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Use of Selenium-enriched olive leaves in the feed of growing rabbits: Effect on oxidative status, mineral profile and Selenium speciation of Longissimus dorsi meat

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ABSTRACT

In the present study the use of Selenium-fortified olive leaves as potential dietary source of Se in rabbit nutrition was evaluated. Sixty New Zealand White rabbits (35 days of age) were randomly assigned to the following dietary treatments: standard diet (C), and C supplemented with either 10% olive leaves (OL) or 10% Selenium-fortified olive leaves (SeOL; 100 mg/L of foliar spray sodium selenate solution). At 70 days of age, 10 rabbits per group were slaughtered and the oxidative status, mineral profile and Selenium speciation of *Longissimus dorsi* meat was analyzed. Meat of the SeOL group exhibited better oxidative status (lower TBARS, higher GPx and α-tocopherol values) and a 5-fold higher Se content compared to that of the other treatments. The main Se form was SeMet (7-fold higher in the SeOL group), followed by $SeCys_2$. The present trial demonstrates the possibility of using agro-industrial by-products as ingredients in rabbit feeds, thereby enriching meat bioactive compound content.

1. Introduction

Selenium (Se) is a trace element that is necessary for human and animal health [1]. The organic forms of Se (i.e. selenocystine – $SeCys_2$, selenomethionine – SeMet, Selenomethylselenocysteine – MeSeCys) form the central structural component of various selenoproteins, including the glutathione peroxidases (GPx) [2] thus contributing to antioxidant defense against reactive molecules and free radicals, as well as complementing the effects of other antioxidant compounds in living systems [1,3].

The recommended dietary intake of Se for human adults is 55 µg/d. However, dietary Se intake in humans varies considerably between countries, largely due to the uneven geographical distribution of Se in soil, water and plants [1]. Although plants are an important source of Se in the human diet, animal products are a more reliable source, since it has been shown that animals readily incorporate Se into edible tissues, thus making it possible to produce Se-enriched products (meat, eggs, milk) [4]. Whereas dietary supplementation of Se has been studied extensively in pigs, poultry, cattle and sheep, information regarding the effect of Se supplementation in rabbits remains scarce. Recently, Papadomichelakis et al. [5] demonstrated that dietary organic Se supplementation (from 0 to 2.5 mg/kg) increases the Se content of rabbit meat in a dose-dependent manner; moreover, supplementation with 0.5 mg/kg Se improved the rabbit muscle fatty acid

Abbreviations: NDF, Neutral Detergent Fiber; ADF, Acid Detergent Fiber; ADL, Acid Digestible Lignin; Ca, calcium; Mg, magnesium; Na, sodium; K, potassium; P, phosphorus; Fe, iron; Cu, copper; Zn, zinc; Se, selenium; Se(IV), selenate; Se(VI), selenite; $SeCys_2$, selenocystine; SeMet, selenomethionine; MeSeCys, Selenomethylselenocysteine; TCA, trichloroacetic acid; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; SH, thiols; GPx, glutathione peroxidases; BHT, butylated hydroxytoluene; DNPH, 2,4-Dinitrophenylhydrazine; HCl, hydrochloric acid; Na_2SeO_3, sodium selenite; Na_2SeO_4, sodium selenate; ROS, Reactive oxygen species; H_2O_2, hydrogen peroxide; ROOH, hydroperoxides

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profile and oxidative stability, with the highest doses appearing to exert a pro-oxidant effect. However, the Authors did not evaluate the different biochemical forms of Se and the possible mechanism of action.

In general, the Se requirements of animals are around 0.10 mg/kg of feed, although many experiments have shown that the amount of Se in animal diets can reach 0.40–0.50 mg/kg [6]. Currently, there is no clear recommendation as to the amount of supplemental Se required for growing rabbits, with only a few indications provided regarding the form of supplementation (inorganic or organic). This is surprising, considering that enrichment in Se could represent further added value for rabbit meat [7].

After studying the real feasibility of olive leaf supplementation on rabbit performance and meat quality [8], the aim of this research was to verify the use of Se-olive leaves derived from the pruning of olive trees treated to reduce drought stress and increase oil Se content [9], as a potential source of this element. In particular, the effect on the oxidative status and mineral profile of rabbit meat, with special consideration as to the biological form in which Se was present, was investigated.

2. Material and methods

2.1. Animals and diets

The experimental protocol was devised according to the Italian directives [10] on animal welfare for experimental and other scientific purposes, and the research was carried out at the experimental farm of the Department of Agricultural, Food and Environmental Science of the University of Perugia (Italy), during Spring 2015.

Sixty New Zealand White mixed-sex rabbits were weaned at 35 days of age, allocated into three homogeneous groups (20 animals/group) in bicellular wire net cages (60 x 25 cm length x 35 cm height), and subjected to the following three different isoenergetic and isonitrogenous dietary treatments (88.86 g/100 g dry matter, 2.78 g/100 g ether extract, 16.21 g/100 g crude protein, 8.21 g/100 g ash, 14.10 g/100 g crude fiber, 27.15 g/100 g NDF, 18.42 g/100 g ADF, 4.29 g/100 g ADL) for 35 days until slaughter:

- Control feed (C);
- Control feed supplemented with 10% olive leaves (OL);
- Control feed supplemented with 10% olive leaves enriched in Se (2.17 mg of Se per kg of dried leaves; SeOL).

