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Effect of phenols extracted from a by-product of the oil mill on the shelf-life of raw and cooked fresh pork sausages in the absence of chemical additives

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ABSTRACT

Replacing chemical additives in meat preparations with natural compounds is a matter of great interest today, both to consumers and to the food sector. The effect of an extract rich in phenols obtained from olive vegetation water (an agricultural by-product) on the pH, weight loss after cooking, diacylglycerols (DAGs), peroxide value (POV), thiobarbituric acid reactive species (TBARS), and cholesterol oxidation products (COPs) of raw and cooked fresh pork sausages prepared without chemical additives was evaluated before and after aerobic storage at 2 ± 2 °C for 14 d. Adding the extract at concentrations of 0.075 and 0.15 g/100 g resulted in a decrease in pH, DAGs, POV, TBARS and COPs; notably, the COPs levels were 4- and 17-fold lower in raw and cooked sausages, respectively. Sensory analysis revealed significant differences between control samples and those enriched with the extract, but the enriched samples were never considered unpleasant by the panellists. Storing the raw sausages for 14 d and subsequently cooking them led to 58% and 49% decreases in phenols, respectively. The purified phenols from olive oil wastewater proved to be an effective antioxidant, thus demonstrating themselves to be a potential ingredient to ensure the quality and safety of meat preparations.

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1. Introduction

The consumption of fresh ground meat preparations is widespread due to their pleasant taste and ease of cooking. The lipid fraction of ground meat can rapidly oxidize due to its large surface/mass ratio. Rancidity causes a general deterioration of the sensory quality while generating free radicals and non-radical reactive derivatives (reactive oxygen species (ROS) and reactive nitrogen species (RNS)). After ingestion, ROS and RNS are decomposed or metabolized into free radicals, which are involved in a series of chronic degenerative pathologies other than cancer (World Cancer Research Fund, 2007). Food additives, such as antioxidants, are generally used with the goal of controlling lipid oxidation during food processing and storage. Even if consumers are aware of the benefits of using additives, they have a strong expectation of foods with the fewest or lowest possible level of additives (Brockman & Beeren, 2011). Although the current regulations do not distinguish between natural and synthetic additives, consumers tend to sharply distinguish between the two (Tarnavölgyi, 2003). When an item existing in both natural and artificial versions (but chemically identical) is submitted to consumer evaluation, those who generally prefer natural products continue to prefer the natural one. However, the preference for natural products appears to be mainly an ideological concept rather than an evaluation of objective and

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measurable superior qualities (Rozin et al., 2004). There is a large and public perception that processed meat is unhealthy (Tobin, O'Sullivan, Hamill, & Kerry, 2014). According to a survey on the health benefits of processed meats, half of the respondents believed that processed meat contains large quantities of harmful chemicals (Tobin et al., 2014).

One alternative approach to the use of chemical additives could be the use of natural antioxidants. Free radical or active oxygen scavenging capacity has been detected in several phenolic compounds (Brown & Rice-Evan, 1998). Virgin olive oil (VOO) is a basic component of the Mediterranean diet with a well-established role in contributing to human health, mainly attributed to the antioxidant actions of a composite class of hydrophilic phenols (Covas, 2008). During VOO production by a three-phase centrifugation system, a by-product known as olive vegetation water (OVW) is formed (between 50 and 90 L of OVW/100 kg of olive paste) (Servili et al., 2011a). OVW is an emulsion made of oil, mucilage and pectin, with organic compounds that range between 3 and 16 g/100 mL of which 0.3–1.1 g/100 mL are phenols. Currently, resolving the OVW disposal problem is fundamental from an ecological viewpoint, and the recovery of the phenols provides an added value to OVW that would otherwise represent only a cost for oil mills. The purified phenolic extract (PE) with a 65 g/100 g concentration of phenolic compounds used in the present study was obtained from fresh OVW arising from Moraiolo Cv. Olives, grown in Umbria region (Central Italy), according to Esposto et al. (2015). The PE was stored a −20 °C in the dark, its qualitative and quantitative composition was evaluated at its using time. It largely contained the aglycone of the hydroxylated form of the tyrosol ester of elenolic acid (3,4-DHPEA-EDA), followed by verbascoside (VB), hydroxytyrosol (3,4-DHPEA) and tyrosol (*p*-HPEA). The OVW is the result of a simple mechanical separation, whereas the membrane filtration process at room temperature allows to obtain the PE. Therefore, its phenolic compounds are the same occurring in olive fruits and in VOO, thus guaranteeing its genuineness and naturalness.

The present study had two main goals. The first was to assess the storage stability of fresh sausages intended for eating after cooking. The sausages were made using only ground meat and salt as ingredients according to a simple recipe and were supplemented with two levels of a PE obtained from OVW. The second goal was to monitor the kinetics of the decrease in the concentration of phenols in the raw sausages during refrigerated storage as well as after cooking. To the best of our knowledge, this is the first study to monitor the kinetics of the transformation and degradation of phenolic compounds during the shelf-life of a refrigerated fresh meat preparation.

