Effect of olive leaves feeding on phenolic composition and lipolytic volatile profile in goat milk

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ABSTRACT

The aim of this study was to evaluate phenolic composition, antioxidant potential, and lipolytic events in raw milk obtained from goat fed a dietary supplementation with olive leaves (OL), a by-product of the olive oil production chain. For this purpose, 30 Saanen goats were randomly allocated into 2 groups of 15 goats each: the control group received a standard diet that was prepared by taking into account the nutritional needs of lactating goats, whereas the experimental group (EG) was fed with an OL-supplemented diet (10% on a dry matter basis). At the end of the 30 d of the trial, the individual milk samples were collected and immediately analyzed for total phenolic content and antioxidant activity (AOA). Subsequently, the individual phenolic compounds have been identified and quantified through an ultra-high-performance liquid chromatography system and a characterization of free fatty acids released in milk has been performed. The results showed a positive effect of dietary OL supplementation in improving total phenolic content and AOA; furthermore, 19 phenolic compounds, including phenolic acids, flavonoids, simple phenols, and secoiridoids, have been identified in EG milk. In addition to this, a reduced accumulation of free fatty acids has been found in EG milk, and this finding leads us to hypothesize an inhibitory action of the identified phenolic compounds toward the enzymes responsible for lipolytic events. The use of the molecular docking approach verified the interactions, defining a fairly interesting framework for cinnamic acid, which should be able to noncovalently bind these enzymes, interfering with the recruitment of the substrate and therefore, slowing down their hydrolytic activity. In any case, this information will be subjected to in vitro evaluations for an accurate characterization of the biochemical mechanisms that can be established in milk naturally enriched with bioactive compounds.

Key words: goat milk, olive leaves, phenolic compounds, lipolysis, molecular docking

INTRODUCTION

Olive oil production represents one of the most widespread and developed agricultural sectors in the Mediterranean area. According to data collected and disseminated by the International Olive Oil Council (IOOC, 2017), over half of global olive oil production takes place in Europe, with Spain, Italy, Greece, and Turkey representing the most productive countries. As evidenced in other agro-industrial sectors, this activity is responsible for the production of considerable amounts of by-products, the disposal of which represents a critical issue with environmental and economic implications. The by-products derived from olive oil production vary in proportion according to the production methods and generally include olive pomace, olive mill wastewaters, and residues of tree pruning and olive defoliation (Souilem et al., 2017). These waste matrices are generally associated with a high biotechnological potential (Ramachandran et al., 2007; Federici et al., 2009), mainly due to the presence of bioactive compounds, especially polyphenols, to which are attributed marked antioxidant and anti-inflammatory functions (Tsao, 2010). For this reason, numerous studies have been published in recent years that focus on the recovery and valorization of these by-products as potentially useful matrices in both human and livestock nutrition (Berbel and Posadillo, 2018; Difonzo et al., 2021. Especially in the zootechnical field, the development of feeding strategies based on the use of by-products of olive oil production evidenced a general improvement in the nutritional properties of animal products (Castellani et al., 2017; Chiofalo et al., 2020) and positive effects on animal health (Iannaccone et al., 2019).

With specific regard to olive leaves (**OL**), there are currently applications in the cosmetic and pharmaceutical sectors, as well as in the food industry for the production of additives characterized by antimicrobial, antioxidant, and anti-inflammatory activities (Şahin et al., 2017). All these properties represent the direct consequence of the content of well-characterized phenolic bioactive compounds endowed of high biological value, such as oleuropeosides, flavones, caffeic acid, tyrosol, and hydroxytyrosol (Rahmanian et al., 2015).

What has been reported justifies the high interest that has developed in the last 2 decades in using OL as an ingredient for feeding farm animals. In particular, recent studies focused their attention on dairy ruminants, highlighting the effectiveness of OL intake in inducing an improvement in the global quality of milk and derived cheeses (Molina-Alcaide and Yáñez-Ruiz, 2008; Innosa et al., 2020a,b), with positive effects especially in the shelf-life of ripened dairy products, presumably as a consequence of the improvement in the oxidative balance induced by dietary phenolic compounds.

However, the potential positive effects of plant polyphenols in animals are widely dependent on their cellular distribution, and metabolism after absorption must be taken into account (Cosme et al., 2020). In this context, a great interest has been developed in investigating the in vivo bioavailability and bioactivity of these compounds in different farm animals, both monogastrics and ruminants. Chedea et al. (2019) reported that a 5% dietary supplementation with red grape pomace is beneficial for piglet health by increasing the polyphenol content in blood plasma and the antioxidant activity (**AOA**) in different organs. In regard to polyphenols coming from olive oil wastes, a study was recently conducted that aimed to evaluate the oxidative status and the presence of polyphenolic compounds in cheese obtained from sheep fed with a spray-dried olive mill wastewater (Branciari et al., 2020).

