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Feed supplemented with byproducts from olive oil mill wastewater processing increases antioxidant capacity in broiler chickens

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ABSTRACT

In the present study, a ceramic membrane microfiltration method was used for the separation of two liquid products, the downstream permeate and the upstream retentate, from olive mill wastewater (OMWW). These liquid products were examined for their antioxidant activity by incorporating them into broilers' feed. Twenty four broilers 13 d old were divided into two feeding groups receiving supplementation with OMWW retentate or permeate for 37 d. Blood was drawn at 17, 27 and 37 d, while tissues (muscle, heart, liver) were collected at 37 d. The antioxidant effects were assessed by measuring oxidative stress biomarkers in blood and tissues. The results showed that broilers given feed supplemented with OMWW retentate or permeate had significantly lower protein oxidation and lipid peroxidation levels and higher total antioxidant capacity in plasma and tissues compared to control group. In both OMWW groups, catalase activity in erythrocytes and tissues was significantly increased compared to control group. OMWW retentate administration increased significantly GSH in erythrocytes in broilers with low GSH, although both OMWW products significantly reduced GSH in broilers with high GSH. Thus, it has been demonstrated for the first time that supplementation with OMWW processing residues could be used for enhancing broilers' redox status.

1. Introduction

Olive oil obtained from olive tree fruit (*Olea europaea* L.) is a main part of the Mediterranean diet. Several studies have shown that olive oil consumption is associated with lower risk of certain diseases such as cardiovascular disease and cancer (Cárdeno et al., 2013; Scoditti et al., 2014). Many of these beneficial effects of olive oil on human health are attributed to its polyphenolic compounds (e.g. tyrosol, hydroxytyrosol, oleuropein and pinoresinol) having potent antioxidant properties (Cárdeno et al., 2013; Scoditti et al., 2014). Apart from olive oil itself, some of the byproducts of olive oil production such as olive oil mill wastewater (OMWW) present important bioactivities (Cardinali et al., 2010, 2012; Hamden et al., 2009).

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 to 0.5–3.25 m^3 per 1000 kg of olives (Kapellakis et al., 2012; Paraskeva and Diamadopoulos, 2006). Since the annual worldwide olive oil production is estimated at about 2×10^6 tons, the OMWW would amount to about 4×10^6 m^3 (Agalias et al., 2007). 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 (3–6) (Paraskeva and Diamadopoulos, 2006). OMWW also contains tannins, lignins, long-chain fatty acids, reduced sugars, proteins and phenolic compounds which are toxic to microorganisms and plants (Paixao and Anselmo, 2002; Paraskeva and Diamadopoulos, 2006). Furthermore, 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 in the sewage system is not possible without any treatment such as aerobic treatment, anaerobic digestion and composting (Aly et al., 2014; Rinaldi et al., 2003). However, an environmentally safe and cost-effective treatment of OMWW has not yet been found (Zagklis et al., 2013).

Abbreviations: 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; GSH, reduced glutathione; OMWW, olive mill wastewater; RO, reverse osmosis; ROS, reactive oxygen species; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid.

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As already mentioned, a large part of the toxic effects of OMWW are attributed to its polyphenolic content causing phytotoxicity, toxicity against aquatic organisms and suppression of soil microorganisms (Aliotta et al., 2002; Fiorentino et al., 2003; Kotsou et al., 2004). The phenolic compounds found in OMWW can be simple phenols or polyphenols resulting from polymerization of simple phenols and their concentration varies from 0.5 to 24 g/L (Frankel et al., 2013).