2. Materials and methods

2.1. Manufacture, storage and cooking of sausages

The entire experiment was performed in three replicates, each one using slightly more than 40 kg of shoulder and belly pork (approximately 50/50, w/w) that was ground with a professional meat mincer equipped with a steel plate with 5 mm diameter holes (Cavalli Meat Processing Machinery Srl, Felino, Italy). The minced meat was mixed with salt (1.5 g/100 g) using an electric mixer (Cavalli Meat Processing Machinery Srl), after which the dough was divided into three batches: i) Control, the minced meat plus salt alone; ii) L1, Control plus PE equivalent to 75 mg of phenols/100 g of dough; and iii) L2, Control plus PE equivalent to 150 mg of phenols/100 g of dough. With the goal of avoiding secondary and non-standardizable antioxidant actions from other ingredients, no spices were added. Each batch was further mixed for 1 min, stuffed into 40 mm diameter bovine casings by an hydraulic piston-type stuffer (Cavalli Meat Processing Machinery Srl). The sausages, nearly 100 g each, were left to drip at 15 ± 1 °C for 6 h and then stored without packaging (to simulate a widespread commercialization mode at retail and butcheries) in a display cabinet under alternating exposure to fluorescent light (12 h dark and 12 h light; Osram Natura De Luxe L36 W/76–1, Munich, Germany) at 2 ± 2 °C for 14 d. At 0, 7 and 14 d, a representative number of sausages from each batch were sampled and frozen in liquid nitrogen before being stored at −80 °C until analysis. At the same sampling times, the same number of sausages from each batch were cooked at 200 °C in a ventilated electrical oven (MPM Instrument Srl, Bernareggio, Italy) to an internal temperature of 85 °C (Testo 700 digital thermometer probe, Testotherm, Postfach 1140, Lenzkirch/Schwartzwald, Germany). After cooking, the sausages were cooled in an ice bath, stored for 72 h at 2–4 °C and then frozen in liquid nitrogen before being stored at −80 °C until analysis.

2.2. Extraction and HPLC evaluation of phenolic compounds in sausages

Ten grams of sausage were mixed with 100 mL of methanol and water (80/20, v/v) containing 20 mg/L of butylated hydroxytoluene (BHT). The system was homogenized using a rod disperser (IKA, T50 Ultra-Turrax, Werke, Staufen, Germany) for 1 min at 7000 rpm, centrifuged at 5000 rpm for 10 min and the supernatant recovered. The operation was repeated twice, and the collected extract was then concentrated by a rotary evaporator (Buchi Rotavapor, R-215, Flawil, Switzerland) until reaching a final volume of 50 mL, which was used for the extraction of phenols by solid-phase extraction (SPE). A C18 SPE cartridge, previously activated with 10 mL of methanol and 10 mL of water, was loaded with 2 mL of aqueous extract. The elution was performed with 50 mL of methanol. After solvent removal under vacuum, the phenolic extract was solubilized in 0.5 mL of methanol and filtered with a 0.2 μm PVDF filter. The extract was submitted to HPLC analysis (Montedoro et al., 1993). Each measurement was done in duplicate.

2.3. Proximate composition and salt

Moisture and crude protein were measured according to AOAC (1990). Crude fat was determined according to Boselli, Velazco, Caboni, and Lercker (2001). The salt content was determined using the Volhard method (AOAC, 1990). Each measurement was done in duplicate.

2.4. pH, cooking loss and diacylglycerols

The pH was determined after blending 10 g of sample with 90 mL of distilled water using a Portamess pH meter (Knick 910, Berlin, Germany) equipped with an INLAB 427 electrode (Mettler Toledo, Urdof, Switzerland). To measure cooking loss, samples were weighed before and after cooking. The cooking loss was calculated as [(fresh weight−cooked weight)/fresh weight] × 100. Diacylglycerols (DAGs) were determined by gas chromatography (Bonoli, Caboni, Rodriguez-Estrada, & Lercker, 2007). Each measurement was done in duplicate.

2.5. Peroxide values and thiobarbituric acid reactive substances

Peroxide values (POV) were determined in 15–40 mg of lipid extract (Shantha & Decker, 1994). Thiobarbituric acid reactive substances (TBARS) were determined in 2 g of sample (Witte, Krause, & Bailey, 1970). Each measurement was performed in duplicate.

2.6. Total cholesterol and cholesterol oxidation products

A 250 mg lipid subfraction of the extracted fat was cold saponified for 18 h (Sander, Addis, Park, & Smith, 1989). The unsaponifiable fraction was extracted with diethyl ether, dried, added of 1 mL of *n*-hexane/isopropanol (4:1, v/v) and kept at −20 °C until analysis. One-tenth of the unsaponifiable matter was dried, silylated, dissolved in 150 μL of *n*-hexane and analysed by GC-FID (Bonoli et al., 2007). The remaining nine-tenths of the unsaponifiable matter were purified by NH_2 SPE; cholesterol oxidation products (COPs) were eluted with acetone, dried, silylated, added of 50 μL of *n*-hexane and analysed by fast GC-mass spectrometry (Cardenia, Rodriguez-Estrada, Baldacci, Savioli, & Lercker, 2012). Each measurement was done in duplicate.

2.7. Sensory evaluation

At the 7th day of storage and on the subsequent two days, an adequate number of sausages were collected and cooked in an electric oven (preheated to 200 °C) until the core temperature reached 85 °C. Samples were kept warm (50 °C) up to the serving to the panelists The evaluation was conducted over three consecutive days with the goal of having three replicates for each comparison. The samples, randomly identified by different three-digit codes, were presented together to the judges. Strong efforts were made to disguise or eliminate the small differences between the samples in terms of the characteristics that were not related to the specific requests. Each judge operated alone in a sensory analysis booth under standardized light and temperature conditions. To verify whether there was a detectable difference between the test samples, a triangle test was used according to an international method (ISO 4120:2004). Twenty trained panellists were involved in the sensory evaluation. The panellists were asked to indicate which was the odd sample. If a significant difference was noticed during the triangle test, the judges were also asked for a simple description to support their observations.

2.8. Statistical analysis

An ANOVA test was used to determine significant differences ($p < 0.05$) among the different storage times for each type of sausage and the effect of the different types of sausage at each storage time considering three replications. When the ANOVA was significant, means were compared using the Tukey *b* posteriori test. The data were processed with SPSS version 15.0 (SPSS Inc., Chicago, Illinois, USA).