Considering the lack of studies on the effect of OL in goats, this trial focused on the determination of the phenolic composition in milk obtained from goats that received a 10% dietary OL supplementation. The objective of this study was to introduce useful information to understand the ability of goats to metabolize the OL phenolic compounds taken by diet, and verify the possibility that these compounds, or their secondary metabolites, can reach the mammary gland and then milk, resulting in a food product with improved health functionality.

MATERIALS AND METHODS

The experimental design has been prepared in accordance with Directive 2010/63/EU of the European Parliament (European Union, 2010) and Directive 86/609/EEC (European Economic Community, 1986), which deal with the protection of animals used for scientific purposes. The experiment was performed in a commercial company whose routine protocol is to enrich the diet of lactating goats with residues obtained from olive tree pruning during the spring months. For this reason, no breeding practices other than those normally adopted have been introduced.

Animals, Feeding Protocol, and Sampling

The applied experimental plan was previously reported (Innosa et al., 2020b). Briefly, 30 lactating Saanen goats, homogeneous for age, weight, lactation days, milk yield, and body condition, were divided into 2 groups of 15 animals each: a control group (**CG**), and an experimental group (**EG**) whose diet was supplemented with OL. Specifically, all animals received polyphite hay ad libitum, and a custom-formulated concentrate was daily offered for a total of 1 kg/head; for EG goats, the concentrate was integrated with OL to obtain a dietary supplementation of about the 10% of the whole diet on a DM basis. The trial lasted for 30 d, then individual milk samples were collected and analyzed for total phenolic content, antioxidant potential, and characterization of phenolic and lipolytic volatile profiles.

Colorimetric Evaluations of Total Phenolic Compounds and Antioxidant Activity in Olive Leaves, Feeds, and Raw Milk

Samples of OL and feeds (n = 5 for OL, CG feed, and EG feed, respectively) were immediately subjected to colorimetric analysis focused on the evaluation of total phenolic content (**TPC**), total flavonoids content, and antioxidant capacity. First, the bioactive compounds were extracted from each matrix (OL and feeds) according to the procedure described by Tsakona et al. (2012), by exploiting a hydroalcoholic solution (ethanol: water 70:30, vol/vol). The obtained extracts were then filtrated and used for the analysis.

The TPC was determined following the protocol previously reported by Singleton and Rossi (1965) with slight modifications. Briefly, 200 µL of extract was mixed with 1 mL of 0.2 N Folin-Ciocalteu reagent and 800 µL of sodium carbonate 7.5% in water. Colorimetric evaluations were done in triplicate at 760 nm (Jenway 6305 UV/Vis spectrophotometer, Jenway) after 30 min of incubation at room temperature (20–22°C) in the dark. A 6-point standard curve, ranging from 1 to 100 µg/mL of gallic acid ($R^2 = 0.9928$) was prepared for

quantification, and results were reported as mg of gallic acid equivalent (**GAE**)/g of sample on a DM basis.

Total flavonoids content was determined through the aluminum chloride spectrophotometric method (Pitz et al., 2016). In brief, 0.5 mL of extract was added to 2.5 mL of ethanol and 0.5 mL of 2% (wt/vol) aluminum chloride methanolic solution. The mixture was incubated for 1 h and protected from light, before recording the absorbance at 420 nm. A 6-point standard curve, ranging from 1 to 100 μg/mL ($R^2 = 0.9964$) of quercetin was constructed for the quantification. Results were expressed as mg of quercetin equivalent/g of sample on a DM basis.

The AOA was instead determined according with the ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) method reported by Abaza et al. (2011) with some modification. One hundred microliters of extract was mixed with 1 mL of ABTS solution properly diluted, and colorimetric evaluations were performed at 734 nm after 4 min of incubation in the dark. An external 6-point calibration curve for Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ranging from 1 to 32 μmol/g, was prepared for quantification; results were reported on a DM basis as μmol/g Trolox equivalent antioxidant capacity (**TEAC**).

In the case of milk, the same colorimetric approaches were applied (n = 15 for CG and EG samples, respectively), with the only difference regarding the initial extraction of the bioactive compounds. Specifically, 5 mL of previously skimmed and deproteinized milk (Zulueta et al., 2009) was mixed with 15 mL of methanol, carefully agitated for 40 min in the dark, and centrifuged at 800 × g for 15 min. The supernatants were then recovered, filtered, and analyzed. The TPC in milk was expressed in ng of GAE/mL, and AOA in μmol of TEAC/mL.

Characterization of Phenolic Profiles in OL, Feeds, and Raw Milk

This analysis was carried out on the phenolic extracts previously obtained for the colorimetric evaluations. The identification and quantification of phenolic compounds (n = 5 for OL and feeds; n = 15 for CG and EG milk samples) was performed through ultra-high-performance liquid chromatography (**UHPLC**)-MS/MS according with the method reported by Simeoni et al. (2018), with some modification due to the different sample treatment. First of all, it was necessary to set up a reliable clean-up of the phenolic extracts by means of solid-phase extraction. Specifically, a Strata-XL 100-μm polymeric reversed phase (Phenomenex) was used,

and the clean-up was performed in 5 steps: (1) conditioning, using 1 mL of MeOH, (2) equilibration, using 1 mL of H_2O:MeOH (90:10; H_2O pH = 3.0), (3) sample loading, using 1 mL of hydroalcolic extract, (4) washing, using 1 mL of H_2O (pH = 3), (5) elution, using 1 mL of MeOH. Then, the eluates were collected in vials and placed into autosampler for subsequent UHPLC-MS/MS analysis.

