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preliminary communication

Antioxidant and Antimicrobial Effects of *Pistacia lentiscus* L. Extracts in Pork Sausages

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Summary

Pistacia lentiscus fruits are ingredients of traditional Cypriot sausages. The objective of this study is to evaluate *P. lentiscus* extracts as natural additives to the sausages. First, the phenolic content and antioxidant activity of fruit and leaf extracts were determined. Results revealed that leaves are richer source of polyphenolic antioxidants than fruits, with methanol being the better extraction solvent. In the next step, the antioxidant and antimicrobial effects of methanolic extracts (300 mg/kg) in the pork sausage formulation were investigated. Peroxide, acid and thiobarbituric acid-reactive substance values demonstrated that both fruit and leaf extracts reduced the rate of lipid oxidation of sausages at 4 °C. Total viable count revealed significant differences on the fifth day of storage, with better microbial inhibition by leaf extract. No significant differences between the extracts were observed after the tenth day of storage. Overall, the extracts can be used to prevent lipid oxidation and reduce microbial spoilage during the first days of storage of fresh traditional pork sausages.

Key words: lipid oxidation, mastic tree, natural antioxidants, phenolic compounds, plant extracts, total viable count

Introduction

Nowadays, the food industry is constantly seeking natural, safe and low-cost antioxidant and antimicrobial agents in an attempt to replace synthetic additives. This growing interest is mainly attributed to (*i*) the plethora of epidemiological studies that has demonstrated the inverse association between the risk of chronic diseases such as cancer and cardiovascular diseases and the consumption of fruits and vegetables, (*ii*) the concerns regarding the safety of the chronic consumption of synthetic compounds traditionally used as preservatives in foods and beverages, and (*iii*) the public's conviction that natural antioxidants are safer than their synthetic analogues. In these efforts, research has been focused on herbs, spices and medicinal plants, since they provide an extraordinary reservoir of phytochemicals with diverse bioactivities (*1*).

In meat and poultry products, several ingredients of plant origin such as spices (rosemary, oregano, *etc*.), fruits (plums, pomegranate, blueberry, *etc*.) and food industry wastes have been evaluated as potential food additives (*2*,*3*). The plant extracts are mainly used in an attempt to prevent lipid and protein oxidation and/or to inhibit the bacterial and yeast growth, therefore providing protection against deterioration and spoilage (*4*,*5*). Their antioxidant and antimicrobial effects have been mainly correlated with their high phenolic content (*6*). In addition, pure phytochemicals such as phenolic acids, flavonoids, diterpenes and tocopherols have been tested as natural additives in meat products (*7*,*8*).

Pistacia lentiscus (Anacardiaceae) known as mastic tree, one of the many evergreen bushes found in the eastern Mediterranean region has a long history in folk medicine dating from the times of the ancient Greeks (*9*). Fruits,

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resins and leaves of *P. lentiscus* have been used to treat many diseases (*10,11*). The potent antioxidant and antimicrobial properties of its extracts have been highlighted and are associated with the presence of flavonoids and phenolic acids (*12*). The ripe *P. lentiscus* L. fruits are also ingredients of traditional Cypriot sausages due to their unique and characteristic taste, but their antioxidant and antimicrobial effects on pork sausages have not been studied yet.

The aim of the present study is to evaluate *Pistacia lentiscus* L. fruit extract as a food additive to control lipid oxidation and investigate potential effects on total viable count of fresh pork sausages during storage at 4 °C. Furthermore, the antioxidant and antimicrobial activities of *P. lentiscus* L. leaf extracts in sausages were also evaluated since they are a plant material easily available at low cost, which could possibly exhibit similar bioactive properties. Four different solvents were evaluated (water, methanol, butanol and acetone) in an attempt to determine the best extraction method.

Materials and Methods

Plant material and reagents

Ripe fruits and leaves of *Pistacia lentiscus* L. were collected from village Pachna (Lemesos, Cyprus; 34°46′N 32°47′E). The plant material was air-dried at room temperature in the dark until constant mass and kept in glass dispensers.

Standards of gallic acid, caffeic acid, rutin, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent, sodium carbonate, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, sodium acetate, 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), iron(III) chloride, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulphate, potassium iodate, phenolphthalein, sodium hydroxide, trichloroacetic acid, 2-thiobarbituric acid and all other common reagents and solvents were also obtained from Sigma-Aldrich.