The olive leaves were obtained from 30-year old Leccino variety trees from Deruta (Umbria, Italy). At the end of April 30 randomly selected trees were sprayed with a solution containing 100 mg/L of selenium. This solution was obtained by dissolving sodium selenate (Sigma-Aldrich, Milan, Italy) in water. For each treatment, 0.5% of wetting agent (Albamilagro International S.p.A., Parabiago, MI, Italy) was added. Each plant was treated with 10 L selenium solution and wrapped with filter paper to prevent the solution from dripping onto the soil. The paper was weighed before and after spraying to calculate the amount of solution that was absorbed by the plant, which was 7.3 ± 1.4 L. Twenty additional randomly selected "control" trees were also sprayed using the same technique, but with a water solution containing only the wetting agent [11].

Leaves were collected from pruned branches (November), dried in a ventilated stove at 60 °C for 24 h and ground.

The rabbit feeding program was adjusted according to previous studies [12,13]. Water was supplied *ad libitum*, with the applied temperature and lighting schedules in the rabbit house equal to 15–18 °C and 16 L:8 D, respectively.

2.2. Slaughtering, carcass dissection and meat sampling

At 70 days of age, 10 rabbits per group were stunned and slaughtered by cutting the carotid artery and jugular veins. The animals did not undergo any transportation.

Longissimus dorsi muscles (LD) (between the 1st and 7th lumbar vertebrae) were excised from the two sides of refrigerated carcasses (24 h at 4 °C), trimmed of all external fat and epimysial connective tissue, and frozen at −80 °C for analyses of oxidative status. About 5 g from each LD was freeze-dried and stored at room temperature (RT) for mineral and Se speciation assessment.

2.3. Analytical determinations

2.3.1. Antioxidant content of leaves and experimental diets

The different isoforms of vitamin E (α-, β + γ and δ-tocopherol, and α-tocotrienol) in olive leaves and diets were quantified according to Zaspel and Csallany [14], using an HPLC system (Perkin Elmer series 200, equipped with an AS 950-10 autosampler, Tokyo, Japan) on a Synergy Hydro-RP column (4 µm, 4.6 × 100 mm; Phenomenex, Bologna, Italy). Identification was performed using a Fluorimetric detector (FD, model Jasco, FP-1525, Tokyo, Japan – excitation and emission wavelengths of 295 and 328 nm, respectively) and quantified based on external calibration curves prepared with increasing amounts of pure standards (Sigma-Aldrich, Steinheim, Germany) in ethanol.

Major carotenoids were determined via HPLC, using a solvent system consisting of solution A (methanol/water/acetonitrile, 10/20/70, v/v/v) and solution B (methanol/ethyl acetate, 70/30, v/v). The volume of injection was 20 µL, the flow rate was 1 mL/min, and the elution program took the form of a gradient starting from 90% A to 100% B over a 20 min time step, followed by a second isocratic step of 10 min. The detector was a UV–vis spectrophotometer (Jasco UV2075 Plus, Tokyo, Japan) set at a wavelength of 450 nm for zeaxanthin, lutein and β-carotene. The different carotenoids were identified and quantified by comparing the sample with pure commercial standards (Sigma Aldrich, Steinheim, Germany) in chloroform.

2.3.2. Antioxidant content and oxidative status of LD meat

The α-tocopherol, α-tocotrienol and retinol contents of the meat were quantified using the HPLC system described above, according to Hewavitharana et al. [15]. Five ml of distilled water and 4 mL of ethanol were added to 2 g of sample and vortexed for 10 s. After mixing, 4 mL of hexane containing BHT (200 mg/L) was added and the mixture was carefully shaken and centrifuged at 8000 *g* for 10 min. An aliquot of supernatant (3 mL) was dried under a stream of nitrogen and dissolved in 200 µL of acetonitrile; 50 µL was then injected into the same HPLC. Retinol was identified as reported for the carotenoids in the diets, using a UV–vis wavelength of 325 nm.

Lipid oxidation was evaluated using a spectrophotometer set at 532 nm (Shimadzu Corporation UV- 2550, Kyoto, Japan) that measured the absorbance of thio-barbituric acid-reactive substances (TBARS), and a 1,1,3,3-tetraethoxypropane calibration curve [16]. Oxidation products were quantified as malondialdehyde equivalents (µg MDA/g of muscle).

Carbonyl derivatives of proteins were detected according to Lushchak et al. [17]. Briefly, the pellets from trichloroacetic acid (TCA) extracts were mixed with 1 mL of 10 mM DNPH in 2 M HCl. Samples were incubated for 1 h at RT, then centrifuged at 13,000 × *g* for 5 min. Supernatants were discarded and pellets were washed three times with 1 mL of ethanol–butylacetate (1:1, v/v) in order to remove unreacted DNPH. Pellets were then dissolved in 1.5 mL of 6 M guanidine − HCl and centrifuged as above to pellet insoluble particles. The carbonyl content of the resulting supernatants was evaluated spectrophotometrically at 370 nm using a molar extinction coefficient of 22,000 1/M*cm; values were expressed as nanomoles of carbonyl per milligram of protein in the guanidine chloride solution. Protein concentrations were measured via the Bradford method with Coomassie Brilliant Blue G-250 [18], using bovine serum albumin as standard. The same TCA extract was also used to evaluate thiol groups based on 5,5′ dithio-bis-2-dinitrobenzoic acid assay, with an extinction coefficient of

13,600 1/M*cm and expressed as μmoL SH – group per g.