3. Results and discussion

3.1. Degradation of phenolic compounds in sausages

The concentrations of the phenolic compounds were evaluated in the PE-enriched sausages to understand their variations during refrigeration and after cooking. The most notable variation was observed for 3,4-DHPEA-EDA, which completely disappeared after seven days of storage at 2 ± 2 °C for both levels of phenols addition. In contrast, a significant increase in 3,4-DHPEA ($p < 0.05$) was detected (+39% in L1 and +49% in L2, Table 1). It can be assumed that, to a certain extent, 3,4-DHPEA-EDA was subjected to hydrolysis, which generates 3,4-DHPEA, as has been found in fermented functional milk (Servili et al., 2011b). As reported elsewhere (Obied et al., 2008), the degradation mechanism of this oleuropein derivative includes enzymatic and non-enzymatic oxidation and hydrolysis. The temporary raising of 3,4-DHPEA at 7 d of storage was due to the hydrolysis of 3,4-DHPEA-EDA, which generates new hydroxytyrosol. Nevertheless, after 14 d of storage, a not negligible level of the added phenols was still present in the raw sausages (38% and 46% of the total initial amount in L1 and L2, respectively). At d 0, cooking caused the complete disappearance of 3,4-DHPEA-EDA at both levels of PE addition, but the other phenolic compounds were only slightly degraded. The total loss after cooking ranged between 36 and 53% of total phenols according to the sampling day. Considering the scenario of 14 days of storage at 2 ± 2 °C, the reduction in total phenols was, on average between L1 and L2, equal to 58%. The cooking caused a further loss of 49%, which means that a total net loss of 78% (average L1-L2) was observed after cooking the sausages previously stored up to 14 d at 2 ± 2 °C. Notably, the reduction in the total concentration of phenols added to the sausages, that was kept at 2 ± 2 °C, showed a decay trend that was strictly linear according to the days of storage. The heat treatment caused a further reduction in the residual concentration of phenols at a rate that, at each sampling day, would result in halving the starting value. Nevertheless, the sum of the concentrations of 3,4 DHPEA and *p*-HPEA in 100 g of cooked sausages was similar to or even higher than the minimum reported effective daily amount of olive polyphenols: "*at least 5 mg hydroxytyrosol and its derivatives, e.g. oleuropein complex and tyrosol, in olive oil should be consumed daily*" to protect LDL particles from oxidative damage (EFSA NDA, 2011). As a comparison, 5 mg of that phenols were still present in the sausages cooked after 14 d of storage at 2 ± 2 °C (the sum of 3,4-DHPEA and *p*-HPEA at 14 d was 5.4 and 9.4 mg/100 g in cooked L1 and L2 sausage, respectively).

Table 1
Concentration (mg/kg) of phenolic compounds from olive vegetation water in sausages enriched at L1: 0.075 g/100 g and L2: 0.15 g/100 g of phenols. Values concerning the sausages at the three sampling times (0, 7 and 14 d of storage at 2 ± 2 °C) before cooking (raw) and after cooking (cooked).

	Days	L1					L2				
		3,4-DHPEA	*p*-HPEA	VB	3,4-DHPEA-EDA	Total phenols	3,4-DHPEA	*p*-HPEA	VB	3,4-DHPEA-EDA	Total phenols
Raw	0	215.5 ± 5.0^{b}	22.1 ± 0.8^{a}	74.4 ± 5.0^{a}	264.5 ± 11.2	576.6 ± 13.3^{a}	336.6 ± 12.1^{b}	40.6 ± 0.4^{a}	167.0 ± 7.7^{a}	453.2 ± 15.5	997.4 ± 26.4^{a}
	7	299.6 ± 1.4^{a}	18.4 ± 0.2^{b}	72.4 ± 0.2^{b}	n.d.	390.4 ± 1.5^{b}	500.8 ± 1.3^{a}	31.0 ± 0.7^{c}	159.5 ± 5.1^{ab}	n.d.	691.3 ± 5.4^{b}
	14	142.7 ± 5.2^{d}	15.9 ± 0.8^{c}	62.3 ± 4.6^{c}	n.d.	220.9 ± 7.0^{e}	274.2 ± 6.7^{c}	35.9 ± 0.3^{b}	151.0 ± 2.7^{bc}	n.d.	461.0 ± 7.2^{cd}
Cooked*	0	202.8 ± 7.9^{bc}	18.9 ± 0.5^{b}	59.7 ± 6.2^{c}	n.d.	281.5 ± 10.1^{c}	277.8 ± 5.9^{c}	37.6 ± 0.5^{b}	155.1 ± 3.7^{ab}	n.d.	470.5 ± 3.9^{c}
	7	191.7 ± 4.5^{c}	9.2 ± 0.8^{d}	48.5 ± 2.6^{d}	n.d.	249.4 ± 5.2^{d}	280.8 ± 6.7^{c}	25.1 ± 1.8^{d}	138.3 ± 3.5^{c}	n.d.	444.2 ± 7.8^{d}
	14	43.3 ± 1.3^{e}	10.4 ± 0.7^{d}	57.3 ± 2.5^{cd}	n.d.	111.0 ± 2.9^{f}	71.7 ± 3.6^{d}	22.5 ± 0.2^{e}	147.8 ± 4.4^{bc}	n.d.	242.0 ± 5.7^{e}

3,4-DHPEA, Hydroxytyrosol; *p*-HPEA, Tyrosol; VB, Verbascoside; 3,4-DHPEA-EDA, Decarbossimetil oleuropeina aglicone. Values in the same column with different superscript letters differed significantly ($p < 0.05$), nd: not detected. Values are mean ± standard deviation (n = 6). * The effect of cooking treatment was evaluated after 0, 7 and 14 days of storage of the fresh sausages at 2 ± 2 °C.