Briefly the analyses were performed an UHPLC system Nexera XR (Shimadzu) coupled to a 4500 Qtrap mass spectrometer (Sciex) with an MRM acquisition; the ionization of the analytes was performed in negative mode, with ion spray voltage set at −4.5 kV. The chromatographic separation was obtained by means of a mixed mode C18-PFP column (Excel 22 μm, 10 cm × 2.1 mm ID-ACE), with aqueous 0.1% formic acid and acetonitrile as mobile phases in gradient elution.

Volatile Compounds Analysis

The identification of volatile compounds (**VOC**) deriving from lipolytic events in raw milk (n = 15 each for CG and EG samples) was performed by solid-phase microextraction (Castellani et al., 2017) followed by GC-MS analysis through a gas chromatographic system (Clarus 580; Perkin Elmer) equipped with an Elite-5MS column (30 × 0.25 mm; 0.25 μm; Perkin Elmer) and coupled with a mass spectrometer (SQ8S; Perkin Elmer). After adsorption time, the extracted VOC were thermally desorbed in splitless mode at 250°C for 1 min. The chromatographic analysis and the identification of individual VOC were carried out as previously described (Innosa et al., 2020b).

Molecular Docking

To evaluate the hypothetical ability of phenolic compounds to influence lipolysis in raw milk, an in silico approach focused on the evaluation of the most probable interactions between the identified phenolic compounds and endogenous lipoprotein lipase (**LPL**) was performed. The analysis was conducted by exploiting SwissDock, a web server (http://www.swissdock.ch/) for modeling evaluations developed by the Swiss Institute of Bioinformatics to predict the most favorable binding modes that may occur between a target protein and a small molecule. This web tool is based on use of the docking software EADock DSS (Grosdidier et al., 2011) and the CHARMM force field method for calculation (Vanommeslaeghe et al., 2010). On the Protein Data Bank (https://www.rcsb.org/), there are no crystallized structures for LPL from *Capra hircus*;

Table 1. Total phenolic compounds (TPC), total flavonoid content (TFC), and antioxidant activity (AOA) in olive leaves (OL) and diets administered to control group (CG) and experimental group (EG)[1]

Item[2]	OL	Diet		
		CG	EG	P-value
TPC (mg GAE/g)	42.45 ± 2.75	9.66 ± 0.53	16.05 ± 0.98	**
TFC (mg QUE/g)	21.72 ± 1.44	6.70 ± 0.49	9.95 ± 0.83	**
AOA (mmol TEAC/g)	14.83 ± 0.38	4.30 ± 0.38	6.01 ± 0.46	**

[1]Data are reported as mean values $\pm$ SD (on a DM basis).

[2]GAE = gallic acid equivalent; QUE = quercetin equivalent; TEAC = Trolox equivalent antioxidant capacity.

**$P < 0.01$.

therefore, the crystal structure of the bovine LPL was used as a model (Protein Data Bank code: 6U7M; Gunn et al., 2020), and the structures of phenolic compounds were exported from ZINC AC (http://zinc.docking .org/substances/; Sterling and Irwin, 2015), a free database of commercially available compounds for virtual screening. The docking clusters related to the most favorable interactions between LDL and phenolic compounds found in EG milk samples were visualized and analyzed by using the ViewDock tool of UCSF Chimera software, version 1.11.2 (Pettersen et al., 2004).

Statistical Analysis

Statistical evaluations were carried out by using SigmaPlot 12.0 Software (Systat Software Inc.) for Windows operating systems. The one-way ANOVA model was applied, and means were compared through the Tukey's test. Statistical significance was attributed in presence of P-values lower than 0.05.

RESULTS

Total Phenolic Compounds, Total Flavonoid Content, and Antioxidant Activity in Olive Leaves, Diets, and Goat Milk

To characterize the phenolic profile, the matrices of interest were tested to determine the TPC and the AOA by means of colorimetric assays. As reported in Table 1, the OL used for the dietary supplementation were found to be particularly rich in phenolic compounds, and this resulted in significantly higher values of these class of compounds in the EG feed (16.05 ± 0.98 mg of GAE/g vs. 9.66 ± 0.53 mg of GAE/g in EG and CG samples, respectively; $P < 0.01$). The subclass of flavonoids had higher values in EG feed samples, which also had higher AOA ($P < 0.01$ for both).