Glutathione peroxidase (GPx, U/mL) enzymatic activity was evaluated in LD samples. Briefly, a cube of tissue (2 g) obtained from each animal was minced - with a surgical scissor in a Petri dish on ice - into smaller pieces that afterwards were individually transferred into 50 mL conical tubes (Falcon, Becton Dickinson and Co., Franklin Lakes, NJ, USA) embeded in 10 mL of cold sodium phosphate buffer (50 mM pH 7.0) containing PMSF 0.4 Mm and 1% Triton x100 (peroxide-free). Samples were then sonicated on ice three times at 12 W for 30 s, with 1 min of interval between each cycle (Misonix Inc S-3000 Sonicator, Cole-Parmer, USA) and then centrifuged (5000 ×g for 15 min at 4 °C) to remove the insoluble material. The supernatants were recovered to fresh tubes pre-chilled on ice. The enzyme activities were measured by recording the oxidation rate of NADPH using the decrease in absorbance at 340 nm (Varian Cary IE, UV–Vis spectrometer, Agilent Technologies, Santa Clara, California, USA) for 3 min and were measured in triplicate [19]. One GPx unit (U) is defined as the amount of enzyme that causes the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25 °C. These cleared tissue lysates were also used to determine the protein concentrations using the Bradford protein assay and a spectrophotometer at 595 nm [20]. Finally, the results of the enzyme activities (U/mL) were divided by the protein concentration of the sample (expressed in mg/mL) to obtain enzyme activities expressed in U/mg of protein [21].

2.3.3. Mineral evaluation of olive leaves and rabbit meat

Experimental diets and meat samples were finely ground and then analyzed following the method of Cubadda et al. [22] for total Se, Ca, Mg, Fe, Na, K, P, Cu and Zn content. About 0.25 g of each sample was microwave-digested (ETHOS one high-performance microwave digestion system; Milestone Inc., Sorisole, Bergamo, Italy) with 8 mL of ultrapure concentrated nitric acid (65%, w/w) and 2 mL of hydrogen peroxide (30% w/w). The heating program for the digestion procedure was 30 min at 1000 W and 200 °C. After cooling, the digests were diluted with Milli-Water (18.2 MΩ) up to 20 mL and then filtered using a 0.22 μm filter. Se, Ca, Mg, Fe, Cu and Zn standard solutions were prepared by diluting the corresponding stock solutions (standards 1000 mg/L for AAS TraceCert) with HPLC-grade water. Ca, Mg, Fe, Cu and Zn were then determined using a Shimadzu AA-6200 atomic absorption spectrophotometer (Shimadzu, Tokyo, Japan).

Total Se concentrations were determined via ICP-MS (Agilent 7900, Agilent Technologies, USA) and an Octopole Reaction System (ORS). Standard solutions of total Se were used by diluting the corresponding stock solutions (Selenium standard 1000 mg/L for AAS TraceCert Sigma Aldrich 89,498). This method was accurately validated using a recovery test (n = 3) by adding from a selenium standard solution (4 mg/L) into a mixture of a Se-enriched sample and nitric acid prior to digestion in tubes and after appropriate dilution according to US-EPA Method 3052B [23]. Na and K were analyzed via flame photometry (Jenway PFP7 flame photometer, Staffordshire, ST15 OSA, UK) on the same acid extract. Total phosphorus determination was carried out using a colorimetric US-EPA method [24]. Results were expressed as mg/kg (Ca, Mg, Na, K, P, Fe, Cu, Zn) and μg/kg (Se) of dry matter for the experimental diets and leaves, and mg/kg of fresh matter for the meat.

2.3.4. Selenium speciation of experimental diets and LD meat

About 0.25 g of diets or freeze-dried meat was accurately weighed and added with 10 mL of solution to 2.0 mg/mL of protease (Protease Type XIV, Sigma Aldrich P5147). Samples were sonicated for 2 min using an ultrasound probe and stirred in a water bath at 37 °C for 4 h, before being cooled at room temperature and centrifuged at 8,600 -X g for 10 min. The supernatant was filtered through 0.22 μm Millex GV filters (Millipore Corporation).

Se standard solutions (Na_2SeO_3, Na_2SeO_4, SeMet, $SeCys_2$, SeMeSeCys) of 1, 5, 10 and 20 μg/L were prepared with ultrapure (> 18 MΩ cm) water.

Speciation of Se was performed by HPLC (Agilent 1100, Agilent Technologies, Santa Clara, CA 95051, United States) using an anion exchange column (Hamilton, PRP-X100, 250 x 4.6 mm, 5 μm particle size; Figure S1). The mobile phase was made by ammonium acetate with gradient elution. The analytical method used in this study was previously described in Fontanella et al. [25]. The chromatographic purity of the anion exchange peak was assessed by orthogonal chromatography, as suggested by do Nascimento da Silva et al. [26]. This analytical control consisted in collecting the fraction eluting from 2 to 4 min after anionic separation and analyzing a little fraction by cation exchange HPLC-ICP-MS with IonoSpher-5C column (3.0 × 100 mm, 5 μm, Agilent; Figure S2 and S3). In this second phase the eluent was composed by A, i.e., 3% (v/v) MeOH at pH 3, and eluent B, i.e., 10 mM pyridinium formate with 3% (v/v) MeOH at pH 3, used at 1 mL/min according to the gradient program: 0–3.5 min, 94% A/6% B; 3.5–9 min, 72% A/28% B; 9–12 min, 94% A/6% B. Results were expressed as μg/kg of dry matter for diets and μg/kg of fresh matter for LD meat.

2.4. Statistical analysis

Statistical analysis was carried out using an ANOVA procedure based on a linear model, with diet considered the fixed effect [27]. Multiple comparisons were performed using Bonferroni's range test and significance levels of $P < 0.05$ and $P < 0.01$.