The polyphenols identified 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 (Frankel et al., 2013). It should be noted that polyphenols in OMWW represent about 50% of the total polyphenols found in olive fruit (Rodis et al., 2002). Despite the toxic effects exhibited at high concentrations, these polyphenols also possess antioxidant activity (Frankel et al., 2013). Therefore, isolation of the polyphenolic content of OMWW could represent an antioxidant source in food, pharmaceutical and cosmetic industry, while reducing the environmental toxicity 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. A major problem of this methodology was that a large quantity of byproducts were produced. These byproducts contain a part of the OMWW polyphenols and have to be discharged in an eco-friendly and sustainable way. For this purpose, in the present study, these byproducts were used for making broilers' feed supplemented with antioxidant compounds as a disposable method. In farm animals, oxidative stress may be involved in several pathological conditions (e.g. pneumonia, sepsis) affecting animal production and general welfare (Basu and Eriksson, 2000; Lauritzen et al., 2003; Lykkesfeldt and Svendsen, 2007). For example, the hot and humid environment in aviaries may cause heat-induced oxidative stress in chickens which in turn it reduces growth and meat quality (Rhoads et al., 2013). Moreover, low feed efficiency in broilers is associated with high oxidative stress in breast muscle, liver and intestine (Iqbal et al., 2004). Thus, administration of natural antioxidant compounds to chickens has been proposed as a means for reducing the oxidative stress-induced adverse effects (Chen et al., 2013; Oskoueian et al., 2014). Although olive oil-supplemented diet has been shown to protect chicken skeletal muscle from heat stress-induced oxidative stress (Mujahid et al., 2009), OMWW has not been used so far in chickens' feed. Thus, the administration of antioxidants to farm animals has been suggested for protecting them from such pathologies or mitigating their symptoms (Abuelo et al., 2014; Deaton et al., 2004; Lykkesfeldt and Svendsen, 2007). The antioxidant effects of supplementation with OMWW of the processing byproducts were assessed by measuring oxidative stress biomarkers in broilers' blood and different tissues (i.e. muscle, cardiac and liver).

2. Materials and methods

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

As a part of a patented OMWW polyphenol powder production scheme, two byproducts containing antioxidant polyphenolic compounds are produced which have to be discharged in an eco-friendly and sustainable way. For this purpose, in this work, the production of animal feed was selected as a disposable technology of these two byproducts. According to the patented production scheme (Fig. 1) the raw OMWW was first passed through a finisher in order to separate suspended particles in the form of a heavy sludge. 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 equipped with a cylindrical stainless steel sieve with openings of 150 μm diameter. 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.

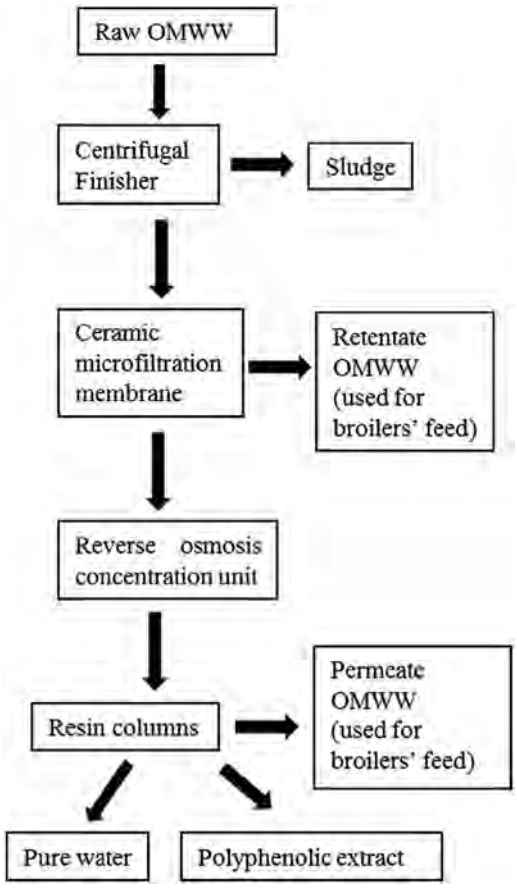

Fig. 1. Patented OMWW polyphenol powder production scheme.

Subsequently, the clarified OMWW was passed through a ceramic microfiltration unit where 30% of the input OMWW stream was separated as retentate (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 which was used as a part of the production of the OMWW liquid was supplied by Jiangu Jiuwu Hi-Tech Co. Ltd (Nanjing, China). This consisted of a membrane module with three pieces of ceramic microfiltration membranes with a total area of 0.69 m^2. The type of membrane was the CMF19040. The unit was fed with a positive displacement pump ensuring fluid velocity of 10 m/s in order to avoid membrane fouling.

