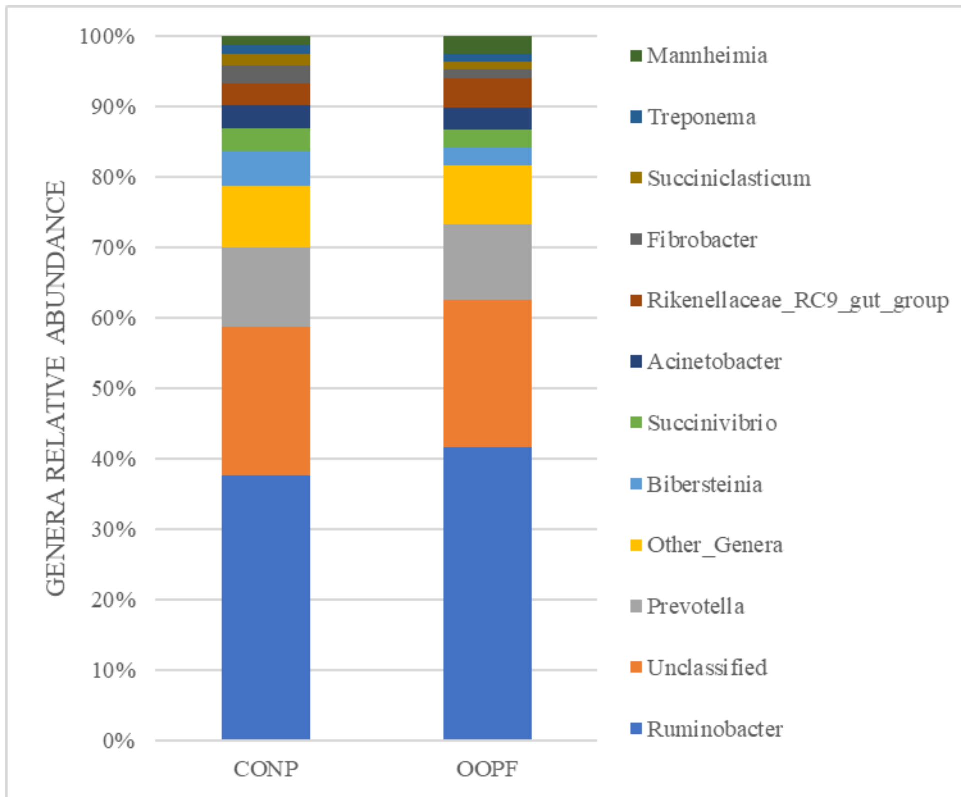

Figure 8. Genera detected in in microbiota from rumen liquor fermented with the control feed without olive oil pomace (CONP) or the treated feed with olive oil pomace (OOPF). Only Genera with a relative abundance > 1 % are reported.

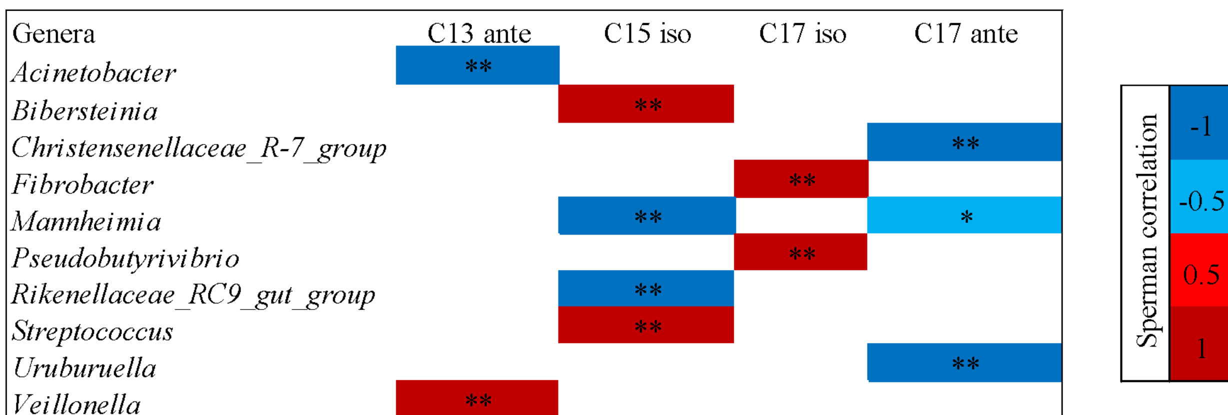

Figure 9. Correlations between odd-branched chain fatty acids and Genera in microbiota from rumen liquor fermented with the control feed without olive oil pomace (CONP) or the treated feed with olive oil pomace (OOPF). Positive correlation is indicated in red and negative correlation is

indicated in blue (* for -0.5 < P value < 0.5; ** for P value < -0.5 and P value > 0.5). Only Genera with a relative abundance > 0.1 % were correlated.