The intake of a diet particularly rich in phenolic compounds seems to have increased these compounds in the milk collected at the end of the trial. In fact, as shown in Figure 1, TPC was significantly higher in EG
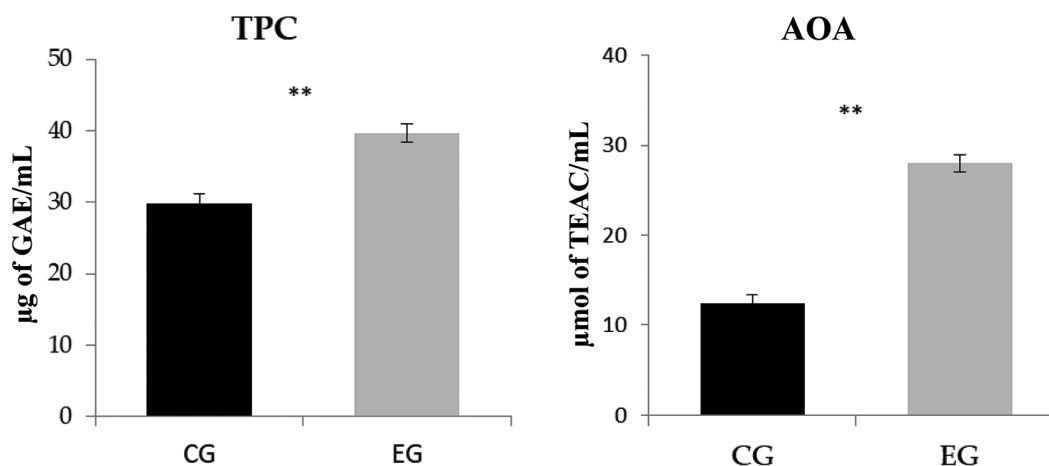

Figure 1. Total phenolic compounds (TPC) and antioxidant activity (AOA) in raw milk samples obtained from goats fed the standard diet (CG) and goats that received the dietary olive leaves (OL) supplementation (EG). Statistical significance is indicated by asterisks (**$P < 0.01$). GAE = gallic acid equivalent; TEAC = Trolox equivalent antioxidant capacity. Error bars represent SD.

Table 2. Characterization of phenolic compounds in olive leaves (OL) and diets administered to control group (CG) and experimental group (EG)[1]

| Phenolic compound | OL | Diet | | P-value |
		CG	EG	
Apigenin	0.10 ± 0.01	0.10 ± 0.1	0.23 ± 0.02	**
Chlorogenic acid	0.14 ± 0.01	ND	0.09 ± 0.01	
Ferulic acid	0.01 ± 0.01	0.04 ± 0.003	0.04 ± 0.01	NS
Hydroxytyrosol	0.20 ± 0.02	ND	0.01 ± 0.002	
3,4-Hydroxybenzoic acid	0.95 ± 0.02	ND	0.25 ± 0.02	
Isoquercetin	0.02 ± 0.001	ND	ND	
Kaempferol	0.56 ± 0.02	0.05 ± 0.02	0.24 ± 0.02	**
Luteolin	0.89 ± 0.04	0.03 ± 0.005	0.34 ± 0.04	**
Oleuropein	22.99 ± 0.65	ND	4.25 ± 0.29	
Quercetin	1.13 ± 0.03	ND	0.73 ± 0.08	
Rosmarinic acid	0.02 ± 0.007	ND	ND	
Rutin	1.11 ± 0.11	0.05 ± 0.008	0.28 ± 0.01	**
Syringic acid	0.02 ± 0.008	0.01 ± 0.005	0.02 ± 0.01	NS
Tyrosol	0.13 ± 0.01	ND	ND	

[1]Data are reported as mean values (mg/g on a DM basis) ± SD; ND = not detectable; NS = not significant.
**$P < 0.01$.

samples (39.53 ± 2.86 ng of GAE/mL vs. 29.75 ± 2.33 ng of GAE/mL for EG and CG, respectively, $P < 0.01$). In addition to this, as previously reported for feeds, an increase in phenolic compounds is accompanied by a significant increase in the antioxidant potential (28.01 ± 1.86 µmol of TEAC/mL vs. 12.45 ± 1.30 µmol of TEAC/mL for EG and CG, respectively, $P < 0.01$).

Phenolic Profiles in OL, Feeds, and Raw Milk

Table 2 shows data concerning the quantification of the single phenolic compounds identified in the OL used for supplementation and in the diets administered to animals. Specifically, 14 compounds were identified, including phenolic acids (syringic acid, ferulic acid, 3,4-hydroxybenzoic acid chlorogenic acid), flavonols (rutin, quercetin, isoquercetin, and kaempferol), flavones (luteolin and apigenin), simple phenols (tyrosol and hydroxytyrosol), an ester deriving from caffeic acid (rosmarinic acid), and a member of the secoiridoids group (oleuropein). Specifically, this latter compound was found to be present in greater concentration in the leaves.

With specific regard to the diets administered to goats, 5 compounds were found to be exclusively present in EG samples (oleuropein, chlorogenic acid, hydroxytyrosol, 3,4-hydroxybenzoic acid, and quercetin), whereas apigenin, kaempferol, luteolin, and rutin were identified in both diets, but with significantly higher concentrations in EG samples ($P < 0.01$).