The microfiltration permeate was then concentrated at low temperature by using a standard reverse osmosis (RO) membrane unit to a concentration factor 4:1 by using a pressure of 20 bar and a spiral type membrane module, thus producing a concentrate rich in polyphenols. Then, this was passed through a XAD4 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 to 30% of the initial polyphenolic content of the input material was the second byproduct (designated hereafter as OMWW permeate) to be used for silage preparation. The above-mentioned two byproducts of the patented polyphenol powder production scheme were then utilized for silage production by mixing them with corn. This processing of the two byproducts ensured a total discharge of the OMWW and complete protection of the ecosystem as only pure water is disposed to the environment.

2.2. Silage and broilers' feed preparation

OMWW retentate and permeate were used for making silage corn. For this purpose, corn was mixed with OMWW retentate or permeate in a ratio of 24:1. Thus, corn was made that contained 56% solids, 4% OMWW retentate or permeate 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 to 40 °C in order to be activated prior to their mixing with corn. After activation, lactic bacteria were mixed with corn (1 g of bacteria with 100 kg of corn). The resulting silages were then mixed with other ingredients for making the final broilers' feed (Table 1).

Table 1
Ingredients and nutrient composition of experimental diets.

Ingredients	Composition (% w/w)
Corn	55.2[a]
Soybean 42/8	31.8
Fat powder (lecithin)	5.0
Fishmeal	4.0
Broiler Balancer Premix	2.5
Marble powder	1.5

[a] Corn contained 60% solids and 40% liquid in control group; 56% solids, 4% retentate OMWW and 40% liquid in OMWW retentate group; 56% solids, 4% permeate OMWW and 40% liquid in OMWW permeate group.

2.3. Animals

The experiment was reviewed and approved by the institutional review board and the appropriate state authority. Thirty six broilers (*Hubbard*) 6 days old were purchased from the 'Kakanoudis' aviary. Chickens were housed under controlled environmental conditions (12-hour light/dark cycle, temperature 18–21 °C, humidity 50–70%) in standard single cages (area 1500 cm^2/cage). Standard ration and water were provided *ad libitum* for one week. Then, chickens were randomly divided into three experimental groups (12 broilers per group) as follows: (a) broilers fed with standard ration (control group), (b) broilers fed with ration containing OMWW ceramic microfiltration permeate (OMWW permeate group), and (c) broilers fed with ration containing OMWW ceramic microfiltration retentate (OMWW retentate group). The broilers were monitored for 37 days.

2.4. Blood and tissue collection

Blood samples were drawn at days 17, 27 and 37. For blood collection, chickens were restrained manually and 4 ml of blood were collected from branchial vein and placed in ethylenediamine tetraacetic acid (EDTA) tubes. Blood samples were centrifuged immediately at 1370 *g* for 10 minutes at 4 °C and the plasma was collected and used for measuring TAC, TBARS and protein carbonyls. The packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged at 4020 *g* for 15 minutes at 4 °C and the erythrocyte lysate was collected for the measurement of GSH and catalase activity.

Tissue collection was made at day 37. Tissues were quickly removed and snap-frozen in liquid nitrogen. For tissue collection, the broilers were sacrificed in a fully automated slaughter complex in 'Kakanoudis' aviary. All relevant procedures (e.g. electric stunning, slaughter, bleeding, removal of feathers, gutting, viscera separation and washing) were executed by special machines and specialized staff. In preparation for tissue biochemical analysis, tissue samples were initially ground using mortar and pestle under 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

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 mol/L Na_2SO4 and 55 mmol/L 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 mmol/L 2,4-dinitrophenylhydrazine (in 2.5 N HCL) for the sample (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 mol/L 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) were added, respectively, to 480 μL or 460 μL of 10 mmol/L sodium potassium phosphate (pH 7.4) and 500 μL of 0.1 mmol/L 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 mmol/L sodium potassium phosphate (pH 8.0) and 330 μL of 1 mmol/L 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 calibration curve made using commercial standards.