Preparation of P. lentiscus extracts

A mass of 3 g of fruits or leaves was extracted with 100 mL of each solvent (water, methanol, butanol and acetone). The mixtures were placed for 20 min in an ultrasonic bath (Raypa UCI-50, 35 KHz; R. Espinar, S.L., Terrassa, Barcelona, Spain). Then, the mixtures were filtered under vacuum and the solvents were evaporated to dryness using a rotary evaporator. The yield of the extraction of leaves and fruits was 12.0 and 14.1 %, respectively.

Sausage preparation

Pork sausages were prepared as described by Georgantelis *et al.* (*7*). Boneless pork meat from the shoulder and pork belly fat were obtained from the local retail market. Pork meat and pork belly were cut into cubes and stored for 24 h in cold storage in order to drain excessive drip. The raw materials were comminuted separately and then mixed at a ratio to achieve a fat content of ((25±1) %). Sausages also contained salt (23 g/kg), corn oil (6 g/kg) and the selected *P. lentiscus* extracts at a mass fraction of 300 mg/kg. Then, the meat blend was stuffed into natural casings from the cleaned small intestine of pigs. The casings were rinsed with tap water and dipped in a lactic acid solution (3 %, by volume) for 15 min for tenderisation. The sausages were air-dried and placed in polyester trays, wrapped with air-permeable polyethene film and stored at 4 °C. All treatments were repeated three times to remove any effects deriving from the initial quality of meat.

Phenolic composition of plant extracts

Determination of total phenolics of extracts using the Folin-Ciocalteu assay

The reaction mixture consisted of 0.5 mL of diluted extract, 5 mL of distilled water and 0.5 mL of the Folin-Ciocalteu reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added. The mixture was thoroughly mixed, allowed to stand for 1 h at room temperature in the dark and the absorbance was measured at 765 nm. Each measurement was repeated three times and total phenolic content was expressed in mg of gallic acid equivalents (GAE) per g of extract (*13*).

Determination of phenolic compounds

The determination of different polyphenolic classes was performed according to Obied *et al*. (*14*). Briefly, 1 mL of diluted extract was mixed with 1 mL of 0.1 % HCl/ethanol solution (0.1 mL of HCl per 100 mL of 95 % ethanol) and 8 mL of 2 % HCl/ethanol solution (2 mL of HCl per 100 mL of 95 % ethanol) in a 10-mL volumetric flask. The absorbance was measured at 280, 320 and 360 nm in order to evaluate the mass fractions of total phenolics, hydroxycinnamic acid derivatives and flavonols, respectively. The corresponding standard curves for the above determinations were prepared using ethanolic solutions of gallic acid, caffeic acid and rutin, respectively.

Antioxidant activity of plant extracts

Determination of DPPH radical scavenging activity

A volume of 2 mL of each diluted extract was mixed with 1 mL of $DPPH^{\bullet}$ solution (0.3 mmol/L). The absorbance of the mixture was measured after 30 min of incubation in the dark at 517 nm and the free radical scavenging activity was expressed in mg of Trolox equivalents (TE) per g of extract (*15*).

Determination of total antioxidant activity by FRAP assay

A sample of 3 mL of freshly prepared ferric reducing antioxidant power (FRAP) solution (0.3 mol/L of acetate buffer, pH=3.6, containing 10 mmol/L of TPTZ and 40 mmol/L of $FeCl_3 \cdot 10H_2O$) and 100 μL of diluted extract were incubated at 37 °C for 4 min and the absorbance was measured at 593 nm. A standard curve of Trolox was prepared and the results were expressed in mg of TE per g of extract (*15*).

Determination of ABTS radical scavenging activity

The ABTS radical cation solution was prepared by mixing 7 mM of ABTS and 2.45 mM of potassium persulphate for 16 h in the dark at room temperature. Then, ABTS solution was diluted with 80 % ethanol to obtain an absorbance of (0.700±0.005) at 734 nm. A volume of 3.9 mL of ABTS solution was added to 0.1 mL of the extract. The reaction mixture was allowed to react for 10 min and the absorbance was measured at 734 nm. The extracts were diluted to give 20–80 % reduction of the blank absorbance. A standard curve of Trolox was used to express the results in mg of TE per g of extract (*16*).

Determination of lipid oxidation

Determination of peroxide value

A mass of 5 g of sausages was weighed into a 250-mL Erlenmeyer flask, followed by the addition of 30 mL of the mixture of acetic acid and chloroform (3:2, by volume) and 0.5 mL of saturated potassium iodate solution. The mixture was allowed to react for 1 min, then 30 mL of deionised water and 1 mL of starch solution, 1 % by mass per volume, were added. The mixture was titrated with 0.01 M $Na_2S_2O_3$ until the blue colour disappeared. Results were expressed in mmol of O_2 per kg of sausages (*17*).