3. Results

3.1. Mineral concentrations and antioxidant compound content of olive leaves and diets

Se supplementation affected the mineral composition of olive leaves (Table 1). Se-enriched leaves presented higher levels of both Se (14-fold) and certain other micronutrients (Ca, Fe, Na, Zn, P); only Cu levels were significantly lower (17.61 ± 2.56 vs 26.22 ± 3.46 mg/kg in Se-enriched and normal olive leaves, respectively).

The mineral content of the three experimental diets also varied accordingly. However, only Fe (1.67-fold higher in SeOL with respect to the other two diets) and, as expected, Se (0.32 ± 0.02, 15.70 ± 1.12, 216.47 ± 20.21 μg/kg in C, OL and SeOL, respectively) concentrations were significantly different between diets.

In the Table 2 is reported the Se speciation of experimental diets. Both organic and inorganic forms were higher in the OL and SeOL group respect to the control, however, the inorganic one was most abundant in OL (111.18 ± 8.62, 44.16 ± 1.50 and 12.25 ± 0.18 μg/kg in OL, SeOL and C respectively), whereas the organic was higher in SeOL (2 and 23-fold, respectively) respect to OL and C, mainly due to the SeMe concentration.

3.2. Antioxidant compound content of olive leaves and diets

The Se treatment of olive trees affected the total vitamin E content of leaves (Table 3), mainly due to increased concentrations of α- and γ-tocopherol (67.71 ± 3.45 and 5.92 ± 1.06 μg/g D.M. vs 38.25 ± 4.26 and 2.04 ± 0.38 μg/g D.M., in Se-enriched and normal leaves, respectively). Se-enriched leaves also contained a significantly higher amount of lutein + zeaxanthin compared to normal leaves.

When leaves were added to the feed, the carotenoid content increased (78.57 ± 11.47 and 63.09 ± 6.25 vs 48.39 ± 7.35 μg/g in SeOL, OL and C, respectively), together with that of most vitamin E isoforms (γ-tocotrienol, δ- and γ-tocopherols). The SeOL group, compared with the other diets, also showed a significantly higher α-tocopherol content (18.60 ± 5.00 vs 14.81 ± 1.56 and 13.15 ± 0.59 μg/g^{-} in SeOL, OL and C, respectively).

Table 1
Minerals profile of olive leaves and experimental diets (mean ± sd).

		Olive Leaves	
		Normal	Se-enriched
Macroelements[1]			
Ca	mg/kg	$8585.20^{A} \pm 123.50$	$11285.00^{B} \pm 150.65$
Mg	"	38.41 ± 4.95	32.60 ± 5.36
Na	"	$8360.23^{a} \pm 115.23$	$9398.50^{b} \pm 98.20$
K	"	2756.40 ± 184.32	2515.32 ± 152.48
P	"	$1439.52^{a} \pm 86.16$	$1872.10^{b} \pm 80.65$
Microelements[2]			
Fe	mg/kg	$12.54^{a} \pm 7.21$	$23.22^{b} \pm 6.15$
Cu	"	$26.22^{b} \pm 3.46$	$17.61^{a} \pm 2.56$
Zn	"	$2.81^{a} \pm 3.12$	$15.55^{b} \pm 2.89$
Se	µg/kg	$154.34^{A} \pm 43.27$	$2162.01^{B} \pm 28.36$

		Experimental Diets[3]		
		C	OL	SeOL
Macroelements[1]				
Ca	mg/kg	6120.00 ± 360.20	6366.52 ± 220.25	6636.11 ± 180.34
Mg	"	60.64 ± 20.31	57.84 ± 19.82	57.26 ± 20.14
Na	"	6150.54 ± 350.11	6371.02 ± 298.72	6474.85 ± 320.25
K	"	5800.32 ± 205.32	5495.64 ± 189.32	5471.53 ± 215.65
P	"	3120.61 ± 89.36	2951.95 ± 77.56	2995.21 ± 70.65
Microelements[2]				
Fe	mg/kg	$45.00^{a} \pm 3.65$	$41.75^{a} \pm 2.65$	$72.82^{b} \pm 5.44$
Cu	"	13.25 ± 3.20	14.32 ± 1.25	13.46 ± 1.96
Zn	"	15.20 ± 3.14	13.78 ± 2.28	15.05 ± 3.21
Se	µg/kg	$0.32^{A} \pm 0.02$	$15.70^{B} \pm 1.12$	$216.47^{C} \pm 20.21$

Values are the mean of three determinations; a, b, on the same row differ at $P < 0.05$; A, B, on the same row differ at $P < 0.01$.
[1] Ca: calcium, Mg: magnesium, Fe: iron, Na: sodium, K: potassium, P: phosphorus.
[2] Fe: iron, Cu: copper, Zn: zinc, Se: selenium.
[3] C: control diet, OL: olive leaves supplemented diet, SeOL: Se-enriched olive leaves supplemented diet.

Table 2
Selenium speciation of experimental diets (mean ± sd).