Catalase activity was determined using the method of Aebi (1984). Briefly, 4 μL of erythrocyte lysate (diluted 1:10) or 40 μL muscle homogenate (diluted 1:2) were added, respectively, to 2991 μL or 2955 μL of 67 mmol/L sodium potassium phosphate (pH 7.4), and the samples were incubated at 37 °C for 10 min. A total of 5 μL of 30% hydrogen peroxide were 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 and 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 analysed by one-way ANOVA followed by Dunnett's test for pairwise comparisons. The level of statistical significance was set at $p < 0.05$. All results are expressed as mean ± SEM. Data were analysed using SPSS, version 13.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Assessment of oxidative stress markers in blood

All oxidative stress biomarkers measured in blood showed that OMWW permeate and retentate improved the redox status of the broiler chickens. Specifically, protein carbonyl levels in plasma were decreased significantly in the groups that received either the OMWW retentate or permeate compared to control group (Fig. 2A). The OMWW retentate group exhibited the highest decrease in protein carbonyl levels by 52.6, 46.3 and 42.7% at days 17, 27 and 37 respectively (Fig. 2A). In OMWW permeate group, protein carbonyls were decreased by 23.1, 19.6 and 27.0% at days 17, 27 and 37 respectively (Fig. 2A). It has been suggested that cleavage of H_2O_2 by iron or copper is a major source of OH• that may oxidize amino acid residues in proteins leading to protein carbonyl formation (Stadtman and Levine, 2003). Thus, polyphenols found in OMWW may inhibit these reactions.

Like protein carbonyls, TBARS levels in plasma were decreased significantly in the groups that received either the OMWW retentate or permeate compared to control group (Fig. 2B). The OMWW retentate was the most potent, exhibiting the greatest decrease in TBARS levels by 69.8, 69.0 and 54.0% at days 17, 27 and 37 respectively (Fig. 2B). In the OMWW permeate group, TBARS were decreased by 53.5, 45.0 and 8.03% at days 17, 27 and 37 respectively (Fig. 2B).

TAC in plasma was increased significantly but only in the group that received the OMWW retentate compared to control group (Fig. 2C). In particular, TAC was increased in OMWW retentate group by 16.8, 16.6 and 17.6% at days 17, 27 and 37 respectively (Fig. 2C).

The administration of either OMWW retentate or permeate increased catalase activity in erythrocytes (Fig. 3A). The OMWW