The same evaluation was performed on raw milk samples, allowing instead to identify 19 phenolic compounds in EG samples (Table 3). In addition to what

was observed for OL and the EG diet, caffeic acid, vanillic acid, gallic acid, *trans*-cinnamic acid (phenolic acids), diosmetin, myricetin (flavonols), catechin (flavanol), *o*-coumaric acid, and *p*-coumaric acid (hydroxy derivatives of cinnamic acid) were also found. On the contrary, no traces were found for apigenin, isoquercetin, syringic acid, and oleuropein. As for the CG milk samples, none of the previously listed compounds has

Table 3. Characterization of phenolic profile detected in raw milk samples obtained from goats that received the dietary olive leaves supplementation (EG)[1]

Phenolic compound	EG milk
3,4-Hydroxybenzoic acid	2.26 ± 0.95
Apigenin	ND[2]
Caffeic acid	0.03 ± 0.01
Catechin	4.68 ± 1.99
Chlorogenic acid	54.77 ± 18.59
Cinnamic acid	361.98 ± 43.04
Diosmetin	1.96 ± 0.89
Ferulic acid	0.13 ± 0.06
Gallic acid	4.58 ± 1.47
Hydroxytyrosol	0.33 ± 0.09
Kampferol	0.20 ± 0.07
Luteolin	0.28 ± 0.16
Myricetin	4.59 ± 1.83
o-Coumaric acid	0.18 ± 0.06
p-Coumaric acid	0.44 ± 0.21
Quercetin	0.22 ± 0.09
Rosmarinic acid	3.90 ± 0.84
Rutin	0.01 ± 0.004
Tyrosol	8.30 ± 2.93
Vanillic acid	2.62 ± 0.43

[1]Data are reported as mean values (ng/mL) ± SD.
[2]ND = not detected.

been identified; only traces of apigenin, ferulic acid, and luteolin were detected in some individual samples.

Identification of Free Fatty Acids in Raw Milk

The GC-MS analysis was useful for the identification of VOC in raw milk samples. The family of free fatty acids (**FFA**) was found to be the most represented in all samples, with significantly higher relative percentages in the CG than in the EG samples (76.95 ± SD 5.13% vs. 66.55 ± 4.88% in CG and EG, respectively, $P < 0.01$). Specifically, 8 FFA were detected in all milk samples: butanoic acid, hexanoic acid, octanoic acid, nonanoic acid, decanoic acid, dodecanoic acid, tetradecanoic acid, and hexadecanoic acid (Table 4).

Decanoic acid was found to be the most represented FFA, with significantly higher percentages in CG milk (61.83 ± 4.15% vs. 49.26 ± 3.06% in CG and EG, respectively, $P < 0.01$). The same result was obtained for hexanoic acid (0.57 ± 0.07% vs. 0.41 ± 0.04% in CG and EG, respectively, $P < 0.05$), whereas nonanoic acid showed higher values in EG samples (0.13 ± 0.02% vs. 0.72 ± 0.06% in CG and EG, respectively, $P < 0.01$).

Molecular Modeling of the Interactions Between Phenolic Compounds and Endogenous Lipoprotein Lipase

The in silico approach was helpful in estimating the probability and the modalities of interaction between phenolic compounds with a LPL, an enzymatic form potentially responsible for the release of FFA in raw milk. In Table 5 are reported all the free energy values associated with the most probable interactions between the single phenolic compound and the LPL. Specifically shown are the values associated with 16 compounds compared with the 19 identified in raw milk, as the

Table 4. Volatile free fatty acids (FFA) detected in raw milk samples obtained from goats fed the standard diet (CG) and goats that received the dietary olive leaves supplementation (EG)[1]

FFA	CG	EG	P-value
Butanoic acid	0.09 ± 0.01	0.09 ± 0.02	NS
Hexanoic acid	0.57 ± 0.07	0.41 ± 0.04	*
Octanoic acid	9.25 ± 0.83	10.06 ± 0.84	NS
Nonanoic acid	0.13 ± 0.02	0.72 ± 0.06	**
Decanoic acid	61.83 ± 4.15	49.26 ± 3.06	**
Dodecanoic acid	4.72 ± 0.46	5.53 ± 0.48	NS
Tetradecanoic acid	0.12 ± 0.02	0.17 ± 0.02	NS
Hexadecanoic acid	0.24 ± 0.06	0.31 ± 0.05	NS

[1]Data are reported as mean relative percentages of total volatile compounds ± SD; NS = not significant.
*$P < 0.05$; **$P < 0.01$.