Determination of acid value

The acid value was determined according to Qi and Zhou (*18*). A mass of 5 g of sausages was mixed with 50 mL of neutralised ethyl alcohol and 0.5 mL of phenolphthalein indicator. The mixture was heated in water bath and then titrated using 0.1 mol/L of NaOH. Results were expressed in mg of NaOH per g of sausages.

Determination of lipid oxidation by TBARS assay

Thiobarbituric acid-reactive substances (TBARS) assay is the most common chemical method for the semi-quantitative estimation of lipid oxidation in foods. A mass of 5 g of sausages was extracted with 30 mL of trichloroacetic acid (7.5 %, by mass per volume). The solution was filtered and aliquots of 5 mL from the filtrate were transferred into tubes together with 5 mL of 0.02 M 2-thiobarbituric acid. For the preparation of the blank sample, 5 mL of 2-thiobarbituric acid and 5 mL of trichloroacetic acid (7.5 %, by mass per volume) were mixed. The reaction was performed in a water bath at 100 °C for 40 min. Then, the absorbance was read at 538 nm. A standard curve of 1,1,3,3-tetramethoxypropane (TMP) was prepared for the determination of malonaldehyde (*17*).

Determination of the total viable count

Plating and enumeration of the total viable count was performed using a modification of the method described by Lorenzo and Franco (*19*) and Botsaris and Taki (*20*). Briefly, from each batch of the three different sausages, 5 g were aseptically weighed and transferred into sterile stomacher bags, where they were mixed with 45 mL of sterile maximum recovery diluent (MRD; Thermo Fischer Scientific Oxoid, Basingstoke, UK) and homogenised in a stomacher for 1 min. Serial dilutions (1:10) were prepared using the MRD down to 10^{-12}. A volume of 0.1 mL of each dilution was inoculated and dispersed onto plate count agar (PCA; Oxoid) in duplicate and incubated at 30 °C for 3 days (*21*). This analysis was repeated every five days for 25 days, starting from day 1 and the total viable count was determined.

Statistical analysis

Statistical analysis was carried out using the software package SPSS v. 20.0 (SPSS Inc., Chicago, IL, USA) and the comparison of average values of each treatment was based on the analysis of variance (one-way ANOVA) according to Tukey's test at significance level of 5 % ($p \leq 0.05$).

Results and Discussion

Phytochemical analysis and antioxidant properties of P. lentiscus extracts

Mature fruits are used in traditional Cypriot sausages, but the leaves were also tested due to their low cost and availability throughout the year. Therefore, solvents of different polarity were used to prepare extracts from *P. lentiscus* fruits and leaves. Results showed that phenolic content of extracts was strongly affected by the solvent and plant material (Table 1). Methanol, a polar protic solvent, was the most suitable for the extraction of phenolic compounds from *P. lentiscus* plant materials among the studied solvents. This finding is in agreement with a previous study about the solubilisation preference of natural phenols to alcohols (*22*). The classification of the phenolic content also showed that these extracts contained significant amounts of flavonols (in fruits from (8.0±0.1) to (33.1±

Table 1. Total phenolics, hydroxycinnamates and total flavonols of *Pistacia lentiscus* leaf and fruit extracts

Solvent	*w*(total phenolics as GAE*) mg/g		*w*(total phenolics as GAE**) mg/g		*w*(hydroxycinnamates as CAE) mg/g		*w*(total flavonols as RE) mg/g	
	Leaves	Fruits	Leaves	Fruits	Leaves	Fruits	Leaves	Fruits
Butanol	(123.2±4.1)^{c}	(74.7±4.4)^{d}	(57.0±2.6)^{b}	(59.1±2.1)^{c}	(8.4±0.3)^{c}	(5.4±0.2)^{c}	(28.7±1.3)^{b}	(8.0±0.1)^{d}
Acetone	(353.7±4.5)^{b}	(178.1±7.8)^{c}	(183.6±7.0)^{a}	(110±2.7)^{b}	(16.5±0.7)^{a}	(12.7±0.2)^{b}	(34.6±1.3)^{a}	(20.8±0.4)^{c}
Methanol	(414.9±3.0)^{a}	(449.3±6.1)^{a}	(173.7±5.2)^{a}	(244.2±8.8)^{a}	(15.5±0.4)^{a}	(22.2±0.8)^{a}	(29.9±0.7)^{b}	(33.1±1.1)^{a}
Water	(397.9±15.9)^{a}	(242.8±13.6)^{b}	(168.5±5.4)^{a}	(117.6±5.7)^{b}	(11.5±0.6)^{b}	(14.2±0.8)^{b}	(20.1±1.1)^{c}	(27.0±1.6)^{b}