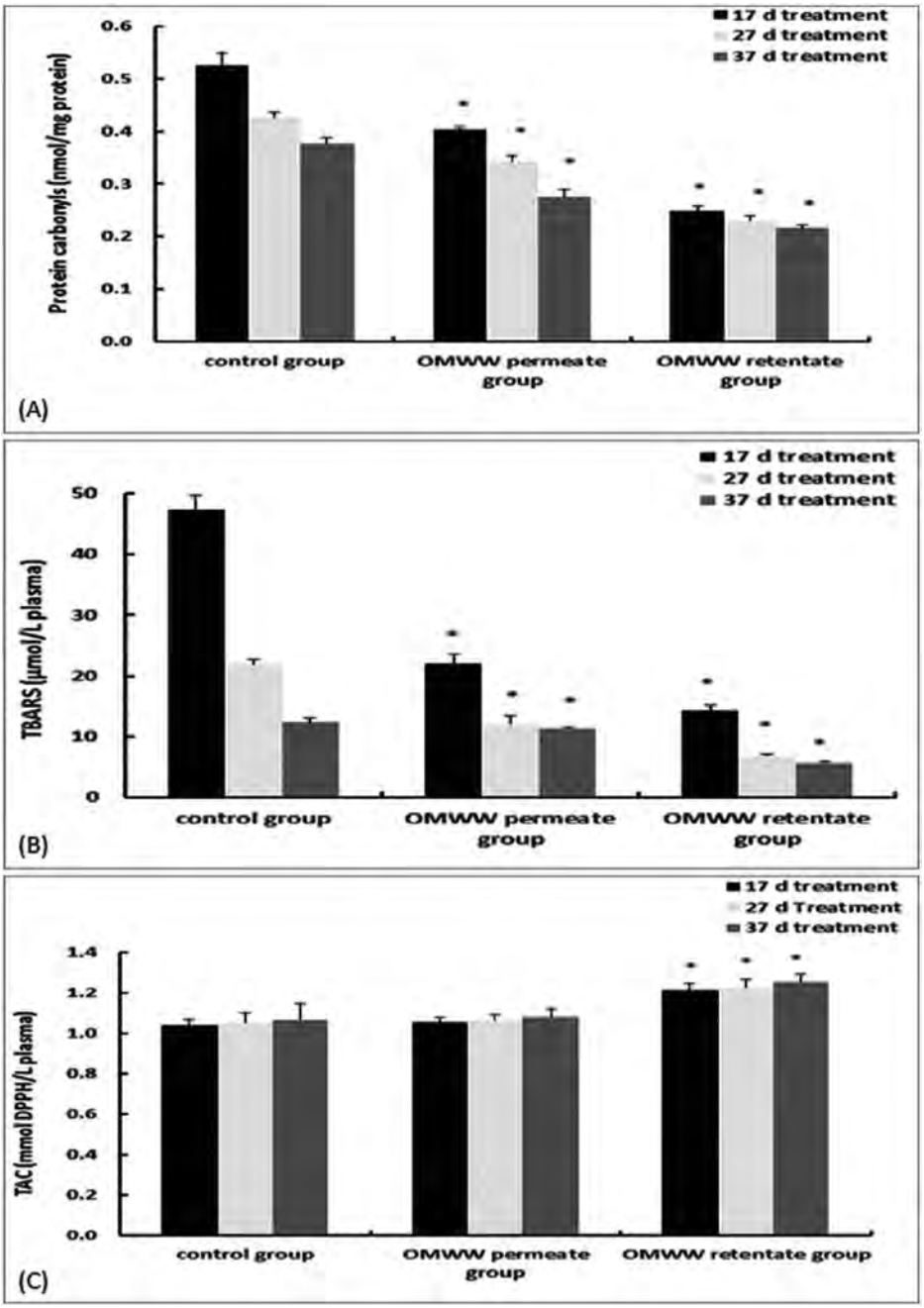

Fig. 2. Effects on oxidative stress markers, (A) protein carbonyls, (B) TBARS and (C) TAC, in plasma of chickens after administration for 17, 27 and 37 d of feed containing standard ration (control group) or OMWW permeate or OMWW retentate. *Significantly different from the values of control group at the same sampling time ($p < 0.05$).

retentate group demonstrated the highest increase in catalase activity by 31.3, 56.6 and 57.9% at days 17, 27 and 37 respectively (Fig. 3A). In the OMWW permeate group, catalase activity was increased significantly by 14.0 and 10.2% at days 27 and 37 respectively (Fig. 3A).

The effect of OMWW permeate and retentate administration on GSH levels in erythrocytes was intriguing. Specifically, in OMWW retentate group, GSH levels were increased by 264% at day 17 compared to control group (Fig. 3B). However, OMWW retentate administration decreased GSH by 36.0 and 25.0% at days 27 and 37 compared to control group (Fig. 3B). Likewise, OMWW permeate administration decreased GSH by 24.2 and 15.3% at days 27 and 37 compared to control group (Fig. 3B).

3.2. Assessment of oxidative stress markers in quadriceps muscle, heart and liver

The administration of OMWW retentate and permeate had similar effects on the oxidative stress markers assessed in quadriceps muscle, heart and liver. In particular, protein carbonyl levels were decreased significantly in the groups that received OMWW retentate and permeate by 52.7 and 16.6% respectively in quadriceps muscle, by 55.4 and 28.6% respectively in heart and by 58.6 and 37.1% respectively in liver compared to control group (Fig. 4A).