Table 5. Free energy values calculated for the most probable interactions between phenolic compounds and lipoprotein lipase

Phenolic compound	ZINC AC entry[1]	ΔG[2]
Caffeic acid	58,172	−7.59
Catechin	119,978	−7.19
Chlorogenic acid	2,138,728	−8.96
Cinnamic acid	16,051,516	−9.06
Diosmetin	5,733,652	−6.95
Ferulic acid	58,258	−9.67
Gallic acid	1,504	−8.91
Hydroxytyrosol	13,108,132	−6.76
Kampferol	3,869,768	−8.80
Luteolin	18,185,774	−8.26
Myricetin	3,874,317	−8.54
p-Coumaric acid	39,811	−8.32
Quercetin	3,669,685	−8.77
Rosmarinic acid	899,870	−8.67
Tyrosol	164,581	−6.67
Vanillic acid	338,275	−9.04

[1]ZINC AC = free database of commercially available compounds for virtual screening (http://zinc.docking.org/substances/).
[2]Data are reported in kcal/mol and are representative of the free energy associated with the most probable interaction between selected ligand and lipoprotein lipase. ΔG = change in Gibbs free energy.

analysis by SwissDock did not give positive results for 3,4-hydroxybenzoic acid, o-coumaric acid, and rutin.

Ferulic acid, vanillic acid, and trans-cinnamic acid showed free energy values lower than −9.00 kcal/mol (−9.67, −9.04, −9.06 kcal/mol, respectively) and, precisely for trans-cinnamic acid, the phenolic compound most represented in milk, the mode of interaction was further evaluated. Specifically, a polar interaction was highlighted between a carbonyl oxygen of cinnamic acid and the amino group of lysine 326, belonging to the C-terminal residue of the enzyme (Figure 2). The distance for the putative bond was estimated to be equal to 2.070 Å.

DISCUSSION

Dietary supplementation with olive leaves showed to be effective in inducing significant changes in the phenolic composition in milk. The characterization however started from OL used for the dietary supplementation and then also the administered diets were analyzed.

The OL used in the trial showed the presence of 14 compounds with a predominance of oleuropein, followed by a derivative of benzoic acid and compounds belonging to the flavonoids group. Oleuropein is notoriously the polyphenolic compound most represented in OL. Other studies focused on the characterization of bioactive phenolic compounds in OL, have highlighted a concentration of oleuropein equal to 37.84 mg/g of dried leaf through an extraction protocol based on the use of a Soxhlet method and exploiting methanol as
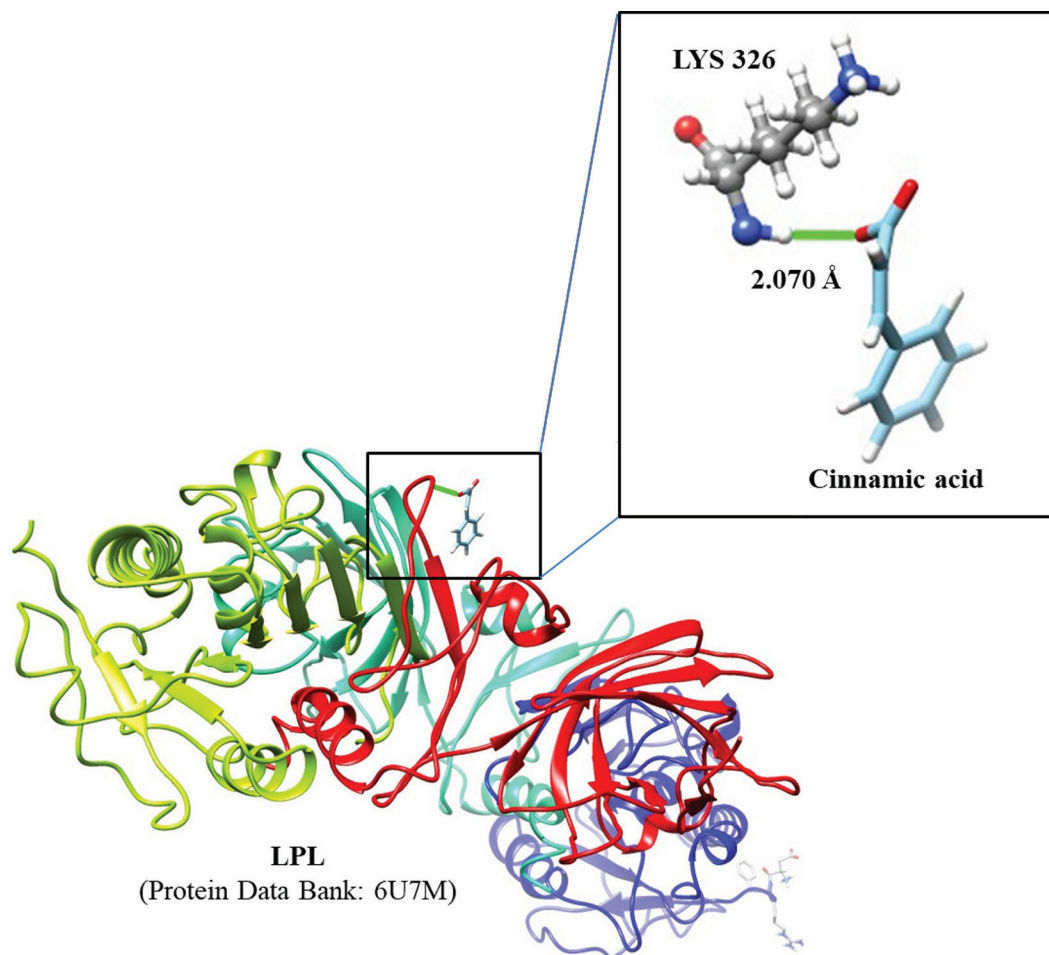
Figure 2. Molecular docking evaluation of the interaction between cinnamic acid and lipoprotein lipase (LPL). The interaction between cinnamic acid and LPL (Protein Data Bank code: 6U7M; https://www.rcsb.org/structure/6U7M) has been analyzed by exploiting the ViewDock tool of UCSF Chimera software (version 1.11.2). The analysis specifically suggests the existence of a polar interaction between the carboxyl oxygen of the ligand with the lysine 326, with an estimated bond distance equal to 2.070 Å.