		Experimental Diets[1]		
		C	OL	SeOL
Se(IV)[2]	µg/kg	$5.24^{A} \pm 0.65$	$65.81^{C} \pm 10.84$	$30.06^{B} \pm 2.13$
Se(VI)[3]	"	$7.01^{a} \pm 0.50$	$45.37^{c} \pm 4.77$	$14.10^{b} \pm 1.50$
Total inorganic Se	"	$12.25^{a} \pm 0.18$	$111.18^{c} \pm 8.62$	$44.16^{b} \pm 3.61$
$SeCys_2$[4]	"	$8.41^{a} \pm 0.82$	$64.67^{c} \pm 2.79$	$53.71^{b} \pm 5.02$
SeMe[5]	"	$4.5^{A} \pm 0.56$	$58.75^{B} \pm 1.32$	$245.53^{C} \pm 4.22$
MeSeCys[6]	"	nd	nd	nd
Total organic Se	"	$13.00^{a} \pm 1.36$	$123.42^{b} \pm 3.54$	$299.24^{c} \pm 7.58$

Values are the mean of three determinations; a, c, on the same row differ at $P < 0.05$; A, C, on the same row differ at $P < 0.01$.
[1] C: control diet, OL: olive leaves supplemented diet, SeOL: Se-enriched olive leaves supplemented diet.
[2] Se (IV): selenite.
[3] Se (VI): selenate.
[4] $SeCys_2$: selenocystine.
[5] SeMet: selenomethionine; nd: not detectable, LOD = 0.1 µg/L.
[6] MeSeCys: Selenomethylselenocysteine.

3.3. Oxidative status and vitamin content of LD meat

Whereas the SeOL group showed lower lipid oxidation of meat than the OL and C groups (Table 4; 0.06 vs 0.09 and 0.11 µg MDA/g, respectively), significant differences in protein oxidation were observed between the OL and C groups (2.98 vs 1.29 nmol/mg proteins, respectively).

Meat retinol concentrations were the same for all diets, although the amount of Vitamin E was higher in SeOL meat followed by C and OL (990.03, 765.79 and 644.46 ng/g, respectively). The main vitamin E isoforms were α-tocopherol (higher in the SeOL group; 892.34 vs 650.46 and 570.35 ng/g in SeOL, C and OL, respectively), and α-tocotrienol (lower in the leaves-supplemented group; 63.08 vs 25.70 and 38.99 ng/g in C, OL and SeOL, respectively).

The GPx activity of rabbit meat did not show significant differences

Table 3
Antioxidant compounds of olive leaves and experimental diets (mean ± sd).

		Olive Leaves	
		Normal	Se-enriched
γ-tocotrienol	µg/g	5.88 ± 0.78	13.73 ± 6.94
α-tocotrienol	"	0.14 ± 0.01	0.10 ± 0.02
δ-tocopherol	"	$0.43^{a} \pm 0.55$	$1.90^{b} \pm 0.20$
γ-tocopherol	"	$2.04^{A} \pm 0.38$	$5.92^{B} \pm 1.06$
α-tocopherol	"	$29.76^{A} \pm 4.21$	$67.71^{B} \pm 3.45$
Σ vitamin E	"	$38.25^{A} \pm 4.26$	$89.36^{B} \pm 10.11$
Lutein + zeaxanthin	"	$115.53^{A} \pm 8.08$	$229.78^{B} \pm 16.54$
β-carotene	"	79.95 ± 10.54	120.46 ± 56.18
Total carotenoids	"	195.48 ± 63.45	350.24 ± 78.15

		Experimental Diets[1]		
		C	OL	SeOL
γ-tocotrienol	µg/g	$0.08^{a} \pm 0.06$	$0.66^{b} \pm 0.37$	$1.44^{b} \pm 0.82$
α-tocotrienol	"	0.11 ± 0.12	0.07 ± 0.08	0.12 ± 0.01
δ-tocopherol	"	$0.18^{a} \pm 0.21$	$0.29^{b} \pm 0.04$	$0.35^{b} \pm 0.02$
γ-tocopherol	"	$0.23^{a} \pm 0.10$	$0.60^{b} \pm 0.03$	$0.52^{b} \pm 0.11$
α-tocopherol	"	$13.15^{a} \pm 0.59$	$14.81^{a} \pm 1.56$	$18.60^{b} \pm 5.00$
Σ vitamin E	"	$13.75^{a} \pm 0.78$	$16.43^{b} \pm 0.90$	$21.04^{c} \pm 3.15$
Lutein + zeaxanthin	"	$25.05^{A} \pm 2.35$	$34.09^{B} \pm 6.47$	$45.52^{C} \pm 6.01$
β-carotene	"	$23.34^{A} \pm 6.69$	$29.00^{B} \pm 0.63$	$33.05^{B} \pm 2.81$
Total carotenoids	"	$48.39^{A} \pm 7.35$	$63.09^{B} \pm 6.25$	$78.57^{B} \pm 11.47$

Values are the mean of three determinations; a, b: on the same row differ at $P < 0.05$; A, B, on the same row differ at $P < 0.01$.
[1] C: control diet, OL: olive leaves supplemented diet, SeOL: Se-enriched olive leaves supplemented diet.

Table 4
Antioxidant compounds and oxidative status of *Longissimus dorsi* meat of growing rabbits (n = 10/group) fed the experimental diets.