Total phenolics were determined by *Folin-Ciocalteu method and **Obied's protocol
GAE=gallic acid equivalent, CAE=caffeic acid equivalent, RE=rutin equivalent
Values with different letters in superscript are significantly different at $p<0.05$

1.1) and in leaves from (20.1±1.1) to (34.6±1.3) mg of rutin equivalents (RE) per g of extract) and hydroxycinnamates (in fruits from (5.4±0.2) to (22.2±0.8) and in leaves from (8.4±0.3) to (16.5±0.7) mg of caffeic acid equivalents (CAE) per g of extract). Previous studies also reported that *P. lentiscus* leaves are rich in phenolics such as tannins and glucogallin, a gallic acid glucoside (*10,23*). Overall, phenolic composition of extracts showed that methanolic extracts are the most promising extracts to determine their antioxidant effect in sausage lipid fraction.

The antioxidant activity of *P. lentiscus* extracts was evaluated using three *in vitro* assays, since an approach with multiple assays for the determination of antioxidant activity is highly advisable for natural products (*24*). DPPH and ABTS radical scavenging activities are based on the reduction of chromogenic radical by antioxidant/reducing compounds (*25*). These assays showed that leaves are a richer source of antioxidants than fruits (Figs. 1a and b). Both assays also suggested that the antioxidant activity of butanolic extracts was the lowest among the studied extracts. Furthermore, the highest antioxidant activity was measured in the methanol extract. In particular, the DPPH radical scavenging activity in fruits was from (70.2±5.3) to (387.6±16.8) and in leaves from (123.9±7.7) to (510.3±6.6) mg of TE per g of extract, while the ABTS radical scavenging activity ranged from (21.3±7.3) to (290.2±28.1) mg of TE per g of extract in fruits and from (92.4±3.9) to (384.6±6.7) mg of TE per g of extract in leaves. FRAP assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-*s*-triazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to the intensely blue-coloured ferrous complex $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ (*25*). A great diversity of antioxidant activities was found among the extracts (from (42.2±2.4) to (518.8±37.2) mg of TE per g of extract). This assay also highlighted the superiority of methanol among the used solvents, and of the antioxidant activity of the leaf extracts, compared to fruits (Fig. 1c).

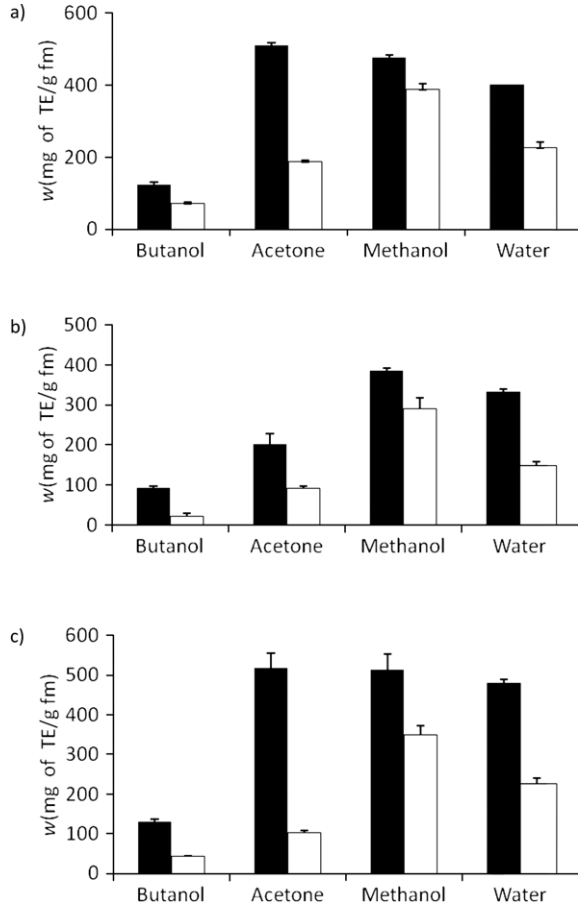

Fig. 1. Antioxidant activity of *P. lentiscus* fruit (□) and leaf (■) extracts as determined by: a) DPPH, b) ABTS, and c) FRAP assays. TE=Trolox equivalents, fm=fresh mass

Overall, methanol was the most appropriate solvent to extract polyphenolic antioxidants from *P. lentiscus* materials. In addition, polar extracts may be more suitable to inhibit lipid oxidation compared to less polar extracts, since the theory of 'polar paradox' describes that polar antioxidants are more effective in non-polar substrates such as pork sausage lipids (*26*).

