Food and Chemical Toxicology 86 (2015) 319–327

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Feed supplemented with polyphenolic byproduct from olive mill wastewater processing improves the redox status in blood and tissues of piglets

Konstantinos Gerasopoulos [a, b], Dimitrios Stagos [a], Konstantinos Petrotos [b], Stylianos Kokkas [b], Dimitrios Kantas [b], Panagiotis Goulas [b], Dimitrios Kouretas [a, *]

[a] Department of Biochemistry and Biotechnology, University of Thessaly, Ploutonos 26 and Aiolou, 41221 Larissa, Greece
[b] Department of Biosystem Engineering, Technical Education Institute of Thessaly, 41110 Larissa, Greece

ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form
5 October 2015
Accepted 5 November 2015
Available online 10 November 2015

Keywords:
Olive mill wastewater
Antioxidant feed
Piglet
Ceramic membrane microfiltration
Natural products
Oxidative stress

ABSTRACT

In the present study, a polyphenolic byproduct from olive mill wastewater (OMWW) was used for making piglet feed with antioxidant activity. For examining the antioxidant capacity of the feed, 30 piglets of 20 d old were divided into two groups receiving basal or experimental feed for 30 d. Blood and tissue samples were drawn at days 2, 20, 35 and 50 post-birth. The tissues collected were brain, heart, kidney, liver, lung, quadriceps muscle, pancreas, spleen and stomach. The antioxidant effects of the experimental feed were assessed by measuring oxidative stress biomarkers in blood and tissues. The oxidative stress markers were total antioxidant capacity (TAC), glutathione (GSH), catalase activity (CAT), protein carbonyls (CARB) and thiobarbituric acid reactive species (TBARS). The results showed that piglets fed with diet supplemented with OMWW polyphenols had significantly increased antioxidant mechanisms in blood and the majority of the tested tissues as shown by increases in TAC, CAT and GSH compared to control group. Moreover, piglets fed with the experimental feed exhibited decreased oxidative stress-induced damage to lipids and proteins as shown by decreases in TBARS and CARB respectively. This is the first study in which OMWW polyphenols were used for making pig feed with antioxidant activity.

1. Introduction

Free radicals are atoms, molecules or ions that have unpaired valence electrons (Halliwell, 2001). Free radicals such as reactive oxygen (ROS) and nitrogen (RNS) species are produced in the living organisms either from normal essential metabolic processes or from external sources (e.g. exposure to X-rays, air pollutants, and industrial chemicals) (Valko et al., 2007). Oxidative stress is defined as an imbalance between the production of free radicals and the ability of the organism to counteract or detoxify their harmful effects through neutralization by antioxidants (Halliwell, 2001). Because free radicals are very reactive species, their overproduction during oxidative stress can cause damage to all biological macromolecules such as DNA, proteins and lipids and thus resulting in cell damage and subsequently in manifestation of pathological conditions (Halliwell, 2001). Several studies have suggested that oxidative stress in farm animals may be involved in pathological conditions and in conditions related to animal production and the general welfare (Lykkesfeldt and Svendsen, 2007).

Oxidative stress in farm animal diseases has primarily been studied in cattle, horses and pigs (Lykkesfeldt and Svendsen, 2007). For example, exposure of piglets to high levels of O_2 and $NO^{\bullet}$ increased inflammation and decreased surfactant function in lungs

Abbreviations: AMPK, AMP-activated protein kinase; ARE, antioxidant response element; BOD, biochemical oxygen demand; COD, chemical oxygen demand; DNPH, 2,4-Dinitro-phenyl-hydrazine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTNB, 5,5′-dithiobis- (2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; GCL, γ-glutamylcysteine ligase; GSH, reduced glutathione; GPx, glutathione peroxidase enzyme; GSSG, oxidized glutathione; OMWW, olive mill wastewater; RO, reverse osmosis; ROS, reactive oxygen species; RNS, reactive nitrogen species; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid.

* Corresponding author.
E-mail address: dkouret@uth.gr (D. Kouretas).

http://dx.doi.org/10.1016/j.fct.2015.11.007
0278-6915/

(Robbins et al., 1995). Moreover, in an experimental model of *Actinobacillus pleuropneumoniae*-induced pneumonia in pigs, the blood levels of the antioxidant ascorbate has been shown to decline in parallel with the progression of the disease, while its levels were normalized after antiobiotic treatment (Lauritzen et al., 2005, 2003a, 2003b). In addition, in a piglet model of deoxycholate-induced gut injury, the concentrations of nitrite, the end product of $NO^{\bullet}$, were increased in parallel with inflammation biomarkers (Miller et al., 1993). Accumulating evidence has also suggested that porcine endotoxaemic shock may be associated with increased oxidative stress and damage, while antioxidant supplementation counteracted these effects (Basu and Eriksson, 2001, 2000, 1998). Of course, it should be noted that antioxidant supplementation may have negative effects on animal health. ROS at low levels are necessary, since they have important roles in cell signaling and homeostasis (Halliwell, 2001). Moreover, ROS at low levels may exhibit the phenomenon of hormesis, that is, the induction of favorable biological responses (Goto and Radak, 2009; Radak et al., 2008). Thus, low levels of ROS may upregulate antioxidant defense mechanisms, and so the organism would be more prepared for counteracting oxidative stress (Goto and Radak, 2009).

Several studies have shown that olive oil due to its phytochemicals such as polyphenols has strong antioxidant activity (Kushi et al., 2012; Klein et al., 2011; Ouyang et al., 2012; Ibiebele et al., 2013). Apart from olive oil itself, some of the byproducts of its production such as olive mill wastewater (OMWW) exhibit also antioxidant activity (Frankel et al., 2013). OMWW is a liquid effluent derived mainly from the water used for the various stages of oil production and vegetable water from the fruit, and amounts of up to 0.5–3.25 m^3 per 1000 kg of olives (Kapellakis et al., 2012; Paraskeva and Diamadopoulos, 2006). Moreover, OMWW has a dark brown colour, high organic content [chemical oxygen demand (COD) 45–170 g/L and biochemical oxygen demand (BOD) 35–110 g/L], suspended solids (SS) 1–9 g/L, strong specific olive oil smell and acidic pH (El-Khateeb et al., 2014). OMWW contains basically tannins, lignins, long-chain fatty acids, reduced sugars, proteins and phenolic compounds (Paixao and Anselmo, 2002; Paraskeva and Diamadopoulos, 2006). The polyphenols found in OMWW include hydroxytyrosol and tyrosol as the major components, as well as p-coumaric acid, homovanillic acid, caffeic acid, protocatechuic acid, 3,4-dihydroxymandelic acid, vanillic acid and ferulic acid that exhibit antioxidant activity (Frankel et al., 2013). The disposal of OMWW causes serious environmental problems such as soil contamination, water body pollution, underground seepage and odour (Rinaldi et al., 2003). Thus, the discharge of large quantities of OMWW into the sewage system is not possible without any treatment such as aerobic treatment or anaerobic digestion and composting (Rinaldi et al., 2003; Aly et al., 2014). However, an environmentally safe and cost-effective treatment of OMWW has not yet been established (Zagklis et al., 2013). The isolation of the polyphenolic content of OMWW could represent a source of antioxidants, while reducing the organic overload of OMWW.