3.2. Moisture and salt composition

Unlike other meat products, sausage does not have a standard formulation, so its composition can vary greatly depending on the local habits, raw material availability and costs. Samples were stored in a refrigerator with only temperature control, and this caused a weight loss of approximately 27% during the two weeks of storage (Table 2). The moisture content at stuffing (>50 g/100 g) significantly decreased, since the unpackaged storage at 2 ± 2 °C did not control the water evaporation, up to residual values less than 40 g/100 g at 14 d ($p < 0.05$). The magnitude of this weigth loss together with phenols exerted a selective pressure on the microbial flora, delaying the growth of several microbial targets among the Gram-negative ones (Fasolato et al., 2016), and thus allowing to extend the usual storage time for this kind of meat preparation (Cocolin et al., 2004). No differences were observed between the moisture values of the three batches. In the cooked sausages, a similar trend was noted in the reduction of the residual moisture as a function of storage days. Although the moisture differences between the sampling days were significant, the moisture reduction appeared to be less linear than that observed in the raw samples, which demonstrates that additional variables were involved during cooking; for instance, it is well known that the salt concentration exerts some effect on water retention during cooking.

3.3. pH, cooking loss and diacylglycerols

The pH of the raw sausage did not change during storage, showing values that were consistent with those of flesh that underwent a regular process of post mortem glycolysis (Table 3). Hayes, Stepanyan, Allen, O'Grady, and Kerry (2011) found a decrease of about one unit of pH in raw sausages that had been enriched with selected plant-derived nutraceuticals, packed under aerobic conditions and stored at 4 °C for 14 d. However, they also found that olive leaf extract had no effect on the pH of fresh sausage or pork patties compared with a control sample. In the present study, the pH values of the PE-enriched samples were at least one-tenth of a point lower than the value of the control ($p < 0.05$). Given that the value at the beginning of the storage time was measured immediately after stuffing, it is likely that the acidification of the dough could be directly attributed to PE. As expected, pH values were higher in the cooked sausages than in the raw ones ($p < 0.001$, not reported in Table 3), likely due to the loss of free acidic groups and the accumulation of soluble substances with a buffer function during heating (Lawrie, 1979). The initial values of the cooking loss varied between 28 and 31%, whereas data at the 14th d of storage were significantly lower than those measured at the beginning ($p < 0.05$). Considering that there was a significant correlation between the dry matter percentage and the cooking loss (r = −0.55, $p < 0.01$, data not shown), it is plausible that the observed trend for cooking loss was due to the progressive reduction in moisture during the refrigerated storage of the raw sausages. The content of diacylglycerols (DAGs) was determined as the sum of 1,2- and 1,3-DAGs, of which diolein was the most abundant. Both raw and cooked sausages exhibited a very low level of DAGs, not exceeding 120 mg/100 g of fat and 30 mg/100 g of fat, respectively (Table 3).

Table 2
Moisture, crude protein, fat and sodium chloride percentage of the sausages (Control; L1: 0.075 g/100 g; L2: 0.15 g/100 g of phenols from olive vegetation water) concerning the three sampling times (0, 7 and 14 d of storage at 2 ± 2 °C) before cooking (raw) and after cooking (cooked).

Treatment	Days	Raw				Cooked*			
		Moisture	Protein	Fat	Salt	Moisture	Protein	Fat	Salt
Control	0	$52.2 \pm 0.6^{a,A}$	$19.8 \pm 1.2^{b,A}$	$24.2 \pm 1.7^{b,A}$	$1.6 \pm 0.3^{b,A}$	$43.8 \pm 0.6^{a,A}$	$22.9 \pm 0.9^{a,A}$	$29.5 \pm 1.6^{b,A}$	$1.5 \pm 0.1^{b,A}$
	7	$43.1 \pm 0.3^{b,A}$	$22.1 \pm 0.6^{a,A}$	$30.7 \pm 3.6^{a,A}$	$2.0 \pm 0.1^{a,A}$	$43.6 \pm 0.6^{a,A}$	$17.8 \pm 0.7^{b,A}$	$36.5 \pm 1.6^{a,A}$	$2.0 \pm 0.0^{a,A}$
	14	$39.1 \pm 0.3^{c,A}$	$22.2 \pm 1.1^{a,A}$	$34.0 \pm 3.1^{a,A}$	$2.1 \pm 0.3^{a,A}$	$39.7 \pm 0.4^{b,A}$	$21.9 \pm 0.1^{a,A}$	$33.9 \pm 4.2^{ab,A}$	$2.2 \pm 0.1^{a,A}$
L1	0	$52.3 \pm 0.6^{a,A}$	$18.2 \pm 0.3^{b,AB}$	$25.5 \pm 2.3^{b,A}$	$1.9 \pm 0.2^{b,A}$	$42.6 \pm 0.7^{a,B}$	$21.9 \pm 0.7^{a,A}$	$31.4 \pm 3.4^{b,A}$	$1.6 \pm 0.5^{b,A}$
	7	$46.5 \pm 2.0^{b,A}$	$17.5 \pm 1.6^{b,B}$	$31.6 \pm 1.5^{a,A}$	$2.1 \pm 0.1^{a,A}$	$40.3 \pm 0.3^{b,B}$	$20.1 \pm 0.1^{b,A}$	$37.2 \pm 2.5^{a,A}$	$2.1 \pm 0.1^{a,A}$
	14	$39.2 \pm 2.7^{c,A}$	$22.7 \pm 2.6^{a,A}$	$33.6 \pm 4.5^{a,A}$	$2.1 \pm 0.1^{a,A}$	$39.6 \pm 0.6^{c,A}$	$21.9 \pm 0.1^{a,A}$	$34.1 \pm 3.0^{ab,A}$	$2.4 \pm 0.1^{a,A}$
L2	0	$54.4 \pm 0.8^{a,A}$	$13.6 \pm 1.3^{c,B}$	$27.9 \pm 3.0^{b,A}$	$1.6 \pm 0.0^{b,A}$	$42.7 \pm 1.4^{a,B}$	$18.4 \pm 0.6^{b,A}$	$34.9 \pm 1.8^{a,A}$	$1.6 \pm 0.1^{b,A}$
	7	$44.9 \pm 1.0^{b,A}$	$19.0 \pm 0.9^{b,B}$	$31.6 \pm 1.1^{ab,A}$	$1.8 \pm 0.2^{a,A}$	$40.5 \pm 1.0^{b,B}$	$19.8 \pm 1.1^{b,A}$	$37.5 \pm 6.6^{a,A}$	$1.9 \pm 0.1^{a,A}$
	14	$38.0 \pm 0.6^{c,A}$	$22.5 \pm 0.5^{a,A}$	$34.8 \pm 3.6^{a,A}$	$2.0 \pm 0.1^{a,A}$	$37.7 \pm 1.3^{c,B}$	$24.9 \pm 1.5^{a,A}$	$33.0 \pm 2.7^{a,A}$	$2.2 \pm 0.1^{a,A}$