The antioxidant effect of P. lentiscus extracts on pork sausage

The potential of *P. lentiscus* extracts to prevent the oxidation of lipids in pork sausages was investigated using three methods (peroxide, acid and TBARS values). Peroxide value is used for monitoring the initial stages of autoxidation, the acid value is correlated with lipid rancidity (*18*), while TBARS quantifies the secondary products of lipid oxidation of unsaturated fatty acids (*27*). Results showed that peroxide values of all samples increased during storage at 4 °C, as expected (Fig. 2a). In general, the rate of formation of peroxides in sausages enriched with *P. lentiscus* extracts was lower than in the sausages without the extract (control). After 20 days of storage at 4 °C, peroxide values compared to the control sausages were 38.2 and 47.2 % lower in sausages with leaf and fruit extract, respectively. Georgantelis *et al*. (*7*) also reported a reduction of 29.1 and 74.8 % of peroxide values in sausages containing α-tocopherol and rosemary extract, respectively, after 20 days cold storage. Statistical analysis showed no significant differences in the antioxidant effect of the two extracts during storage at 4 °C.

The acid values were also determined since they are an indicator of lipid rancidity that affects odour and taste of meat products. Both triglycerides and phospholipids contribute to the development of rancidity, although the contribution of phospholipids is the most important (*28*). The acid value of control sausages increased progressively during cold storage; an increase of acid value was monitored during 20 days of storage at 4 °C (Fig. 2b). The acid value of sausages containing fruit extract changed less in the initial stages of storage and increased after 20 days of cold storage. On the other hand, the acid value of sausages enriched with leaf extract did not change during storage. Specifically, at 20 days of cold storage, the acid values of sausages containing fruit and leaf extract were 9 and 31 %, respectively, lower compared to the control, highlighting the potential of leaf extract.

The measurement of TBARS demonstrated that the addition of *P. lentiscus* to pork sausages had a significant effect on their lipid oxidation. TBARS of control sausages increased during storage; the highest rate of TBARS formation was found between 10 and 15 days of cold storage

in sausages with and without extract (Fig. 2c). On the 15th day, TBARS value of control sausages was 1741 mg/kg, while sausages with fruit and leaf extracts had significantly lower TBARS values (<869 mg/kg). Results did not show any significant differences between leaf and fruit extracts.

Overall, peroxide and TBARS values showed that both fruit and leaf extracts prevented significantly the lipid oxidation of pork sausages. In addition, the acid value confirmed the strong antioxidant effect of the leaf extract, but the fruit extract did not inhibit lipid oxidation. This fact could be attributed to the presence of fatty acids such as oleic and linoleic acids in *P. lentiscus* fruit (*29*), which gives higher acidity to sausages with added fruit extracts.

Antimicrobial effect of P. lentiscus extracts on pork sausage

The results of the total viable count are graphically presented in Fig. 3 in the form of a box and whisker plot.