Our research group has developed a patented methodology (patent application number: 20120100569 – Greek Industrial Property Organisation) for obtaining polyphenols from OMWW based on the use of ceramic membrane microfiltration. Byproducts produced by this method contain a part of the OMWW polyphenols. In a previous study, we have shown that feed supplemented with these byproducts increased antioxidant status in broiler chickens (Gerasopoulos et al., 2015). In the present study, one of these byproducts was utilized for making piglet feed supplemented with antioxidant compounds. The antioxidant effects of feed supplemented with polyphenols from OMWW byproduct were assessed by measuring oxidative stress biomarkers in piglets' blood and in nine different tissues (i.e. brain, heart, kidney, liver, lung, pancreas, stomach, spleen and quadriceps muscle).

2. Materials and methods

2.1. Preparation and isolation of byproducts containing polyphenolic compounds from OMWW processing

The isolation of two byproducts containing polyphenolic compounds from OMWW processing was based on a patented OMWW polyphenol powder production scheme that has been previously described (Gerasopoulos et al., 2015). In brief, raw OMWW was first passed through a finisher in order to separate suspended particles in the form of a heavy sludge (Fig. 1). The finisher that was used for this first clarification step of the OMWW was a standard one step butterfly type finisher, operating at 1200 rpm/min and was equipped with a cylindrical stainless steel sieve with 150 μm diameter openings. The suspended particles from the OMWW raw material were removed by the finisher, in order to avoid clogging of the ceramic microfiltration membranes in the subsequent steps. Afterwards, the purified OMWW was passed through a ceramic microfiltration unit (Jiangu Jiuwu Hi-Tech Co. Ltd, Nanjing, China) where 30% of the input OMWW stream was separated as retentate

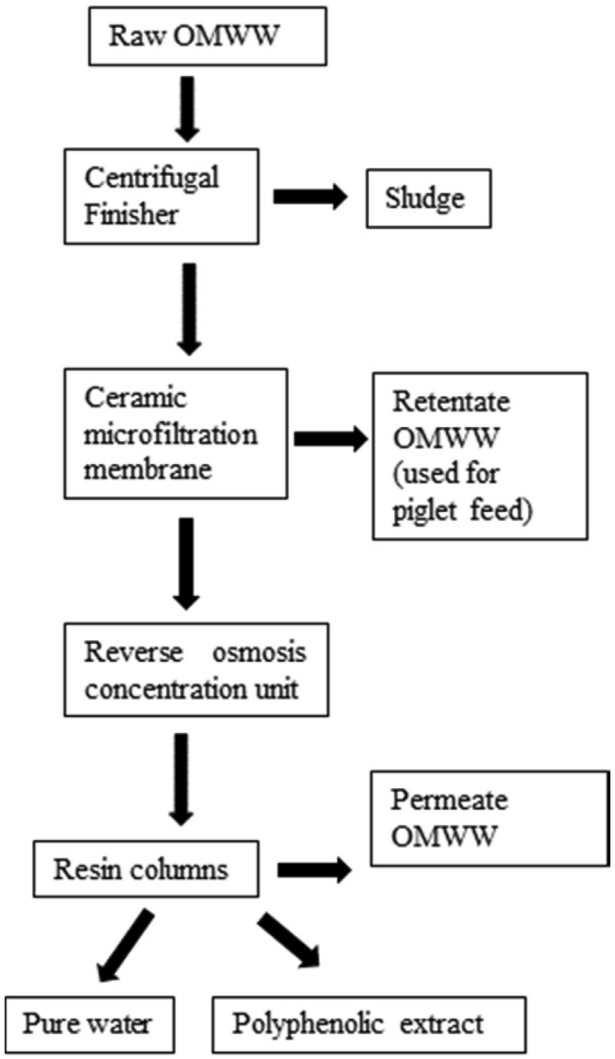

Fig. 1. Patented OMWW polyphenol powder production scheme.

(upstream - this was used for silage production and designated hereafter as OMWW retentate) and 70% as permeate (downstream) in a tangential mode of operation. The microfiltration unit consisted of a membrane module with three pieces of ceramic microfiltration membranes (type CMF19040) with a total area of 0.69 m^2. The unit was fed with a positive displacement pump ensuring fluid velocity of 10 m/sec in order to avoid membrane fouling. The microfiltration permeate was then concentrated at a low temperature by using a standard reverse osmosis (RO) membrane unit to a concentration factor of 4:1 by using a pressure of 20 bar and a spiral type membrane module, thus producing a polyphenol-rich concentrate. Then, this was passed through a XAD-4 resin column to isolate the main product which was the polyphenol extract that could be used as raw material for antioxidant powder production. The liquid material which was the leftover of the polyphenol absorption from the resins and which contained approximately 20–30% of the initial polyphenolic content of the input material was the second byproduct. From the above mentioned two byproducts of the patented polyphenol powder production scheme, the OMWW retentate was used for silage production by mixing it with corn. This processing ensured a total discharge of the OMWW and protection of the ecosystem as only pure water is disposed to the environment.

2.2. Silage and piglet feed preparation

OMWW retentate was used for making silage corn. For this purpose, corn was mixed with OMWW retentate at a ratio of 24:1. Thus, silage corn was made that contained 56% solids, 4% OMWW retentate and 40% liquid. Then, standard commercial formulation of lactic bacteria was used for the lactic fermentation of corn. The lactic bacteria had been dissolved in water (10% w/v) by stirring and warmed at 40 °C in order to be activated prior to mixing with corn. After activation, lactic bacteria were mixed with corn (1 g of bacteria with 100 kg of corn). For producing the silage, the mixture of lactic bacteria and corn was placed in special airtight-seal plastic bags and was fermented for 3–4 weeks. To prevent the bags from rupturing due to the inflation caused by the carbon dioxide production during fermentation, the material was repackaged in new plastic bags every two to three days. Finally, the resulting silages were mixed with other ingredients to make the final piglet feed (Table 1).

2.3. Animals

The experiment was reviewed and approved by the institutional review board and the appropriate state authority. Thirty piglets were used that were from the pigsty of the Technical Education Institute of Thessaly. All piglets came from Landrace (mother) x Large White - Duroc - Pietrain (father) cross. The piglets were housed under controlled environmental conditions (12-h light/dark cycle, temperature 27–33 °C, humidity 50–70%) in standard single cages (for each group). All the newborn pigs were fed exclusively with breast milk for 20 d post birth. Then, they were separated into two groups (15 piglets per group) as follows: i) Control group fed with basal ration and ii) polyphenolic group fed with ration supplemented with OMWW retentate. However, up to 35 d post birth (i.e. feeding with ration for 15 days) the piglets of both groups were fed with both breast milk and the respective ration. After the 35 d post birth, the piglets were fed with ration only for 15 days.

Table 1
Ingredients and nutrient composition of experimental diets.