		Experimental Diets[1]			*P* value[2]	Pooled SE[3]
		C	OL	SeOL		
TBARS[4]	μg MDA[5]/g	0.11[b]	0.09[b]	0.06[a]	0.052	0.18
Thiols	μmol SH-group/g of wet tissue	12.33	9.76	11.90	0.248	1.60
Carbonyls	nmol/mg of proteins	1.29[a]	2.98[b]	1.62[ab]	0.030	8.33
Retinol	ng/g	96.33	80.13	72.81	0.484	19.96
γ-tocotrienol	"	28.70	22.96	33.36	0.525	9.11
α-tocotrienol	"	63.08[b]	25.70[a]	38.99[a]	0.042	16.01
δ-tocopherol	"	7.04	8.48	8.69	0.319	1.31
γ-tocopherol	"	16.51	16.97	16.65	0.986	2.45
α-tocopherol	"	650.46[a]	570.35[a]	892.34[b]	0.045	26.00
Total vitamin E	"	765.79[b]	644.46[a]	990.03[c]	0.032	31.01
GPx[6]	U/mg of protein	67.97	67.45	83.71	0.264	16.27

[1] C: control diet, OL: olive leaves supplemented diet, SeOL: Se-enriched olive leaves supplemented diet.
[2] a, b: on the same row differ at $P < 0.05$.
[3] SE: standard error.
[4] TBARS: thio-barbituric acid-reactive substances.
[5] MDA: malondialdehyde.
[6] GPx: glutathione peroxidases.

between groups, however the value in the SeOL one was higher respect to the others (67.97 and 67.45 vs 83.71 U/mg of protein in C, OL and SeOL, respectively).

3.4. Mineral concentrations and selenium speciation of LD meat

Regarding macroelement content (Table 5), LD meat of the OL group contained lower levels of Mg (169.80 vs 182.99 and 175.12 mg/kg in C and SeOL, respectively); no significant differences were found for Ca, Na, K and P concentrations.

In contrast, the microelement profile of the tested rabbit meat was much more influenced by dietary treatment. Whereas SeOL meat presented Se concentrations 5-fold higher than those of the other two groups (1.88 vs 0.35 and 0.40 mg/kg in C and OL, respectively), Cu and Zn levels were lower in both leaves-supplemented groups.

Table 5
Minerals profile of *Longissimus dorsi* meat of growing rabbits (n = 10/group) fed the experimental diets.

		Experimental Diets[1]			*P* value[2]	Pooled SE[3]
		C	OL	SeOL		
Macroelements[4]						
Ca	mg/kg	236.52	222.58	221.64	0.391	25.32
Mg	"	182.99[b]	169.80[a]	175.12[b]	0.030	10.37
Na	"	158.07	153.36	145.60	0.854	46.12
K	"	504.49	494.46	519.52	0.665	64.68
P	"	794.34	723.50	825.03	0.129	65.36
Microelements[5]						
Fe	mg/kg	6.66[A]	11.35[B]	9.01[AB]	0.001	1.41
Cu	"	11.44[B]	5.58[A]	5.82[A]	0.002	1.32
Zn	"	12.02[b]	10.24[a]	10.28[a]	0.040	0.98
Se	"	0.35[a]	0.40[a]	1.88[b]	0.001	0.21

[1] C: control diet, OL: olive leaves supplemented diet, SeOL: Se-enriched olive leaves supplemented diet.
[2] a, b: on the same row differ at $P < 0.05$; A, B, on the same row differ at $P < 0.01$.
[3] SE: standard error.
[4] Ca: calcium, Mg: magnesium, Fe: iron, Na: sodium, K: potassium, P: phosphorus.
[5] Fe: iron, Cu: copper, Zn: zinc, Se: selenium.

Table 6
Selenium speciation of *Longissimus dorsi* meat of growing rabbits (n = 10/group) fed the experimental diets.

		Experimental Diets[1]			*P* value[2]	Pooled SE[3]
		C	OL	SeOL		
Se(IV)[4]	μg/kg	17.3	11.7	15.7	0.145	3.85
Se(VI)[5]	"	22.8[b]	9.8[a]	7.7[a]	< 0.001	0.84
Total inorganic Se	"	40.1[b]	21.5[a]	23.4[a]	0.032	4.69
$SeCys_2$[6]	"	51.6[a]	109.6[c]	80.3[b]	< 0.001	8.70
SeMet[7]	"	245.9[a]	253.1[a]	1763.3[b]	< 0.001	39.16
MeSeCys[8]	"	16.6	17.9	18.3	0.058	0.35
Total organic Se	"	314.1[a]	380.6[a]	1861.9[b]	< 0.001	43.12

[1] C: control diet, OL: olive leaves supplemented diet, SeOL: Se-enriched olive leaves supplemented diet.
[2] a, b: on the same row differ at $P < 0.05$; A, B, on the same row differ at $P < 0.01$.
[3] SE: standard error.
[4] Se (IV): selenite.
[5] Se (VI): selenate.
[6] $SeCys_2$: selenocystine.
[7] SeMet: selenomethionine.
[8] MeSeCys: Selenomethylselenocysteine.

The Fe contents were higher in OL than C meat, with an intermediate value detected in SeOL meat.

Table 6 presents the Se speciation results for the analyzed LD rabbit meat. Whereas the amount of organic Se in SeOL meat was 5-fold that of the OL and C groups, the amount of inorganic Se was lower in the OL and SeOL groups with respect to C (21.5 and 23.4 vs 40.1 μg/kg, respectively), mainly due to differences in Se (VI).

Selenomethionine (SeMet) was the most representative organic form, reaching levels of about 1.8 mg/kg in SeOL meat. Selenocystine content followed the order OL > SeOL > C (109.6, 80.3 and 51.6 μg/kg, respectively).

Considering the distribution of selenium species (Fig. 1), the organic form was more abundant in leaves-supplemented groups (98.8 and 94.7 vs 88.7% in SeOL, OL and C, respectively), with the inorganic form thus less abundant (1.2 and 5.3 vs. 11.3% in SeOL, OL and C, respectively).