solvent; a value of 14.26 mg/g of dried leaf was instead achieved by using supercritical fluid extraction (Şahin et al., 2011). It is therefore evident that the value of 22.99 mg/g of dried OL, reported in the present study, falls within the order of magnitude just described. The minimal differences that can be observed from this point of view may partly depend on the applied extraction protocol, and partly on the type of cultivar being analyzed; in this regard, many studies have shown that different cultivars, grown in different geographical areas, can be characterized by different phenolic profiles, both from a qualitative and quantitative point of view (Talhaoui et al., 2014).

The only benzoic acid derivative identified in the OL was the 3,4-hydroxybenzoic acid; this compound belongs to the broad class of phenolic acids and has been identified as one of the most effective antifungal agents that can be isolated in OL (Korukluoglu et al., 2008).

With regard to the flavonoid group, OL analyzed in this study were characterized by the presence of apigenin, luteolin, rutin, quercetin and kaempferol. Apigenin and luteolin belong to the subclass of flavones and, together with rutin, a flavonoic glycoside, they are generally considered the most represented flavonoids in OL (Wang et al., 2018). In lower concentrations were instead found compounds belonging to the subgroup of flavonols, such as quercetin and kaempferol. Precisely with regard to this latter compound, various studies have shown kaempferol, and its isomers, to be present in highly variable concentrations depending on the reference cultivar and on the time of the year when leaf sampling was performed (Fabbri et al., 2004). The

non-negligible concentration found in OL used in this study, higher than 0.5 mg/g of dried OL, represents an interesting aspect if it is kept into account the high bioactive potential of this compound, especially for its anti-inflammatory action (Devi et al., 2015). What was highlighted in the OL had direct repercussions on the phenolic composition observed in the diets administered to animals. In particular, the enrichment with 10% OL on a DM basis, resulted in the presence of significantly higher concentrations of all the compounds cited above.

The goats fed with the EG diet therefore assumed greater quantities of bioactive compounds and this undoubtedly influenced the phenolic profile found in milk; in particular have been identified 19 compounds divided between simple phenols, flavonoids, cinnamic acid derivatives and benzoic acids. First of all, it should be pointed out that most of the compounds found in the EG samples are totally absent or present in traces in the CG milk, thus generating a fairly defined picture.

The most represented compound in EG samples was *trans*-cinnamic acid, a phenolic acid credited with biological functions mostly associated with antidiabetic and anti-inflammatory effects, as well as with the ability to stimulate white fat browning and brown adipocytes activation (Kang et al., 2019). However, in our study, this compound is important because it is the main precursor of other phenolic compounds, such as benzoic and coumaric acids. In our case, the biosynthetic pathway that originates with the formation of *p*-coumaric acid is particularly interesting. This compound is produced by a catalytic mechanism mediated by the *trans*-cinnamic acid 4-hydroxylase in presence of NADPH as an electron donor, and its accumulation is responsible for the downstream synthesis of flavonoids and phenolic acids (Li et al., 2018).

Flavonoids found in the EG milk samples belong to the subgroups of flavonols (quercetin, isoquercetin and kaempferol), flavones (apigenin, luteolin, diosmetin and myricetin), and flavonoic glycosides, as in the case of rutin. All these compounds are commonly found in OL extracts and are particularly important due to the numerous benefits for human health. The intake of natural matrices rich in these compounds seems to be associated with significant antioxidant, anti-inflammatory, and protective effects in the context of the onset and progression of neoplastic events (Perez-Vizcaino and Fraga, 2018).