Ingredients	Composition (% w/w)
Corn	46.5[a]
Soybean	21.0
Milk powder	20.0
Pig grower concentrate	10.0
Balancer (piglet corn)	2.5

[a] Corn contained 60% solids and 40% liquid in control feed; 56% solids, 4% OMWW retentate and 40% liquid in feed supplemented with OMWW polyphenols.

2.4. Blood and tissue collection

Blood samples were drawn at days 2, 20, 35 and 50 post birth. The first two blood samplings (2 and 20 d post birth; at each time point blood was collected from 2 piglets) were made in order to determine the redox status at a very young age without the administration of ration. At 35 d post birth (i.e. after piglets had been fed with both breast milk and ration for 15 days) 6 blood samples were taken from each group. At 50 d post birth (i.e. after piglets had been fed with ration only for 15 days), 7 blood samples were taken from each group. For blood collection, piglets were restrained manually and 4 mL of blood was collected from the anterior vena cava and placed in ethylenediamine tetraacetic acid (EDTA) tubes. Blood samples were centrifuged immediately at 1370 g for 10 min at 4 °C and the plasma was collected and used for measuring TAC, TBARS and CARB. The packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged at 4020 g for 15 min at 4 °C and the erythrocyte lysate was collected for the measurement of GSH and CAT.

Tissue collection was also performed at the same time points that blood samples were drawn. The tissues that were collected were brain, heart, kidney, liver, lung, quadriceps muscle, pancreas, spleen and stomach. For tissue collection, the piglets were sacrificed in a fully automated slaughter complex (Slaughterhouses of Larissa S.A., Girtoni, Greece). All relevant procedures (e.g. CO_2 stunning, slaughter, bleeding, skin removal, gutting, viscera separation and washing) were executed by special machines and specialized staff. Tissues were quickly removed and snaped-frozen in liquid nitrogen. In preparation for tissue biochemical analysis, mortar and pestle were used for crushing and grinding the samples with the assistance of liquid nitrogen. One part of tissue powder was then homogenized with two parts (weight/volume) of 0.01 M phosphate buffered saline pH 7.4 (138 mM NaCl, 2.7 mM KCl, and 1 mM EDTA) and a cocktail of protease inhibitor tablet (complete mini, Roche, Germany) was added. The homogenate was vigorously vortexed and a brief sonication treatment on ice was applied. The homogenate was then centrifuged at 12,000 g for 30 min at 4 °C and the supernatant was collected. Plasma, erythrocyte lysate and tissues were then stored at −80 °C until biochemical analysis.

2.5. Oxidative stress biomarkers

Oxidative stress biomarkers were assessed as described previously (Gerasopoulos et al., 2015). In particular, for thiobarbituric acid–reactive substances (TBARS) determination, a slightly modified assay of Keles et al. (2001) was used. According to this method, 100 μL of plasma or 50 μL of muscle homogenate (diluted 1:2) was mixed with 500 μL of 35% TCA and 500 μL of Tris–HCl (200 mmol/L; pH 7.4), and incubated for 10 min at room temperature. One milliliter of 2 M Na_2SO_4 and 55 mM thiobarbituric acid solution was added, and the samples were incubated at 95 °C for 45 min. The samples were cooled on ice for 5 min, and were vortexed after 1 mL of 70% TCA was added. The samples were centrifuged at 15,000 g for 3 min, and the absorbance of the supernatant was read at 530 nm. A baseline shift in absorbance was taken into account by running a

blank along with all samples during the measurement. Calculation of TBARS concentration was based on the molar extinction coefficient of malondialdehyde.

Protein carbonyls were determined based on the method of Patsoukis et al. (2004). In this assay, 50 μL of 20% TCA was added to 50 μL of plasma or muscle homogenate (diluted 1:2), and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000 g for 5 min at 4 °C. The supernatant was discarded, and 500 μL of 10 mM 2,4-dinitrophenyl hydrazine (DNPH), in 2.5 N HCl for the sample or 500 μL of 2.5 N HCl for the blank was added in the pellet. The samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min, and were centrifuged at 15,000 g for 5 min at 4 °C. The supernatant was discarded, and 1 mL of 10% TCA was added, vortexed, and centrifuged at 15,000 g for 5 min at 4 °C. The supernatant was discarded, and 1 mL of ethanol-ethyl acetate (1:1 v/v) was added, vortexed, and centrifuged at 15,000 g for 5 min at 4 °C. This washing step was repeated twice. The supernatant was discarded, and 1 mL of 5 M urea (pH 2.3) was added, vortexed, and incubated at 37 °C for 15 min. The samples were centrifuged at 15,000 g for 3 min at 4 °C, and the absorbance was read at 375 nm. Calculation of protein carbonyls concentration was based on the molar extinction coefficient of DNPH. Total plasma protein was assayed using a Bradford reagent (Sigma–Aldrich Ltd.).

The determination of TAC was based on the method of Janaszewska and Bartosz (2002). Briefly, 20 μL of plasma or 40 μL muscle homogenate (diluted 1:10 with PBS) were added, respectively, to 480 μL or 460 μL of 10 mM sodium potassium phosphate (pH 7.4) and 500 μL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, and the samples were incubated in the dark for 30 min at room temperature. The samples were centrifuged for 3 min at 20,000 g, and the absorbance was read at 520 nm. TAC is presented as mmol of DPPH reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH:H) by the antioxidants of plasma and muscle.

Reduced glutathione (GSH) was measured according to the methods of Reddy et al. (2004). In particular, 20 μL of erythrocyte lysate or muscle homogenate (diluted 1:2), treated with 5% TCA, was mixed with 660 μL of 67 mM sodium potassium phosphate (pH 8.0) and 330 μL of 1 mM 5,5′-dithiobis-2 nitrobenzoate (DTNB). The samples were incubated in the dark at room temperature for 45 min, and the absorbance was read at 412 nm. GSH concentration was calculated on the basis of a calibration curve made using commercial standards.

Catalase activity was determined using the method of Aebi (1984). Briefly, 4 μL of erythrocyte lysate (diluted1:10) or 40 μL muscle homogenate (diluted 1:2) were added, respectively, to 2991 μL or 2955 μL of 67 mM sodium potassium phosphate (pH 7.4), and the samples were incubated at 37 °C for 10 min. A total of