Different lowercase letters within the column denote significant differences among different storage times (0, 7 and 14 d) for each type of sausage (Control, L1 and L2) ($p < 0.05$). Different capital letters within the column denote significant differences among different type of sausage at the same storage time ($p < 0.05$). Values are mean ± standard deviation (n = 6). * The effect of cooking treatment was evaluated after 0, 7 and 14 days of storage of the fresh sausages at 2 ± 2 °C.

Table 3
pH, Cooking loss (%), Total Diacylglycerols content (DAGs) (mg/100 g of fat), Peroxide Value (POV) (meq O_2/kg of fat) and Thiobarbituric Acid Reactive Substances (TBARS) (mg malondialdehyde/kg of product) of the sausages (Control; L1: 0.075 g/100 g; L2: 0.15 g/100 g of phenols from olive vegetation water) concerning the three sampling times (0, 7 and 14 d of storage at 2 ± 2 °C) before cooking (raw) and after cooking (cooked).

		Control			L1			L2		
		Days								
		0	7	14	0	7	14	0	7	14
pH	raw	$5.9 \pm 0.0^{a,A}$	$5.8 \pm 0.0^{a,A}$	$5.8 \pm 0.0^{a,A}$	$5.8 \pm 0.0^{a,AB}$	$5.8 \pm 0.0^{a,B}$	$5.8 \pm 0.0^{a,AB}$	$5.7 \pm 0.0^{a,B}$	$5.8 \pm 0.1^{a,B}$	$5.7 \pm 0.0^{a,B}$
	cooked*	$6.1 \pm 0.0^{a,A}$	$6.1 \pm 0.0^{a,A}$	$6.1 \pm 0.0^{a,A}$	$6.1 \pm 0.0^{a,B}$	$6.0 \pm 0.1^{a,B}$	$6.0 \pm 0.0^{a,B}$	$6.0 \pm 0.0^{a,C}$	$6.0 \pm 0.0^{a,B}$	$6.0 \pm 0.0^{a,C}$
Cooking loss		$31.2 \pm 6.3^{a,A}$	$19.4 \pm 1.8^{b,A}$	$18.0 \pm 7.9^{c,A}$	$29.5 \pm 1.4^{a,A}$	$22.2 \pm 1.3^{b,A}$	$14.4 \pm 1.3^{c,A}$	$28.7 \pm 4.0^{a,A}$	$24.7 \pm 0.6^{b,A}$	$18.2 \pm 1.5^{c,A}$
DAGs	raw	$17.2 \pm 1.3^{c,B}$	$59.7 \pm 6.2^{b,A}$	$118.6 \pm 19.2^{a,A}$	$22.7 \pm 2.0^{c,B}$	$37.9 \pm 5.8^{b,B}$	$68.3 \pm 4.8^{a,C}$	$45.9 \pm 2.8^{b,A}$	$59.3 \pm 6.2^{b,A}$	$91.6 \pm 8.3^{a,B}$
	cooked*	$15.5 \pm 1.9^{a,A}$	$22.0 \pm 1.2^{a,A}$	$20.2 \pm 2.4^{a,A}$	$15.7 \pm 1.8^{a,A}$	$23.3 \pm 2.5^{a,A}$	$19.4 \pm 0.8^{a,A}$	$17.8 \pm 1.3^{b,A}$	$20.8 \pm 2.0^{b,A}$	$26.6 \pm 2.1^{a,A}$
POV	raw	$1.4 \pm 0.1^{c,A}$	$7.2 \pm 0.7^{b,A}$	$13.6 \pm 1.3^{a,A}$	$1.2 \pm 0.2^{c,A}$	$3.7 \pm 1.7^{b,B}$	$6.6 \pm 0.9^{a,B}$	$0.8 \pm 0.1^{c,B}$	$3.1 \pm 0.4^{b,B}$	$3.0 \pm 0.1^{a,C}$
	cooked*	$22.4 \pm 1.6^{b,A}$	$19.2 \pm 1.6^{c,A}$	$27.1 \pm 3.1^{a,A}$	$4.2 \pm 0.3^{a,B}$	$1.5 \pm 0.3^{b,B}$	$3.6 \pm 0.9^{a,B}$	$3.0 \pm 0.2^{a,B}$	$1.3 \pm 0.3^{b,B}$	$2.9 \pm 1.3^{a,B}$
TBARS	raw	$0.6 \pm 0.1^{c,A}$	$2.0 \pm 0.1^{b,A}$	$2.5 \pm 0.3^{a,A}$	$0.4 \pm 0.1^{c,B}$	$1.1 \pm 0.2^{a,B}$	$0.7 \pm 0.1^{b,B}$	$0.1 \pm 0.0^{c,C}$	$0.5 \pm 0.0^{b,C}$	$0.7 \pm 0.1^{a,B}$
	cooked*	$4.6 \pm 0.5^{a,A}$	$3.7 \pm 0.3^{b,A}$	$4.2 \pm 0.3^{ab,A}$	$0.4 \pm 0.0^{a,B}$	$0.2 \pm 0.0^{b,B}$	$0.4 \pm 0.0^{a,B}$	$0.3 \pm 0.0^{a,B}$	$0.2 \pm 0.0^{b,B}$	$0.3 \pm 0.0^{a,B}$

Different lowercase letters within the row denote significant differences among different storage times (0, 7 and 14 d) for each type of sausage (Control, L1 and L2) ($p < 0.05$). Different capital letters within the row denote significant differences among different type of sausage at the same storage time ($p < 0.05$). Values are mean ± standard deviation (n = 6). * The effect of cooking treatment was evaluated after 0, 7 and 14 days of storage of the fresh sausages at 2 ± 2 °C.