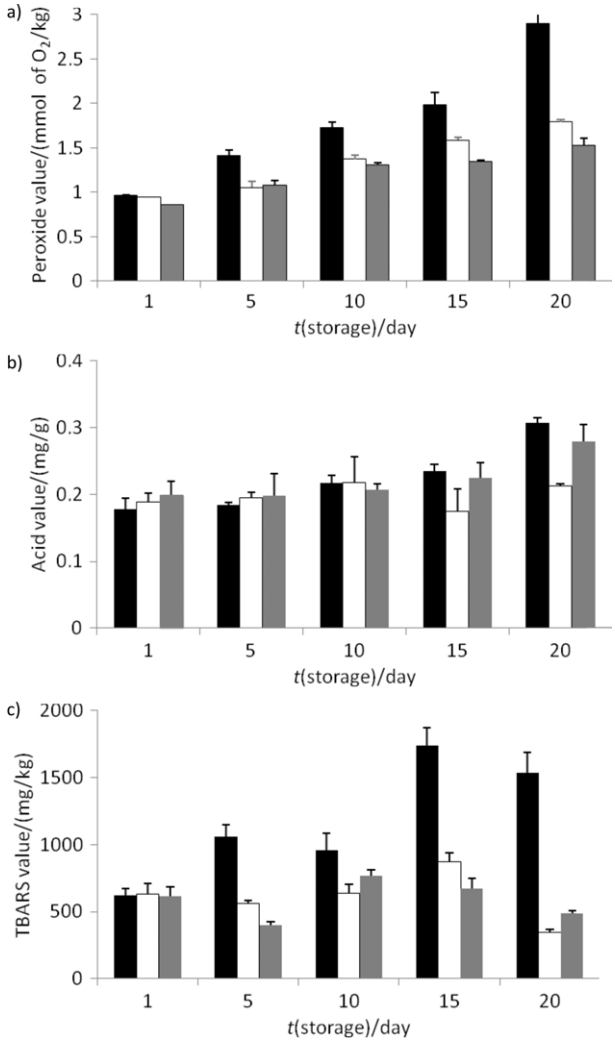

Fig. 2. Effect of *P. lentiscus* fruit and leaf extracts on pork sausage lipids as measured by: a) peroxide value, b) acid value, and c) TBARS value. (■) sausages without extract (control), (□) sausages with leaf extract, and (■) sausages with fruit extract

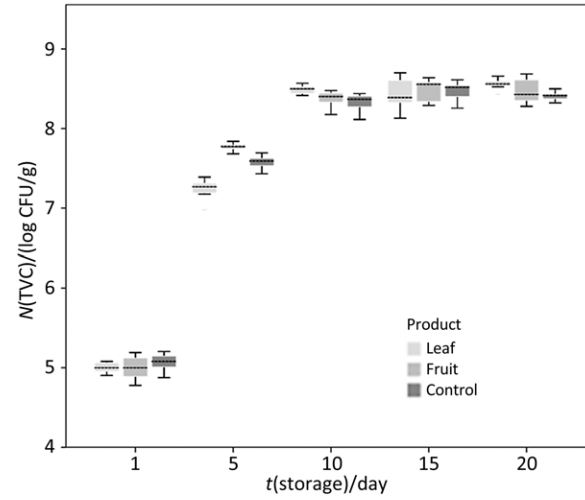

Fig. 3. Effect of *P. lentiscus* fruit and leaf extracts on total viable count (TVC) of pork sausages stored at 4 °C

The box represents 50 % of the population of each count and the remaining 50 % is covered by the whiskers. The horizontal dotted line crossing the box represents the median value of each of the three different samples tested (leaf, fruit and control). The initial mean count of the control±standard error on day 1 was (5.07±0.03) log CFU/g. Sausages prepared with leaf and fruit extracts had initial counts on day 1 of (5.00±0.02) and (5.00±0.04) log CFU/g, respectively. Analysis of variance revealed no significant differences between the initial counts ($p=0.145$). At the second point of sampling, which was after five days of refrigerated storage, a statistically significant difference was observed between the samples ($p<0.0005$), with the sausage made with the leaf extract exposing the lowest total viable count of (7.25±0.03) log CFU/g. Statistically significant differences were not observed during storage at 4 °C for 10, 15 and 20 days. On day 10 all samples had mean viable count above 8 log CFU/g, which was considered to be the threshold for microbial rejection of the sausages. At this point spoilage was visible in all samples, even those containing plant extracts. The addition of leaf extract was successful in inhibiting the growth of aerobic microorganisms during the first five days of storage. This is an important technological improvement of a product that usually has less than a week for display on the shelf. Previous research using extracts has also revealed important improvement of microbial quality (*30*), but in some cases the intense odour produced by the extract was a major drawback for the industrial application. In the case of the traditional sausages presented here, the application of the extract did not result in off-odours as *P. lentiscus* is a traditional additive to the sausage.

Conclusions

Phenolic content and antioxidant properties of *Pistacia lentiscus* are strongly affected by extraction solvent. In general, methanol was the most appropriate solvent to extract antioxidants from this plant material. The use of methanolic extracts as food additives in pork sausages inhibited significantly the lipid oxidation during storage at

4 °C. On the other hand, the methanolic extracts reduced microbial spoilage of fresh traditional pork sausages in the initial stages of storage. Overall, although leaves were richer in phenolics than fruits, both extracts are an excellent source of natural antioxidants and are candidates as additives in sausages and meat products. This is the first time that *P. lentiscus* fruits and leaves have been evaluated as food additives in meat products. Further studies are needed to fractionate the extracts and to isolate individual compounds and/or fraction with potent antioxidant and antimicrobial activities.

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