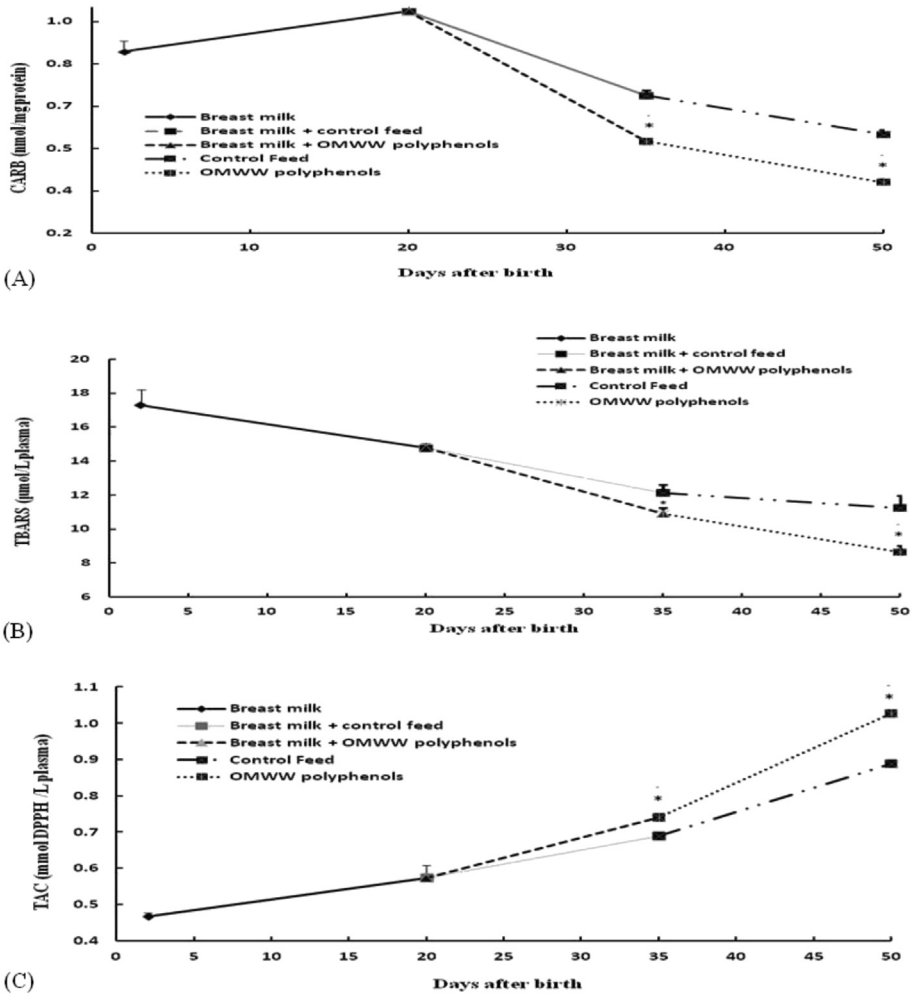

Fig. 2. Effects on oxidative stress markers, (A) CARB, (B) TBARS and (C) TAC, in plasma of chickens at 2, 20, 35 and 50 d post birth. The feeding of the piglets was as follows: up to 20 d, breast milk; from 21 to 35 d, breast milk + basal feed or feed supplemented with OMWW retentate; from 36 to 50 d, basal feed or feed supplemented with OMWW retentate. *Significantly different from values of control group at the same sampling time ($p < 0.05$). The results are presented as the mean ± SEM.

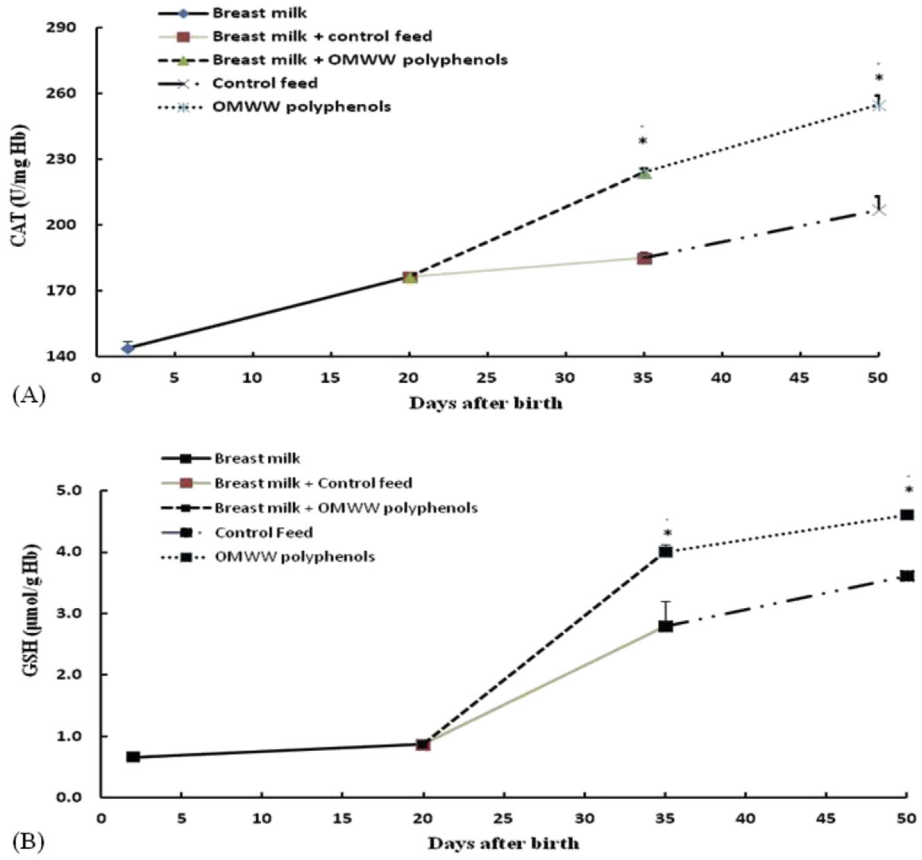

Fig. 3. Effects on oxidative stress markers, (A) CAT activity and (B) GSH in erythrocytes of piglets at 2, 20, 35 and 50 d post birth. The feeding of the piglets was as follows: up to 20 d, breast milk; from 21 to 35 d, breast milk + basal feed or feed supplemented with OMWW retentate; form 36–50 d, basal feed or feed supplemented with OMWW retentate. *Significantly different from values of control group at the same sampling time ($p < 0.05$). The results are presented as the mean ± SEM.

5 μL of 30% hydrogen peroxide was added to the samples, and the change in absorbance was immediately read at 240 nm for 1.5 min. Calculation of catalase activity was based on the molar extinction coefficient of H_2O_2.

Each assay was performed in triplicate within 3 months of the blood collection. Blood samples were stored in multiple aliquots at −80 °C, and thawed only once before analysis. All reagents were purchased from Sigma–Aldrich (St. Louis, Mo.).

2.6. Statistical analysis

Data were analyzed by one-way ANOVA. The level of statistical significance was set at $p < 0.05$. All results are expressed as mean ± SEM. Data were analyzed using SPSS, version 13.0 (SPSS Inc., Chicago, Ill).

3. Results

3.1. Assessment of oxidative stress markers in blood

Administration to piglets of feed supplemented with OMWW retentate improved their redox status as indicated by the tested oxidative stress biomarkers. In particular, CARB levels in plasma were decreased significantly by 23.6 and 30.7% at days 35 and 50 post birth respectively compared to the control group (Fig. 2A). TBARS levels in plasma were also decreased significantly in the group received OMWW retentate compared to the control group, by 10.1 and 23.1% at days 35 and 50 post birth respectively (Fig. 2B). TAC in plasma was increased significantly by 15.7% in the group received OMWW retentate at 50 d post birth compared to the control group (Fig. 2C).