Table 4
Single and total cholesterol oxidation products (μg COPs/g of fat), cholesterol (mg/100 mg of fat) and cholesterol oxidation rate (OR, %) of the sausages (Control; L1: 0.075 g/100 g; L2: 0.15 g/100 g of phenols from olive vegetation water) concerning the three sampling times (0, 7 and 14 d of storage at 2 ± 2 °C) before cooking (raw) and after cooking (cooked).

Treatment	Days	7α-HC	7β-HC	β-EC	α-EC	CT	25-HC	7-KC	Total COPs	Cholesterol	OR
Raw											
Control	0	$8.31 \pm 2.06^{c,A}$	$2.71 \pm 0.74^{c,A}$	$1.62 \pm 0.32^{b,A}$	$0.75 \pm 0.24^{b,A}$	$0.52 \pm 0.18^{a,A}$	$0.09 \pm 0.01^{b,B}$	$3.50 \pm 1.33^{c,A}$	$17.50 \pm 3.36^{c,A}$	$0.22 \pm 0.01^{a,A}$	$0.79 \pm 0.19^{a,A}$
	7	$16.14 \pm 1.22^{b,A}$	$6.97 \pm 0.60^{b,A}$	$4.87 \pm 0.99^{a,A}$	$2.25 \pm 0.74^{a,A}$	$0.58 \pm 0.08^{a,A}$	$0.13 \pm 0.01^{a,A}$	$9.64 \pm 0.44^{b,A}$	$40.58 \pm 2.94^{b,A}$	$0.21 \pm 0.03^{a,A}$	$2.00 \pm 0.38^{b,A}$
	14	$21.98 \pm 1.38^{a,A}$	$9.45 \pm 1.10^{a,A}$	$4.33 \pm 0.53^{a,A}$	$1.34 \pm 0.40^{ab,A}$	$0.61 \pm 0.07^{a,A}$	$0.10 \pm 0.01^{b,A}$	$16.01 \pm 2.27^{a,A}$	$53.81 \pm 4.28^{a,A}$	$0.20 \pm 0.01^{a,A}$	$2.79 \pm 0.18^{c,A}$
L1	0	$2.54 \pm 0.23^{b,B}$	$0.69 \pm 0.11^{b,B}$	$0.73 \pm 0.09^{b,B}$	$0.30 \pm 0.06^{b,A}$	$0.56 \pm 0.05^{a,A}$	$0.12 \pm 0.02^{a,A}$	$0.83 \pm 0.15^{b,B}$	$5.90 \pm 0.31^{b,B}$	$0.21 \pm 0.02^{a,A}$	$0.29 \pm 0.02^{a,B}$
	7	$5.34 \pm 0.39^{a,B}$	$1.47 \pm 0.12^{a,B}$	$1.73 \pm 0.08^{a,B}$	$1.18 \pm 0.20^{a,AB}$	$0.51 \pm 0.11^{a,A}$	$0.11 \pm 0.01^{a,B}$	$2.38 \pm 0.65^{a,B}$	$12.71 \pm 1.05^{a,B}$	$0.20 \pm 0.01^{a,A}$	$0.64 \pm 0.05^{b,B}$
	14	$4.65 \pm 0.66^{a,B}$	$1.31 \pm 0.19^{a,B}$	$1.58 \pm 0.37^{a,B}$	$0.98 \pm 0.23^{a,A}$	$0.56 \pm 0.13^{a,AB}$	$0.12 \pm 0.02^{a,B}$	$2.31 \pm 0.04^{a,B}$	$11.49 \pm 1.28^{a,B}$	$0.20 \pm 0.02^{a,A}$	$0.59 \pm 0.02^{b,B}$
L2	0	$2.59 \pm 0.62^{a,B}$	$0.72 \pm 0.21^{a,B}$	$0.60 \pm 0.06^{b,B}$	$0.39 \pm 0.09^{b,A}$	$0.42 \pm 0.13^{a,A}$	$0.09 \pm 0.01^{a,B}$	$1.06 \pm 0.18^{b,B}$	$5.85 \pm 0.92^{b,B}$	$0.22 \pm 0.03^{a,A}$	$0.28 \pm 0.03^{a,B}$
	7	$3.36 \pm 0.32^{a,C}$	$0.94 \pm 0.16^{a,B}$	$1.02 \pm 0.20^{ab,B}$	$0.84 \pm 0.09^{a,B}$	$0.42 \pm 0.09^{a,A}$	$0.11 \pm 0.01^{a,B}$	$1.13 \pm 0.16^{b,B}$	$7.80 \pm 0.62^{a,C}$	$0.20 \pm 0.01^{a,A}$	$0.40 \pm 0.04^{b,B}$
	14	$3.68 \pm 0.26^{a,B}$	$1.02 \pm 0.03^{a,B}$	$1.66 \pm 0.39^{a,B}$	$1.01 \pm 0.09^{a,A}$	$0.42 \pm 0.06^{a,B}$	$0.10 \pm 0.01^{a,B}$	$1.66 \pm 0.25^{a,B}$	$9.54 \pm 0.76^{a,B}$	$0.19 \pm 0.02^{a,A}$	$0.50 \pm 0.05^{c,B}$
Cooked*											
Control	0	$78.75 \pm 7.97^{a,A}$	$38.16 \pm 4.84^{a,A}$	$9.76 \pm 0.80^{a,A}$	$2.56 \pm 0.40^{a,A}$	$0.67 \pm 0.16^{a,A}$	$0.56 \pm 0.05^{a,A}$	$38.29 \pm 4.36^{a,A}$	$168.75 \pm 16.96^{a,A}$	$0.22 \pm 0.01^{a,B}$	$7.88 \pm 0.72^{a,A}$
	7	$42.88 \pm 5.28^{b,A}$	$20.19 \pm 2.45^{b,A}$	$5.23 \pm 0.67^{b,A}$	$0.92 \pm 0.14^{b,A}$	$0.37 \pm 0.04^{b,A}$	$0.40 \pm 0.04^{b,A}$	$22.27 \pm 4.92^{b,A}$	$92.25 \pm 12.98^{b,A}$	$0.22 \pm 0.02^{a,A}$	$4.18 \pm 0.57^{b,A}$
	14	$52.82 \pm 11.25^{b,A}$	$21.99 \pm 6.07^{b,A}$	$6.36 \pm 1.51^{b,A}$	$1.19 \pm 0.20^{b,A}$	$0.33 \pm 0.06^{b,A}$	$0.36 \pm 0.02^{b,A}$	$23.73 \pm 5.35^{b,A}$	$106.77 \pm 23.70^{b,A}$	$0.23 \pm 0.02^{a,A}$	$4.20 \pm 0.52^{b,A}$
L1	0	$4.14 \pm 0.92^{a,B}$	$1.18 \pm 0.32^{a,B}$	$1.03 \pm 0.29^{a,B}$	$0.62 \pm 0.29^{a,B}$	$0.44 \pm 0.09^{a,B}$	$0.29 \pm 0.06^{a,B}$	$1.65 \pm 0.29^{a,B}$	$9.32 \pm 1.80^{a,B}$	$0.24 \pm 0.01^{a,A}$	$0.39 \pm 0.07^{a,B}$
	7	$2.14 \pm 0.28^{b,B}$	$0.59 \pm 0.10^{b,B}$	$0.35 \pm 0.03^{b,B}$	$0.00 \pm 0.00^{b,B}$	$0.30 \pm 0.08^{b,B}$	$0.24 \pm 0.05^{a,B}$	$0.62 \pm 0.14^{c,B}$	$4.24 \pm 0.50^{c,B}$	$0.20 \pm 0.02^{b,A}$	$0.22 \pm 0.03^{b,B}$
	14	$3.12 \pm 0.32^{ab,B}$	$0.89 \pm 0.08^{ab,B}$	$0.80 \pm 0.15^{a,B}$	$0.15 \pm 0.04^{b,B}$	$0.22 \pm 0.01^{b,B}$	$0.24 \pm 0.02^{a,B}$	$1.02 \pm 0.03^{b,B}$	$6.43 \pm 0.41^{b,B}$	$0.25 \pm 0.02^{a,A}$	$0.27 \pm 0.03^{b,B}$
L2	0	$2.78 \pm 0.29^{a,B}$	$0.73 \pm 0.09^{a,B}$	$0.90 \pm 0.17^{a,B}$	$0.37 \pm 0.01^{a,B}$	$0.36 \pm 0.06^{a,B}$	$0.25 \pm 0.05^{a,B}$	$1.10 \pm 0.16^{a,B}$	$6.48 \pm 0.65^{a,B}$	$0.22 \pm 0.01^{a,B}$	$0.30 \pm 0.03^{a,B}$
	7	$1.87 \pm 0.30^{b,B}$	$0.59 \pm 0.06^{a,B}$	$0.25 \pm 0.08^{c,B}$	$0.17 \pm 0.06^{b,B}$	$0.25 \pm 0.06^{a,B}$	$0.20 \pm 0.03^{a,B}$	$0.68 \pm 0.07^{b,B}$	$4.00 \pm 0.45^{b,B}$	$0.23 \pm 0.02^{a,A}$	$0.18 \pm 0.01^{b,B}$
	14	$2.39 \pm 0.26^{ab,B}$	$0.69 \pm 0.12^{a,B}$	$0.61 \pm 0.13^{b,B}$	$0.21 \pm 0.05^{ab,B}$	$0.25 \pm 0.07^{a,B}$	$0.21 \pm 0.01^{a,B}$	$1.07 \pm 0.25^{a,B}$	$6.12 \pm 1.71^{a,B}$	$0.24 \pm 0.02^{a,A}$	$0.22 \pm 0.03^{b,B}$