TBARS levels were also decreased significantly in the groups that received OMWW retentate and permeate by 50.70 and 13.60% respectively in quadriceps muscle, by 33.5 and 11.8% respectively in

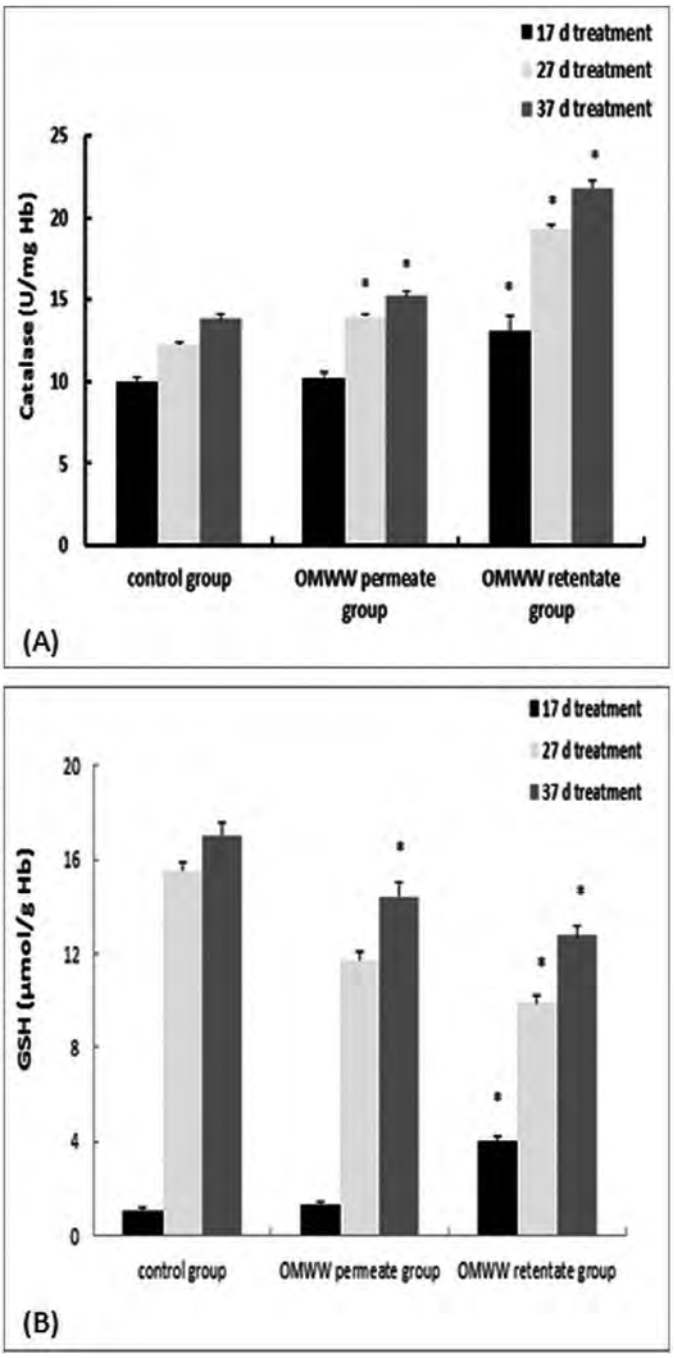

Fig. 3. Effects on oxidative stress markers, (A) catalase activity and (B) GSH, in erythrocytes of chickens after administration for 17, 27 and 37 d of feed containing standard ration (control group) or OMWW permeate or OMWW retentate. *Significantly different from the values of control group at the same sampling time ($p < 0.05$).

heart and by 21.4 and 12.5% respectively in liver compared to control group (Fig. 4B).

TAC was increased significantly in the groups that received OMWW retentate and permeate extracts by 18.1 and 10.5% respectively in quadriceps muscle, and by 19.2 and 7.6% respectively in heart (Fig. 4C). In liver, there was a statistically significant increase by 14.8% only in OMWW retentate group (Fig. 4C).