In OMWW retentate group, CAT was increased significantly in erythrocytes by 21.1 and 23.2% at days 35 and 50 post birth compared to the control group (Fig. 3A). Like CAT, GSH levels in erythrocytes were increased in OMWW retentate group by 43.5 and 27.7% at days 35 and 50 post birth respectively compared to the control group (Fig. 3B).

3.2. Assessment of oxidative stress markers in tissues

The administration of OMWW retentate had similar effects on the oxidative stress markers assessed in different tissues (i.e. brain, heart, kidney, liver, lung, quadriceps muscle, pancreas, spleen and stomach). In particular, CARB levels were decreased in all tissues in the group received OMWW retentate for 15 and 30 d post birth, compared to the control group (Fig. 4A and B). Specifically, at 35 d after birth (i.e. 15 days after OMWW retentate administration), CARB levels were decreased significantly in brain by 11.0%, in heart by 19.8%, in kidneys by 22.8%, in liver by 24.0%, in lungs by 17.4%, in quadriceps muscle by 18.9%, in pancreas by 26.7%, in spleen by 32.9% and in stomach by 9.6% compared to the control group (Fig. 4A). CARB levels were also decreased significantly at 50 d after birth (i.e. 30 days after OMWW retentate administration) in brain by 27.2%, in heart by 31.4%, in kidneys by 29.1%, in lungs by 29.7%, in quadriceps muscle by 26.1%, in pancreas by 36.6%, in spleen by 44.9% and in stomach by 39.2% compared to the control group (Fig. 4B).

Like CARB levels, TBARS levels were decreased in all tissues in

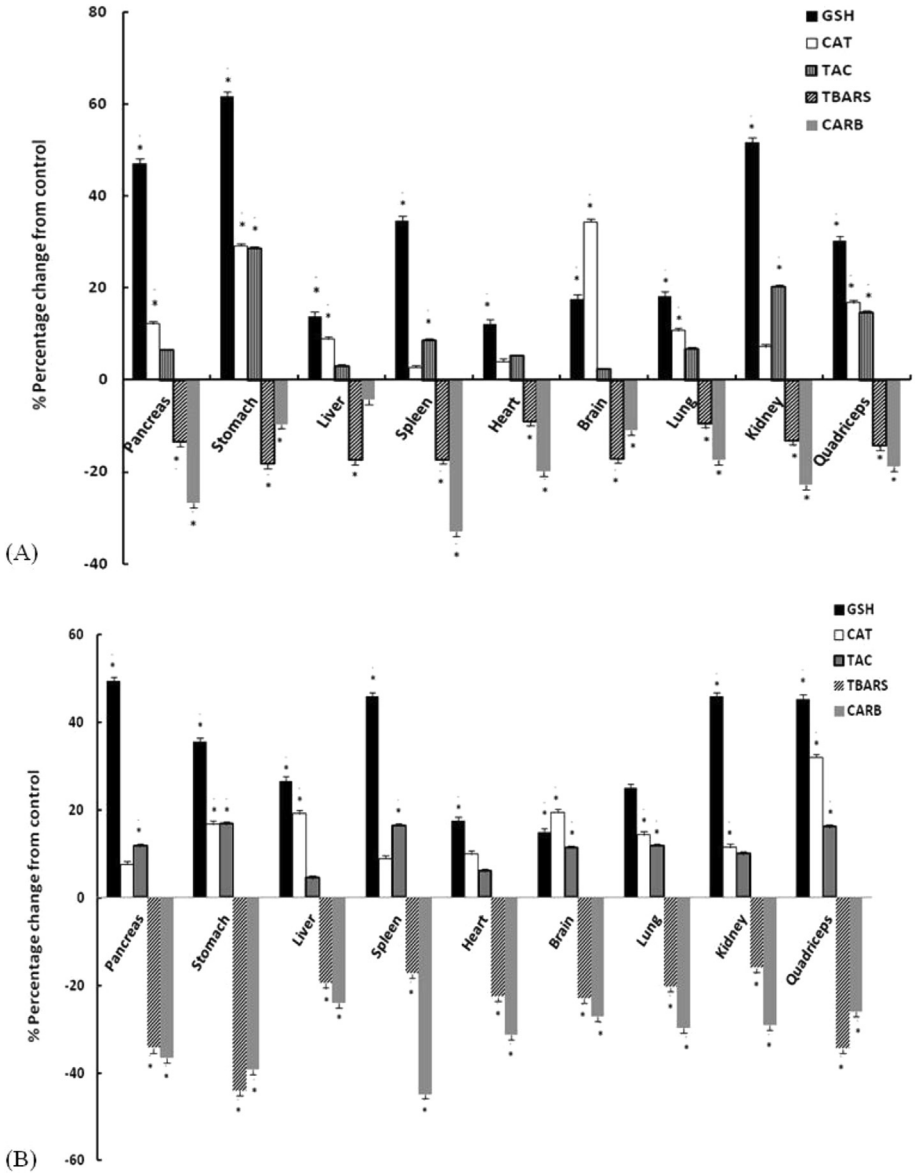

Fig. 4. Effects on GSH, CAT activity, TAC, TBARS and CARB in tissues of piglets fed with diet supplemented with OMWW retentate for (A) 15 d (i.e. at 35 d post birth) and (B) for 30 d (i.e. at 50 d post birth). *Significantly different from the values of control group at the same sampling time ($p < 0.05$). The results are presented as the mean ± SEM.

the group received OMWW retentate for 15 and 30 d compared to the control group (Fig. 4A and B). So, at 35 d after birth, TBARS levels were decreased significantly in brain by 17.1%, in heart by 9.0%, in kidneys by 13.1%, in liver by 17.4%, in lungs by 9.3%, in quadriceps muscle by 14.3%, in pancreas by 13.4%, in spleen by 17.3% and in stomach by 18.3% compared to the control group (Fig. 4A). TBARS levels were also decreased significantly at 50 d after birth in brain by 23.0%, in heart by 23.0%, in kidneys by 16.0%, in liver by 19.5%, in lungs by 20.4%, in quadriceps muscle by 34.6%, in pancreas by 34.4%, in spleen by 17.4% and in stomach by 44.1% compared to the control group (Fig. 4B).

TAC was increased in all tissues in the group received OMWW retentate for 15 and 30 d compared to the control group (Fig. 4A and B). Thus, TAC levels were increased significantly at 35 d after birth in kidneys by 20.4%, in quadriceps muscle by 14.8%, in spleen by 8.7% and in stomach by 28.7% compared to the control group (Fig. 4A). At 50 d after birth, TAC levels were increased significantly in brain by 11.3%, in kidneys by 9.9%, in lungs by 11.8%, in quadriceps muscle by 16.2%, in pancreas by 11.7%, in spleen by 16.4% and in stomach by 16.8% compared to the control group (Fig. 4B).