The enzymatic process mediated in plants by p-coumaric 3-hydroxylase is instead responsible for the synthesis of caffeic acid, a phenolic acid in turn identified as a substrate for the enzymatic production of ferulic, gallic, rosmarinic, and chlorogenic acids (Döring and Petersen, 2014). Therefore, the fact that these compounds are almost exclusively present in the EG samples represents an important finding in the potential benefits for consumers' health. In the last decade, numerous studies have been focused on obtaining food products rich in these phenolic acids or on the identification of protocols useful for the production of high amounts of these compounds without necessarily resorting to extraction from vegetable matrices. From this perspective, there is a noteworthy study recently conducted by Li et al. (2018), who identified a plausible procedure for the de novo biosynthesis of caffeic acid from glucose by exploiting engineered *Saccharomyces cerevisiae*. With specific regard to chlorogenic acid, a notable study was conducted by Jiang et al. (2018), who evaluated the effects of the interaction between the phenolic compound and milk proteins, both whey proteins and caseins. Specifically, the chlorogenic acid was demonstrated to bind with more affinity the whey proteins compared with caseins and, in both conditions, the interaction was effective in inducing significant structural changes that led to an increase in digestibility and improved protein functionalities.

Tyrosol and hydroxytyrosol are compounds normally found in matrices coming from products and by-products of the olive oil supply chain; therefore it is not surprising that they are present in milk samples as a consequence of the OL dietary supplementation in goats. At the same time, it should be emphasized that in the same samples oleuropein is totally absent, although it was present in considerable concentration in the leaves used for the trial and, therefore, in the EG feed. Those findings can be correlated by taking into account the study conducted on humans by Vissers et al. (2002), who measured the absorption of these compounds in healthy ileostomy subjects. Data showed oleuropein to be subjected to extensive hydrolysis with consequent accumulation of tyrosol and hydroxytyrosol and advanced the consideration that the poor polarity of oleuropein structure was responsible for low absorption efficiency in the small intestine, contrary to what was observed for tyrosol and hydroxytyrosol.

The GC-MS analysis showed a greater accumulation of FFA in the CG milk samples. The production of FFA may be generally attributed to the activity of esterases and endogenous lipoprotein lipases, which are naturally present in raw milk and catalyze the hydrolysis of the ester bond of tri-, di-, and monoglycerides into FFA and glycerol (McSweeney and Sousa, 2000). These enzymes can come from the animals' blood plasma or be released by leucocytes (somatic cells) and secretory cells through the apical membrane or cytoplasm (Fox and Kelly, 2006). The presence of high concentrations of these compounds generally represents a not very

positive aspect as accumulates substrate that can be exploited by oxidative events responsible for the release of compounds in turn capable of inducing negative effects on the aroma and product shelf-life (Bertuzzi et al., 2018; Ianni et al., 2020). In addition to what has been reported, it should be mentioned that milk lipids in mammals also represent nonimmunoglobulin protective factors. As previously described, the antimicrobial activity by milk lipids is mainly due to FFA and monoglycerides released following the catalytic action of lipases. Specifically, studies performed on human milk evidenced that lipids do not initially have antimicrobial activity, but they acquire this potential following digestion in the gastrointestinal tract (Isaacs and Thormar, 1991). In the light of this evidence, the lipolytic profile obtained in this study in goat milk must be investigated.

The lower accumulation of FFA in samples of raw milk obtained from goats fed the dietary OL supplementation led to the hypothesis that phenolic compounds could interfere with this process. Assuming that the mechanisms responsible for the release of FFA in raw milk are mostly associated with the function of endogenous lipases, we explored the possibility of the onset of spontaneous interactions between the identified compounds and these class of enzymes. Specifically, the interaction was evaluated through bioinformatics tools taking as reference target the crystallized structure of a bovine LPL. For 3 phenolic compounds found in milk (ferulic, vanillic, and *trans*-cinnamic acids), molecular docking highlighted free energy values that could justify the establishment of spontaneous interactions with the target protein. Given its predominant concentration, we decided to perform further evaluations on *trans*-cinnamic acid, characterizing the possible ways of interaction with the LPL structure. Interestingly, an interaction with lysine 326 appeared probable, through a polar interaction between a carbonyl oxygen of the ligand and the amino group of the amino acid. In addition to this, the distance of this presumed interaction, estimated to be equal to 2.070 Å, could justify the presence of a hydrogen bond. Understanding the possible repercussions that this interaction could have on the kinetics of the enzyme is not immediate and certainly further and more specific evaluations would be necessary. However, the fact that the interaction can take place at the level of the C-terminal residue of the protein, could lead to advance the hypothesis of the ability of the phenolic compound to interfere with the recruitment of the substrate by the enzyme, rather than with the catalytic activity, which instead occurs at the level of the N-terminal residue, by a catalytic triad consisting of a serine, a histidine, and an aspartic

acid (Mead et al., 2002). From this point of view, only the definition of the kinetic parameters obtained from native or recombinant enzyme inhibition assays will be able to clarify the exact mechanism.

CONCLUSIONS

This experimental feeding strategy showed effective in inducing an enrichment of phenolic compounds in goat milk samples, a finding that has been associated with an improvement in antioxidant potential and a significant reduction of lipolytic events. Furthermore, such a breeding approach highlighted a viable strategy of valorization of one of the major by-products derived from the olive oil production, as a feeding ingredient for lactating dairy ruminants.

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