7α-HC: 5-cholesten-3β,7α-diol; 7β-HC: 5-cholesten-3β,7β-diol; β-EC: 5-cholestan-5β,6β-epoxy-3β-ol; α-EC: 5-cholestan-5α,6α-epoxy-3β-ol; CT: 5-cholestane-3β,5α,6β-triol; 25-HC: 5-cholesten-3β,25-diol: 7-KC: 5-cholesten-3β-ol-7-one; Total COPs: Total Oxidation Products; OR: Oxidation Ratio.
Different lowercase letters within the column denote significant differences among different storage times (0, 7 and 14 d) for each type of sausage (Control, L1 and L2) that must be read separately for raw and cooked sausages ($p < 0.05$). Different capital letters within the column denote significant differences among different type of sausage at the same storage time that must be read separately for raw and cooked sausages ($p < 0.05$). Values are mean ± standard deviation (n = 6). * The effect of cooking treatment was evaluated after 0, 7 and 14 days of storage of the fresh sausages at 2 ± 2 °C.

These data are lower than those reported by Summo, Caponio, and Bilancia (2005) for sausages ripened for 28 d (27 g/kg). In the present study, lipolysis and the formation of DAGs was moderately evident only in the raw sausages, which showed the maximum level after 14 d, with the DAG contents of L1 and L2 lower than those of the control batch. This difference might be due to an inhibitory effect of phenols on microorganisms and a consequent reduction in lipolytic activity (Fasolato et al., 2016).