4. Discussion and conclusion

The Se treatment is often employed to improve the capacity of certain vegetable species to tolerate oxidative stress by increasing their ability to remove reactive oxygen substances (ROS) [9]. In particular this practice is known to positively affect the quality of some vegetable products (olive oil [28], cucumber, lettuce, tomato [29], soybean [30]). The novelty of the present study was to further increase the value of the derived by-products, such as Se-enriched leaves obtained from the

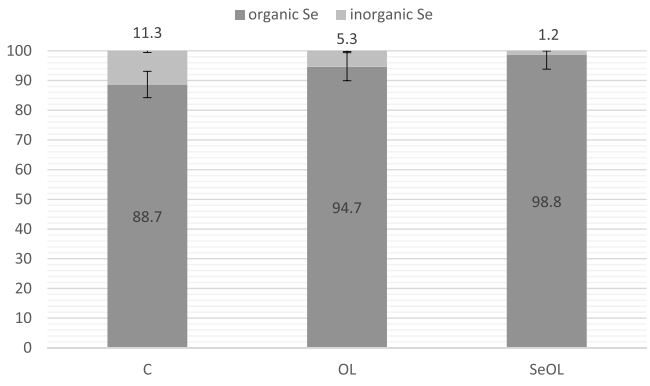

Fig. 1. Organic and inorganic Se form (%) of *Longissimus dorsi* meat of growing rabbits (n = 10/group) fed the experimental diets (mean ± sd).

pruning of olive trees.

Here, the dietary supplementation of olive leaves provided a higher amount of some bioactive compounds, with Se supplementation also preserving their content in the diets. Some Authors have suggested that Se has the ability to reduce inorganic (H_2O_2) and organic (ROOH) peroxide levels, with the formation of seleninic acid occurring as an intermediate product; its role in these processes is approximately equivalent to that of tocopherols [31,32].

The Se-enriched olive leaves contained about the double concentration of both vitamin E and lutein + zeaxanthin, with the resulting rabbit feed characterized by significantly higher concentrations of α-tocopherol. Such trend was probably related to the antioxidant effect operated by Se.

In their study of young water-stressed olive trees, Proietti et al. [9] reported that Se addition increased some antioxidant enzymes (ascorbate peroxidase, catalase and glutathione peroxidase) and reduced the content of MDA, which otherwise accumulates during oxidative perturbations. Furthermore, other minerals, over the Se (i.e. Fe and Zn, which reach higher levels in Se-enriched olive leaves), could cooperate to antioxidant defense, because they are cofactors of some enzymes active against free radicals [33].

In the present study, the SeOL diet positively affected the oxidative status of LD meat, producing lower lipid oxidation and higher concentrations of vitamin E (mainly α-tocopherol). Conversely, OL meat exhibited worse oxidative status, with lipid and protein oxidation higher than that of the control and SeOL groups ($P > 0.05$), and the antioxidant content lower (α-tocotrienol, α-tocopherol) or not different (thiols, retinol, γ-tocotrienol, *δ*-tocopherol, α-tocopherol). In agreement, the Se administration *via* olive leaves, improved also the enzyme activity of GPx ($P > 0.05$), confirming what was found in other studies [9,34].

However, various conflicting reports regarding the effect of Se dietary supplementation on rabbit meat oxidative status can be found in the literature. Whereas Ebeid et al. [35] reported that supplemental Se significantly reduced MDA values during 6 days of storage, Marounek et al. [1] did not observe similar results. In agreement with the latter study, Dokoupilovà et al. [36] found no effect on GPx activity and TBARS levels in loin meat obtained from rabbits fed a Se supplemented diet (0.50 mg/kg). Such discrepancies are probably related to the form in which Se was administered and metabolized.

For example, as Se in $SeCys_2$ is the fixed fragment of the GPx active site, GPx activity in this case depends to the incorporation of a $SeCys_2$ residue into each of its polypeptide chains [37]. In contrast, SeMet is incorporated into general proteins via the same codon as that of methionine, and thus it is feasible to enrich meat with Se by providing animals with additional SeMet in their feed [38].

In the present study, the Se content of rabbit meat was greatly increased (5-fold higher value) in the SeOL group due to Se fortification. Such results are particularly interesting considering that the recommended Se intake for humans could be achieved by consuming about 30 g of this meat. Indeed, the Se content of SeOL meat is double that generally reported for fresh rabbit meat (170 μg/kg) [39].

Many Authors have reported positive effects on meat quality when Se supplementation was carried out at concentrations of 2.5 mg/kg [5], higher than that adopted in the present experiment (about 0.20 mg/kg). In the cited experiment, however, Se was added directly to the diet in either organic or inorganic forms, which are metabolized differently by the animal body. In our case, Se administration was performed by olive leaf supplementation, and thus probably in a much more bioavailable form.

It is well known that the bioavailability of Se depends on its form of occurrence and food composition, with selenite(IV), selenate(VI), SeMet, $SeCys_2$ and Se compounds containing amino groups associated with the best assimilation rates [38]. Kieliszek et al. [38] suggested that the assimilation of Se increases when the diet is rich in low molecular weight proteins (enzymes, immuno-proteins, peptide-hormones) and certain antioxidants (mainly vitamins B, C and D). In light of this, it has to be stressed that olive leaves are constituted by many small proteins (by 29 to 63 kDa) [40], and also contain high levels of antioxidant compounds (tocopherols, carotenoids, phenols) [41].