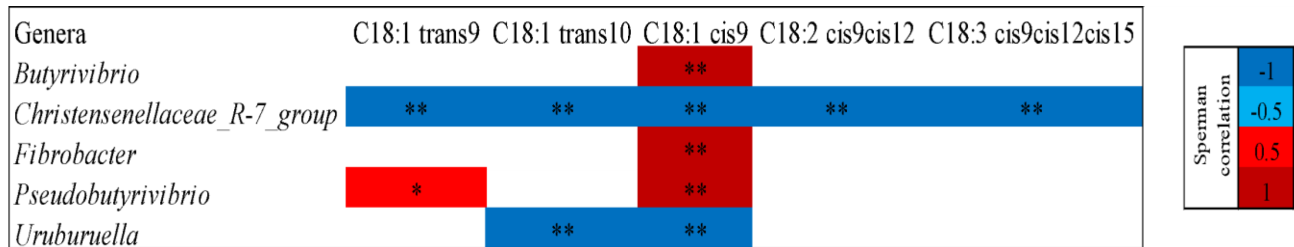

Figure 10. Correlations between C18 biohydrogenation chain and Genera in microbiota from rumen liquor fermented with the control feed without olive oil pomace (CONP) or the treated feed with olive oil pomace (OOPF). Positive correlation is indicated in red and negative correlation is indicated in blue (* for -0.5 < P value < 0.5; ** for P value < -0.5 and P value > 0.5). Only Genera with a relative abundance > 0.1 % were correlated.

Conclusions

The thesis was carried out to evaluate the possible use of by-products from the olive oil production in feeding strategy for dairy ruminants. The richness in PUFA, such as OA and LNA, and PPs as oleuropein or hydroxytyrosol and tyrosol determines the high quality of the nutritional profile of post-milling leaves and olive oil pomace.

Olive oil pomace at 8% on DM was evaluated in the dairy cow feeding strategy using a multifunctional approach. Productive performance as milk quantity and milk proximate profile were not affected by the alternative feed ingredient inclusion, and results were consistent with typical values for dairy cows at middle lactation. The possible detrimental effect of PPs expected *in vivo* did not occur. In fact, no alteration on DMI (caused by the strong flavour of PPs) or on milk yield (caused by the lack of nutrients absorption for protein binding by PPs) were observed. Moreover, the milk nutritional quality was improved increasing the content of several important functional FAs (i.e., C18:2c9c12, C18:2c9t11, C18:2c9, C18:1t11). Certainly, the quality of the diet and the mammary gland physiology affected this finding, but also PPs involvement could be suppose in rumen metabolism in a selective manner (Vasta et al. 2019). At the rumen level, the increment of PUFAs was related to a decrease of biohydrogenation rate. Considering FAs and rumen bacteria as biomarkers of rumen metabolism, an analysis on rumen microbial community was conducted using a metagenomic approach. In fact, data on rumen microbiota highlighted finding discussed at now. No differences between groups were observed (see α- and β-diversity) and the only differing taxonomic level is the genus one. Interesting, the abundance of the genera *Acetobacter* and *Eubacterium* increased with OOP dietary inclusion. *Eubacterium* is an ammonia-hyperproducing genus that use H_2 to produce ammonia during amino acid fermentation while *Acetobacter* uses H_2 as substrate during the acetogenesis (Wallace et al., 2003; Kersters et al., 2006). They are normally thermodynamically less efficient in H_2 utilization as consequence of a less favourable Gibbs free energy (-32.68 kj/mol H2 vs -24.94 kj/mol H2; pH = 7).

The *in vitro* experiment involving OOP as feed alternative ingredient confirmed the dietary effect on the quality of rumen liquor fatty acid profile (OA) and the perturbative effect of PPs on rumen metabolism exerted in a selective manner. In fact, beyond the PUFA rumen liquor content increment linked to a general lowering of BH rate, the isomerization of several cis form to the trans one seems to be lower in several pathway than other (C18:1 c12 to C18:1 t11 or C18:1 c9 to C18:1 t9).

Otherwise, when OTL were fermented as feed alternative ingredient, the OA content increased in rumen liquor as dietary effect on the rumen FA profile. As above mentioned, the perturbative effect of PPs on rumen metabolism was observed, despite the way of action is slightly different and less evident. Since PPs effect depend on their own chemical structure, probably OOP and OTL act differently on the base of the PPs profile.

In vitro trial did not evidence the effect of OOP on rumen microbial community, however the observed effect *in vivo* evidenced slightly differences between the control and the treated group. Probably, the level of inclusion of the alternative ingredients tested were well fitted for improving FA profile without altering animal metabolism and the *in vitro* approach, being only likely to the living animal, need to be stressed for highlighting the same finding.

Concluding, by-products of the olive oil milling process are rich in functional molecules such as PPs that can modulate rumen fermentation and improve the ruminant quality of the end products. Particularly, destoned and fresh olive oil pomace from a two-phase milling process, with a high PPs and PUFA content, may be successfully included in the feeding strategy of dairy cows. Not only, the animal performances were not altered but milk nutritional quality increased because of OA, LA, VA and CLA high content. Moreover, focusing on the rumen metabolism, olive tree leaves and olive oil pomace affect FA profile and microbial community ratio in different ways, confirming the modulating effect of agro-industrial co-products applied as alternative ingredients in ruminant feeding strategy thanks to their richness in functional molecules.