CAT was also increased in all tissues in the group received OMWW retentate for 15 and 30 d compared to the control group (Fig. 4A and B). Specifically, CAT levels were increased significantly at 35 d after birth in brain by 34.4%, in liver by 8.8%, in lungs by 10.7%, in quadriceps muscle by 16.8%, in stomach by 29.1% and in pancreas by 12.2% compared to the control group (Fig. 4A). At 50 d after birth, CAT levels were increased significantly in brain by 19.6%, in liver by 19.2%, in heart by 9.9%, in kidneys by 11.5%, in lungs by 14.4%, in quadriceps muscle by 32.1% and in stomach by 16.8% compared to the control group (Fig. 4B).

Finally GSH levels were also increased in all tissues in the group received OMWW retentate for 15 and 30 d compared to the control group (Fig. 4A and B). In particular, GSH levels were increased significantly at 35 d after birth in brain by 17.6%, in heart by 12.2%, in kidneys by 51.7%, in liver by 13.9%, in lungs by 18.2%, in quadriceps muscle by 30.2%, in spleen by 34.6%, in stomach by 61.6% and

in pancreas by 47.1% compared to the control group (Fig. 4A). GSH levels were also increased significantly at 50 d after birth in brain by 15.0%, in liver by 26.7%, in heart by 17.5%, in kidneys by 46.0%, in lungs by 25.0%, in quadriceps muscle by 45.5%, in pancreas by 49.5%, in spleen by 46.0% and in stomach by 35.7% compared to the control group (Fig. 4B).

4. Discussion

Accumulating evidence suggests that oxidative stress affects farm animal wellness and productivity (Lykkesfeldt and Svendsen, 2007). For example, weaning has been shown to be a stressful condition for piglets and is associated with pathological conditions such as gastrointestinal disorders (Boudry et al., 2004). Interestingly, Zhu et al. (2012) have shown recently that weaning of piglets was also associated with reduction of antioxidant mechanisms. This hypothesis has been supported by our findings, since all the oxidative stress markers in blood of the control group were improved post weaning. Namely, markers showing antioxidant capacity (i.e. TAC, GSH and CAT) were increased, while markers indicating oxidative damage (i.e. protein oxidation and lipid peroxidation) were decreased in the control group post weaning. Thus, it seems that during weaning oxidative stress is induced and/or antioxidant mechanisms have not been adequately developed. Several studies have suggested the supplementation of feeds with antioxidants as a means for reducing the detrimental effects of oxidative stress on animal health (Zhu et al., 2012; Fragou et al., 2004; Eicher et al., 2006). In a previous study, we have shown that incorporation of antioxidant polyphenols from byproducts of OMWW to chicken broiler feed improved their redox status (Gerasopoulos et al., 2015). In the present study, a byproduct from OMWW processing was used for making a piglet feed with antioxidant activity.

Thus, the administration of feed supplemented with OMWW retentate increased TAC in plasma and most of the tested tissues indicating an enhancement of the piglets' total antioxidant mechanisms. This increase was more profound after 30 d administration of the experimental feed, since at this time point there was a significant increase in TAC in plasma as well as in 7 out of 9 tested tissues compared to the control group.

The increase in piglets' TAC after consumption of feed supplemented with polyphenols from OMWW retentate may be attributed to the direct free radical scavenging, since OMWW polyphenols are strong antioxidants (Frankel et al., 2013). However, it also seems that feed supplemented with OMWW retentate enhanced piglets' TAC through increase in antioxidant molecules. Thus, in piglets fed diet supplemented with OMWW retentate, the CAT activity, one of the most important antioxidant enzymes, was increased in erythrocytes and tested tissues, especially after 30 d feeding. A cellular study in porcine pulmonary artery endothelial cells has suggested that hydroxytyrosol, one of the main polyphenols found in OMWW, increased catalase mRNA and protein expression and activity through phosphorylation of AMP-activated protein kinase (AMPK) leading to activation of FOXO3a transcription factor (Zrelli et al., 2011). Moreover, administration of diet supplemented with extra-virgin olive oil rich in polyphenols to mice increased mRNA expression of CAT in pancreatic islets (Oliveras-Lopes et al., 2008). CAT, an enzyme catalyzing the decomposition of hydrogen peroxide to water and oxygen, is one of the most important antioxidant enzymes protecting the cell from ROS (Halliwell, 2001). Importantly, a recent study has shown that piglets affected by rotaviral enteritis, a major cause of morbidity and mortality during their post-natal life, had low serum levels of CAT (Kumar et al., 2014).

Apart from increase in CAT activity, the administration of feed supplemented with OMWW retentate for both 15 and 30 d increased GSH levels, one of the most important antioxidant molecules in living organisms (Halliwell, 2001), in erythrocytes and all tested tissues. Moreover, the assessment of GSH levels during weaning (especially until 20 d post birth) showed that the piglets had very low GSH, and so they may be more vulnerable to oxidative stress-induced diseases, as has been suggested by previous studies (Zhu et al., 2012; Fragou et al., 2004; Eicher et al., 2006). Indeed, as mentioned above, weaning has been shown to be a stressful condition for piglets and is associated with oxidative stress and subsequent pathological conditions such as gastrointestinal disorders (Boudry et al., 2004; Zhu et al., 2012). However, the administration of an antioxidant blend including among different antioxidants tea polyphenols to piglets increased their antioxidant status and improved morphological and histological evaluation of intestinal tissues and maintained intestinal barrier function (Zhu et al., 2012). Zhu et al. (2012) have also shown that one of the antioxidant mechanisms that was increased was the glutathione peroxidase enzyme (GPx) which buffers H_2O_2 produced during cellular metabolism by converting oxidized glutathione (GSSG) to reduced form (i.e. GSH) (Zhu et al., 2012). In our study, feed supplemented with OMWW retentate may also increase GSH levels through i) increase in GPx activity, ii) increase in enzymes which are responsible for GSH synthesis such as γ-glutamylcysteine ligase (GCL) and GSH synthetase (Aquilano et al., 2014) and iii) reserve GSH from reaction with free radicals by their direct scavenging (Frank et al., 2013). Interestingly, Masella et al. (2004) have shown that two polyphenols of olive oil, protocatechuic acid and oleuropein, enhanced the antioxidant capacity of murine J774 A.1 macrophage-like cells through direct scavenging of ROS, and thus resulting in the preservation of GSH levels. Moreover, these two polyphenols increased the expression and the activity of GPx and glutathione reductase (GR; regenerates GSH from GSSG) enzymes (Masella et al., 2004).

The enhancement of piglets' antioxidant mechanisms seemed to protect them from oxidative stress-induced damage. Thus, in piglets administered feed supplemented with OMWW retentate, there was a reduction in protein oxidation as shown by the decrease in CARB levels in plasma and all tested tissues compared to the control group. Moreover, the reduction of protein oxidation was time dependent especially in tissues, since the percentage decrease in CARB levels was higher after 30 d feeding with OMWW retentate than after 15 d feeding. It was especially interesting the reduction of protein oxidation in muscle tissue, since muscles are the edible part of the animal. Studies have shown that ROS-induced oxidation of muscle proteins results in the loss of essential amino acids (e.g. tryptophan), and affects water-holding capacity of meat proteins, the color and texture of processed meat products, and the digestibility of muscle foods leading to the reduction of their nutritional value (Villaverde et al., 2014; Lund et al., 2011). Oxidation of porcine myofibrillar proteins has also been shown to reduce their gelation which is important for the textural and structural characteristics of meat products (Zhou et al., 2014).