Speciation analysis of the tested rabbit meat revealed that the main form of Se was SeMet, levels of which were 7-fold higher in the SeOL group, followed by $SeCys_2$, which was significantly higher in OL rabbit meat. Not only is SeMet the most abundant Se form in humans [42], both SeMet and $SeCys_2$ can be converted by cells from inorganic forms of Se such as selenite (Se(IV)) or selenate (Se(VI)) [43]. Vegetal sources rich in inorganic Se, which is rapidly metabolized to the organic form, include cereals (wheat, maize, rice), fruits, vegetables (garlic bulbs, onion, broccoli), coconut fruits, and seedlings [44,45].

It should be noted that the Se profile of the LD meat followed those of experimental diets: $SeCys_2$ was the highest in the OL diet and meat, whereas the SeMe was the major in both SeOL matrixes. The SeMet form is considered the most active Se-protein (as ubiquitous) and together with vitamin E is widely utilized for free radical neutralization [1,3].

Conversely, both inorganic forms of Se were higher in OL experimental diets, but not in the rabbit meat, where inorganic Se concentrations were lower in the SeOL and OL groups (1.2% and 5.3%, respectively) respect to the control, probably as consequence of the intense Se conversion towards the organic form (98.8% and 94.7%, respectively) in the animal body. Similarly, in healthy humans, whereas the overall efficiency of organic Se absorption is reported to be close to 70–80%, that of the inorganic form does not exceed 60% [46,47].

The reasons of this highest concentration of $SeCys_2$ found in OL meat could be multiple: firstly, it is correlated with the $SeCys_2$ concentration of OL diet; moreover, one possible explanation could be that the lower incorporation of $SeCys_2$ in GPx causes a $SeCys_2$ accumulation and, at the same time, a lower enzymatic activity, in agreement it is reported that the GPx activity is negative correlated with the $SeCys_2$ abundance [40]; finally this value is also likely related to meat oxidative status.

Besides Se enrichment, levels of other minerals in the tested rabbit meat were also positively affected by olive leaf supplementation.

It is well known that rabbit meat offers excellent nutritive and dietetic properties [48], mainly due to the nutritional composition of the meat and the low cholesterol content (45–50 mg/100 g) [7].

However, there is scarce information available regarding the mineral content (both macro- and microelements) of rabbit meat. Hernández et al. [49] mention only that rabbit meat has a low Na content and contains levels of Fe (~ 9 mg/kg) slightly lower than those found in bovine meat (~ 13–16 mg/kg).

In the present study the mineral found in the highest concentrations in olive leaves was Ca; similar values (9250 mg/kg of D.M.) were reported by Bahloul et al. [50]. Although leaf K levels were also high (from 1439 to 2162 mg/kg D.M.), no significant differences were found between experimental diets and LD meat samples.

Adequate intake of Ca (~ 1 mg/d) and K (~ 5 mg/d) contributes to the prevention of cardiovascular diseases [51]. Furthermore, Ca not only helps develop and maintain strong bones and teeth, but also enhances the use of other elements (i.e., P, K). Considering these values, about 10 g of rabbit meat may be considered an excellent source of Ca and K that is sufficient to meet human daily requirements. In the present study the only macroelement affected by dietary supplementation was Mg, with levels in OL group meat lower than those found in the other groups ($P < 0.05$).

The Mg is an activator of several key enzymes, including kinases, mutases, and muscle ATPases, as well Cu and Zn. Through its role in enzyme activation, Mg stimulates muscle and nerve contraction, as well as playing an important role in carbohydrate, protein and lipid metabolism.

Probably, the lower amounts of Mg found in the OL group reflect this element's involvement in the described metabolic pathways,

considering that concentrations in normal and Se-enriched olive leaves are almost the same. In agreement with these findings, Andreini et al. [52] reported that the metal ion most frequently involved in enzyme activation is Mg, followed by Zn and Fe.

The average LD meat Zn concentration was 10.80 mg/kg, slightly lower than the reference values reported previously for rabbit meat (15.7 mg/kg) [53].

Furthermore, Cu concentrations in the meat of leaf-supplemented groups were half (5.70 mg/kg on average) those of the C group (11.44 mg/kg), which in turn were higher than the values reported by Lombardi-Boccia et al. [54] (about 1 mg/kg). However, considering the scarcity of bibliographic sources, it is not easy to provide definitive reference data.

In addition to Se, the mineral content of olive leaves and rabbit feed also partially affected the microelement profile of the produced meat.

Although the Fe content of olive leaves and the Se-supplemented diet was higher than that of the other groups, no significant differences were found in LD meat. Such a discrepancy was probably due to Fe bioavailability, which may be affected by the presence of antioxidants and/or other ions (i.e., Mg, Se) [55].

In conclusion, the addition of Se-supplemented olive leaves to rabbit feed improved both the oxidative status and antioxidant content of the resulting rabbit meat. Se fortification thus introduces considerable added value, both as antioxidant defense and as an essential mineral with positive effects on human health.

An average-sized portion of such rabbit meat (~100 g) would correspond to about 340% of the recommended daily requirement of Se, yet remaining well below the maximum tolerability limit (400–800 µg/d) [56].

In light of the data reported above, the contribution of the present experimental trial is dual-fold: on the one hand, it has the demonstrated the possibility of using agro-industrial by-products (that may otherwise be difficult to dispose of) as an ingredient in rabbit feed, and on the other, it has described a potential method with which to improve the already excellent nutritional characteristics of rabbit meat. Furthermore, the present research may be considered original, because no literature studies investigated extensively the mineral composition (macro and microelements) of rabbit meat, thus, it added a further value to the nutritional characterization of this product.

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Conflict of interest

None conflict of interest to declare.

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Appendix A. Supplementary data

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