3.4. Evaluation of oxidation

The assessment of the oxidation process was monitored by POV and TBARS (Table 3), as well as by COPs (Table 4). POV and TBARS showed similar trends in both the raw and cooked products. In the raw products, all values were low at d 0. Storage increased both oxidation parameters, but the PE contributed to maintaining significantly lower values (POV < 7 meq O_2/kg fat and TBARS $\leq$ 1 mg MDA/kg; $p < 0.05$). In addition, POV values exhibited a dose response effect at the end of storage. Garrido, Auqui, Marti, and Belen Linares (2011) studied the antioxidant activity of red grape pomace in raw pork burgers stored at 4 °C under fluorescent light, concluding that the vegetable extract was effective at preventing lipid oxidation. In cooked sausages, control samples showed much higher values of POV and TBARS at all tested times. At d 0, cooking already caused severe oxidation in the control products, which was limited by the incorporation of the PE. Cooked L1 and L2 sausages maintained low and constant POV and TBARS, confirming that the PE was also effective at preventing oxidation after cooking. If the POV and TBARS levels of the control raw products are compared with those of their cooked counterparts, it is evident that the cooked sausages reached much higher values of both oxidation parameters (POV 27.1 vs. 13.7 meq O_2/kg fat, and TBARS 4.2 vs. 2.5 mg MDA/kg at 14 d), which confirms the well-known pro-oxidizing effect of cooking. It should be noted that the PE used in this study promoted good oxidative stability; TBARS values remained below the limit of 1 mg MDA/kg product that would suggest the development of rancid flavour (Gray & Pearson, 1987). The effective antioxidant activity of hydroxytyrosol has been tested in frankfurters and was found to be even higher than the inhibitory activity displayed by BHA/BHT (Cofrades et al., 2011). Recently, it was demonstrated that olive leaf extract (200 μg/g meat) could be used as an effective natural functional ingredient to suppress lipid oxidation in raw and cooked pork sausages (Hayes et al., 2011) and in lamb meat patties using an olive waste extract (Muíño et al., 2017). In the present study, it is noteworthy that the presence of residual amounts of phenols ensured the efficient control of oxidation even after 14 d of storage (levels of 220.9 and 111 mg/kg in L1 and 461 and 242 mg/kg in L2 for raw and cooked sausages, respectively) (Table 1). Available information regarding the protective effects of plant phenols against cholesterol oxidation is limited. Successful results were obtained by using a mixture of lipophilic (quercetin and rutin) and hydrophilic (caffeic acid and carnosic acid) compounds in sausages (Capitani, Hatano, Marques, & Castro, 2013). As with POV and TBARS, total COPs showed an increase during the storage time for raw sausage, with a larger increase in control samples, whereas the addition of the PE significantly inhibited the formation of COPs during storage ($p < 0.05$). L1 and L2 sausages had 4- and 17-fold lower amounts of total COPs than their control counterparts in raw and cooked products, respectively. No significant differences were observed between L1 and L2 types at any storage time ($p > 0.05$). Temperature also favoured cholesterol oxidation in control cooked samples with respect to raw samples (COPs, 106.8 vs. 53.8 μg COPs/g of fat at 14 d). The most abundant COP was 7α-HC, followed by 7-KC and 7β-HC, thus demonstrating that 7-derivatives are more favoured to form than epoxy and triol derivatives (Ferioli, Dutta, & Caboni, 2010).

Table 5
Sensory evaluation (triangle test) for comparison of Control vs L1, Control vs L2 and L1 vs L2 of cooked sausages (L1: 0.075 g/100 g and L2: 0.15 g/100 g phenols from olive vegetation water).

Comparisons triangular test	Total answers	Right answers	p
Control/L1	25	20	<0.0001
Control/L2	30	22	<0.0001
L1/L2	28	13	0.1039

3.5. Sensory evaluation

As shown in Table 5, the panellists could discriminate between the controls and the sausages with added PE. However, no substantial differences were noted between L1 and L2. The colour was never mentioned as a discriminating factor by the panellists. Under these experimental conditions, the added phenols did not darken during refrigerated storage and/or while cooking (Table 7 Supplementary material). The PE-enriched sausages were perceived as less cohesive, more acidic, more spicy, and with a taste that resembled wine. The control sample was perceived as having a greater aroma of meat and/or sausage and being more oily, a little rancid and less spicy. In the study of Hayes et al. (2011), both sausages and meat patties with an added olive leaf extract could not be differentiated from control samples from a sensory standpoint; it should be noted that the added amounts of PE in that study were 250 and 200 mg/kg for the sausages and patties, respectively, and a curing mix was also used for the sausages. Reduced cohesion could be partly due to the protective effect of phenols against the oxidation of lipids and proteins, which otherwise would have caused coagulation between lean and fat particles, which was more evident in the control sample (Karel, Schaich, & Roy, 1975).

4. Conclusions

In this study, a purified extract rich in phenols obtained from olive vegetation water showed efficacy at preventing both primary and secondary lipid oxidation and at limiting the oxidative degradation of cholesterol in raw and cooked fresh pork sausages. Refrigerated storage for 14 d led to a 58% decrease in phenols concentration. The cooking treatment caused a further loss of 49%, which means that a net loss of 78% was observed in the cooked samples with respect to the measured concentration of the PE added to the meat dough. The PE concentrations used in this study modified the sensory perception of the samples, but they were never considered unpleasant by the panellists. Effectively counteracting the oxidative process, with TBARS values lower than 1 mg/kg and COPS's oxidation ratios consistently low, as achieved in this study, requires the use of an appropriate quantity of a PE to be in proper balance with the sensory quality of the end product. The concentration of 0.075 g/100 g is effective for this purpose, but lower levels may well be equally efficient. The advantages of this approach are many. For the meat industry, in a perspective of a progressive reduction in the use of chemical additives, toward the so-called clean label. For the olive oil industry, in a more sustainable approach to managing their by-products. For consumers, for which there may be the availability of meat products with high added value from the health point of view.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2017.07.001.

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