Administration of feed supplemented with OMWW retentate also reduced lipid peroxidation as demonstrated by the reduction in TBARS levels in plasma and all tissues. Similar to reduction in protein oxidation, decrease in lipid peroxidation was also time dependent, that is, it was higher in piglets fed for 30 d with diet supplemented with OMWW retentate than in those fed for only 15 d. Inhibition of lipid peroxidation is important, since it has been reported to affect pig productivity and health (Shurson et al., 2015). Moreover, lipid peroxidation is one of the primary causes leading to meat's quality deterioration, while it may also result in production of toxic compounds (Rey et al., 2001). It is also believed that lipid peroxidation and protein oxidation, especially when occurring in meat products, are interrelated processes at which the former

probably takes place faster and subsequently induces the latter (Zhou et al., 2014).

In conclusion, the present results indicated that feeding of piglets after weaning with diet supplemented with polyphenolic byproduct from OMWW processing using ceramic membrane microfiltration enhanced antioxidant mechanisms as shown by increases in TAC, GSH and CAT markers. Moreover, feed supplemented with OMWW polyphenols reduced oxidative stress-induced damage to proteins and lipids as shown by decreases in CARB and TBARS markers respectively. This finding is important, since as mentioned above piglets' weaning is a stressful event that may cause oxidative stress and subsequently manifestation of pathological conditions (Zhu et al., 2012; Lykkesfeldt and Svendsen, 2007). Although products from olive oil have been used previously for making pig feed (Gonzalez et al., 2012; Park et al., 2012), this is the first study in which OMWW was used. The use of byproducts from OMWW processing for making animal feeds is also interesting, since OMWW cause serious environmental problems. In future studies, we also intend to investigate the possible transfer through diet of the antioxidant polyphenolic compounds or their metabolites from feed to pig meat and subsequently to humans.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research has been co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funding Program: ARCHIMEDES III investing in knowledge society through the European Social Fund. The work was also funded by the 'Biotechnology-Nutrition & Environment' and 'Molecular Biology and Genetics Applications' MSc programmes in the Department of Biochemistry & Biotechnology at the University of Thessaly.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2015.11.007.

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Aly, A.A., Hasan, Y.N., Al-Farraj, A.S., 2014. Olive mill wastewater treatment using a simple zeolite-based low-cost method. J. Environ. Manag. 145, 341–348.
Aquilano, K., Baldelli, S., Ciriolo, M.R., 2014. Glutathione: new roles in redox signaling for an old antioxidant. Front. Pharmacol. 26, 5–196.
Basu, S., Eriksson, M., 1998. Oxidative injury and survival during endotoxemia. FEBS Lett. 438, 159–160.
Basu, S., Eriksson, M., 2000. Vitamin E in relation to lipid peroxidation in experimental septic shock. Prostaglandins Leukot. Essent. Fat. Acids 62, 195–199.
Basu, S., Eriksson, M., 2001. Retinol palmitate counteracts oxidative injury during experimental septic shock. Ann. Acad. Med. 30, 265–269. Singapore.
Boudry, G., Peron, I., Le Huerou-Luron, J., Lalles, P., Seve, B., 2004. Weaning induces both transient and longlasting modifications of absorptive, secretory, and barrier properties of piglet intestine. J. Nutr. 134, 2256–2262.
Eicher, S.D., McKee, C.A., Carroll, J.A., Pajor, E.A., 2006. Supplemental vitamin C and yeast cell wall betaglucan as growth enhancers in newborn pigs and as immunomodulators after an endotoxin challenge after weaning. J. Anim. Sci. 84, 2352–2360.
El–Khateeb, M.A., Tantry, B.A., Mohammed, A.H., Rahiman, S., 2014. Phenotypic characterization of phenol degrading microorganisms isolated from olive mill waste. J. Microbiol. Res. 8 (21), 2131–2136.
Fragou, S., Fegeros, K., Xylouri, E., Baldi, A., Politis, I., 2004. Effect of vitamin E supplementation on various functional properties of macrophages and neutrophils obtained from weaned piglets. J. Vet. Med. A Physiol. Pathol. Clin. Med. 51, 178–183.
Frankel, E., Bakhouche, A., Lozano-Sánchez, J., Segura-Carretero, A., Fernández-Gutiérrez, A., 2013. Literature review on production process to obtain extra virgin olive oil enriched in bioactive compounds. Potential use of byproducts as alternative sources of polyphenols. J. Agric. Food. Chem. 61 (22), 5179–5188.
Gerasopoulos, K., Stagos, D., Kokkas, S., Petrotos, K., Kantas, D., Goulas, P., Kouretas, D., 2015. Feed supplemented with byproducts from olive oil mill wastewater processing increases antioxidant capacity in broiler chickens. Food Chem. Toxicol. 82, 42–49.
González, E., Hernández-Matamoros, A., Tejeda, J.F., 2012. Two by-products of the olive oil extraction industry as oleic acid supplement source for Iberian pigs: effect on the meat's chemical composition and induced lipoperoxidation. J. Sci. Food Agric. 92 (12), 2543–2551.
Goto, S., Radák, Z., 2009. Hormetic effects of reactive oxygen species by exercise: a view from animal studies for successful aging in human. Dose Response 8 (1), 68–72.
Halliwell, B., 2001. Free radicals and other reactive species in disease. In: Wiley, J., & Sons (Ed.), Encyclopedia of Life Sciences. Nature Publishing Group, London, pp. 1–7.
Ibiebele, T.I., Hughes, M.C., Nagle, C.M., Bain, C.J., Whiteman, D.C., Webb, P.M., 2013. Dietary antioxidants and risk of Barrett's esophagus and adenocarcinoma of the esophagus in an Australian population. Int. J. Cancer 133 (1), 214–224.
Janaszewska, A., Bartosz, G., 2002. Assay of total antioxidant capacity: comparison of four methods as applied to human blood plasma. Scand. J. Clin. Lab. Investig. 62, 231–236.
Kapellakis, I.E., Paranychianakis, N.V., Tsagarakis, K.P., Angelakis, A.N., 2012. Treatment of olive mill wastewater with constructed wetlands. Water 4, 260–271.
Keles, M.S., Taysi, S., Sen, N., Aksoy, H., Akçay, F., 2001. Effect of corticosteroid therapy on serum and CSF malondialdehyde and antioxidant proteins in multiple sclerosis. Can. J. Neurol. Sci. 28 (2), 141–143.
Klein, E.A., Thompson, I.M., Tangen, C.M., Crowley, J.J., Lucia, M.S., Goodman, P.J., Minadian, L.M., Ford, L.G., Parnes, H.J., Gaziano, J.M., Karp, D.D., Lieber, M.M., Walther, P.J., Klotz, L., Parsons, J.K., Chin, J.L., Darke, A.K., Lippman, S.M., Goodman, G.E., Meyskens, F.L., Baker, L.H., 2011. Vitamin E and the risk of prostate cancer the selenium and vitamin E cancer prevention trial (SELECT). JAMA 306 (14), 1549–1556.
Kumar, De U., Mukherjee, R., Nandi, S., Patel, B.H., Dimri, U., Ravishankar, C., Verma, A.K., 2014. Alterations in oxidant/antioxidant balance, high-mobility group box 1 protein and acute phase response in cross-bred suckling piglets suffering from rotaviral enteritis. Trop. Anim. Health Prod. 46 (7), 1127–1133.
Kushi, L.H., Doyle, C., McCullough, M., Rock, C.L., Demark-Wahnefried, W., Bandera, E.V., Gapstur, S., Patel, A.V., Andrews, K., Gansler, T., 2012. Nutrition and physical activity guidelines Advisory Committee American Cancer Society guidelines on nutrition and physical activity for Cancer prevention reducing the risk of cancer with healthy food choices and physical activity. CA Cancer J. Clin. 62, 30–67.
Lauritzen, B., Lykkesfeldt, J., Friis, C., 2003a. Evaluation of a single dose versus divided dose regimen of danofloxacin in treatment of Actinobacillus pleuropneumoniae infection in pigs. Res. Vet. Sci. 74, 271–277.
Lauritzen, B., Lykkesfeldt, J., Skaanild, M.T., Angen, O., Nielsen, J.P., Friis, C., 2003b. Putative biomarkers for evaluating antibiotic treatment: an experimental model of porcine Actinobacillus pleuropneumoniae infection. Res. Vet. Sci. 74, 261–270.
Lauritzen, B., Lykkesfeldt, J., Friis, C., 2005. Evaluation of a single dose versus a divided dose regimen of amoxicillin in treatment of Actinobacillus pleuropneumoniae infection in pigs. Res. Vet. Sci. 79, 61–67.
Lund, M.N., Heinonen, M., Baron, C.P., Est'evez, M., 2011. Protein oxidation in muscle foods: a review. Mol. Nutr. Food Res. 55, 83–95.
Lykkesfeldt, J., Svendsen, O., 2007. Oxidants and antioxidants in disease: oxidative stress in farm animals. Vet. J. 173 (3), 502–511.
Masella, R., Varì, R., D'Archivio, M., Di Benedetto, R., Matarrese, P., Malorni, W., Scazzocchio, B., Giovannini, C., 2004. Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes. J. Nutr. 134 (4), 785–791.
Miller, M.J., Zhang, X.J., Sadowska-Krowicka, H., Chotinaruemol, S., McIntyre, J.A., Clark, D.A., Bustamante, S.A., 1993. Nitric oxide release in response to gut injury. Scand. J. Gastroenterol. 28, 149–154.
Oliveras-López, M.J., Berná, G., Carneiro, E.M., López-García de la Serrana, H., Martín, F., López, M.C., 2008. An extra-virgin olive oil rich in polyphenolic compounds has antioxidant effects in OF1 mice. J. Nutr. 138 (6), 1074–1078.
Ouyang, L., Shi, Z., Zhao, S., Wang, F.-T., Zhou, T.-T., Liu, B., Bao, J.-K., 2012. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell Prolif. 45, 487–498.
Paixao, S.M., Anselmo, A.M., 2002. Effect of olivemill wastewaters on the oxygen consumption by activated sludge microorganisms: an acute toxicity test method. J. Appl. Toxicol. 22, 173–176.
Paraskeva, P., Diamadopoulos, E., 2006. Technologies for olive mill wastewater (OMW) treatment: a review. J. Chem. Technol. Biotechnol. 81, 475e1485.
Park, J.C., Kim, S.C., Lee, S.D., Jang, H.C., Kim, N.K., Lee, S.H., Jung, H.J., Kim, I.C., Seong, H.H., Choi, B.H., 2012. Effects of dietary fat types on growth performance, pork quality, and gene expression in growing-finishing pigs. Asian-Australas. J. Anim. Sci. 25 (12), 1759–1767.
Patsoukis, N., Zervoudakis, G., Panagopoulos, N.T., Georgiou, C.D., Angelatou, F., Matsokis, N.A., 2004. Thiol redox state (TRS) and oxidative stress in the mouse hippocampus after pentylenetetrazol-induced epileptic seizure. Neurosci. Lett. 357, 83–86.
Radak, Z., Chung, H.Y., Goto, S., 2008. Systemic adaptation to oxidative challenge

induced by regular exercise. Free Radic. Biol. Med. 44 (2), 153–159.
Reddy, Y.N., Murthy, S.V., Krishna, D.R., Prabhakar, M.C., 2004. Role of free radicals and antioxidants in tuberculosis patients. Indian J. Tuberc. 51, 213–218.
Rey, A.I., Kerry, J.P., Lynch, P.B., López-Bote, C.J., Buckley, D.J., Morrissey, P.A., 2001. Effect of dietary oils and alpha-tocopheryl acetate supplementation on lipid (TBARS) and cholesterol oxidation in cooked pork. J. Anim. Sci. 79 (5), 1201–1208.
Rinaldi, M., Rana, G., Introna, M., 2003. Olive-mill wastewater spreading in southern Italy: effects on a durum wheat crop. Field Crops Res. 84, 319–326.
Robbins, C.G., Davis, J.M., Merritt, T.A., Amirkhanian, J.D., Sahgal, N., Morin, F.C., Horowitz, S., 1995. Combined effects of nitric oxide and hyperoxia on surfactant function and pulmonary inflammation. Am. J. Physiol. 269 (4 Pt 1), L545–L550.
Shurson, G.C., Kerr, B.J., Hanson, A.R., 2015. Evaluating the quality of feed fats and oils and their effects on pig growth performance. J. Anim. Sci. Biotechnol. 6 (1), 10.
Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell. B 39, 44–84.
Villaverde, A., Morcuende, D., Estévez, M., 2014. Effect of curing agents on the oxidative and nitrosative damage to meat proteins during processing of fermented sausages. J. Food Sci. 79 (7), 1331–1342.
Zagklis, D.P., Arvaniti, E.C., Papadakis, V.G., Paraskeva, C.A., 2013. Sustainability analysis and benchmarking of olive mill wastewater treatment methods. J. Chem. Technol. Biotechnol. 88, 742–750.
Zhou, F., Zhao, M., Zhao, H., Sun, W., Cui, C., 2014. Effects of oxidative modification on gel properties of isolated porcine myofibrillar protein by peroxyl radicals. Meat Sci. 96 (4), 1432–1439.
Zhu, L.H., Zhao, K.L., Chen, X.L., Xu, J.X., 2012. Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. J. Anim. Sci. 90 (8), 2581–2589.
Zrelli, H., Matsuoka, M., Kitazaki, S., Zarrouk, M., Miyazaki, H., 2011. Hydroxytyrosol reduces intracellular reactive oxygen species levels in vascular endothelial cells by upregulating catalase expression through the AMPK-FOXO3a pathway. Eur. J. Pharmacol. 660 (2–3), 275–282.