Catalase activity was increased significantly in the groups that received OMWW retentate and permeate by 44.3 and 38.5% respectively in quadriceps muscle, by 33.9 and 9.30% respectively in heart and by 23.0 and 17.9% respectively in liver compared to control group (Fig. 5A).

As regards GSH levels, similar to erythrocytes at day 37, OMWW retentate and permeate administration decreased GSH levels by 17.2 and 8.20% respectively in quadriceps muscle, by 43.8 and 26.2% respectively in heart and by 38.2 and 13.2% respectively in liver compared to control group (Fig. 5B).

4. Discussion

The findings of the present study suggested that the incorporation of OMWW byproducts into the feed improved the chickens' redox status. In particular, the feed supplemented with either OMWW retentate or permeate reduced protein oxidation by ROS as indicated by the reduction in protein carbonyl levels in plasma and all tested tissues. The OMWW retentate-induced decrease in protein carbonyl levels was similar between plasma and tissues, indicating that its antioxidant compounds could prevent protein oxidation in all tested body parts with similar efficiency. Recent studies have shown that protein oxidation can induce protein polymerization and aggregation, thus affecting their digestibility, which reduces the nutritional value of muscle foods (Zhang et al., 2013). Moreover, when myofibrillar protein isolate was incubated with oxidants, the amount of cysteine, tyrosine, methionine, glycine, histidine, alanine, leucine and lysine, and the total amounts of amino acids were decreased (Park and Xiong, 2007). Thus, the oxidation of these essential amino acids of meat proteins could decrease its nutritional value (Zhang et al., 2013).

Apart from protein oxidation, OMWW retentate and permeate consumption also decreased lipid peroxidation as shown by the reduction in TBARS levels, a biomarker of lipid peroxidation. Other studies have shown that some of the major polyphenolics found in OMWW (e.g. hydroxytyrosol, verbascoside and isoverbascoside) are effective to inhibit lipid peroxidation (Cardinali et al., 2012; Rubio-Senent et al., 2014). The reduction of lipid peroxidation could improve the quality of chicken meat, since lipid oxidation in poultry meat is one of the primary causes for limiting its quality (Arshad et al., 2013). Specifically, lipid oxidation leads to the production of off-flavour and decrease nutritional values of meat and meat products (Nam and Ahn, 2003). Thus, the use of natural antioxidants such as polyphenols that are safer than the synthetic ones has been used in broiler feed for reducing lipid oxidation (Arshad et al., 2013; Starčević et al., 2015). Moreover, there has been evidence that oxidation products from proteins and lipids can further increase the oxidation in a reciprocal manner (Faustman et al., 2010). For example, consumption of oxidized oil has been reported to increase levels of protein carbonyl and decrease meat quality, including lipid oxidation and drip loss in breast meat of broiler chickens (Zhang et al., 2011).

The observed reduction of protein and lipid oxidation in chickens that consumed feed supplemented with byproducts from OMWW processing may be attributed to increase in their TAC. Indeed, OMWW retentate especially increased TAC in plasma and tissue chickens. Furthermore, both OMWW retentate and permeate increased catalase activity, one of the most important antioxidant enzymes, in erythrocytes as well as in the tested chicken tissues. Other studies have also reported that OMWW extract increases catalase activity in plasma, liver and kidney of rats (Hamden et al., 2009).

The effects of OMWW retentate or permeate on all tested oxidative stress biomarkers exhibited a consistency (i.e. increase in antioxidant capacity) apart from those on GSH levels, which were intriguing. In particular, OMWW retentate increased GSH levels in erythrocytes at 17 d after initiation of feeding. Another study conducted in humans has also shown that consumption of OMWW extract increased GSH levels in plasma (Visioli et al., 2009). However, both OMWW retentate and permeate reduced GSH levels in erythrocytes at 27 and 37 d after feeding initiation. Similarly, OMWW

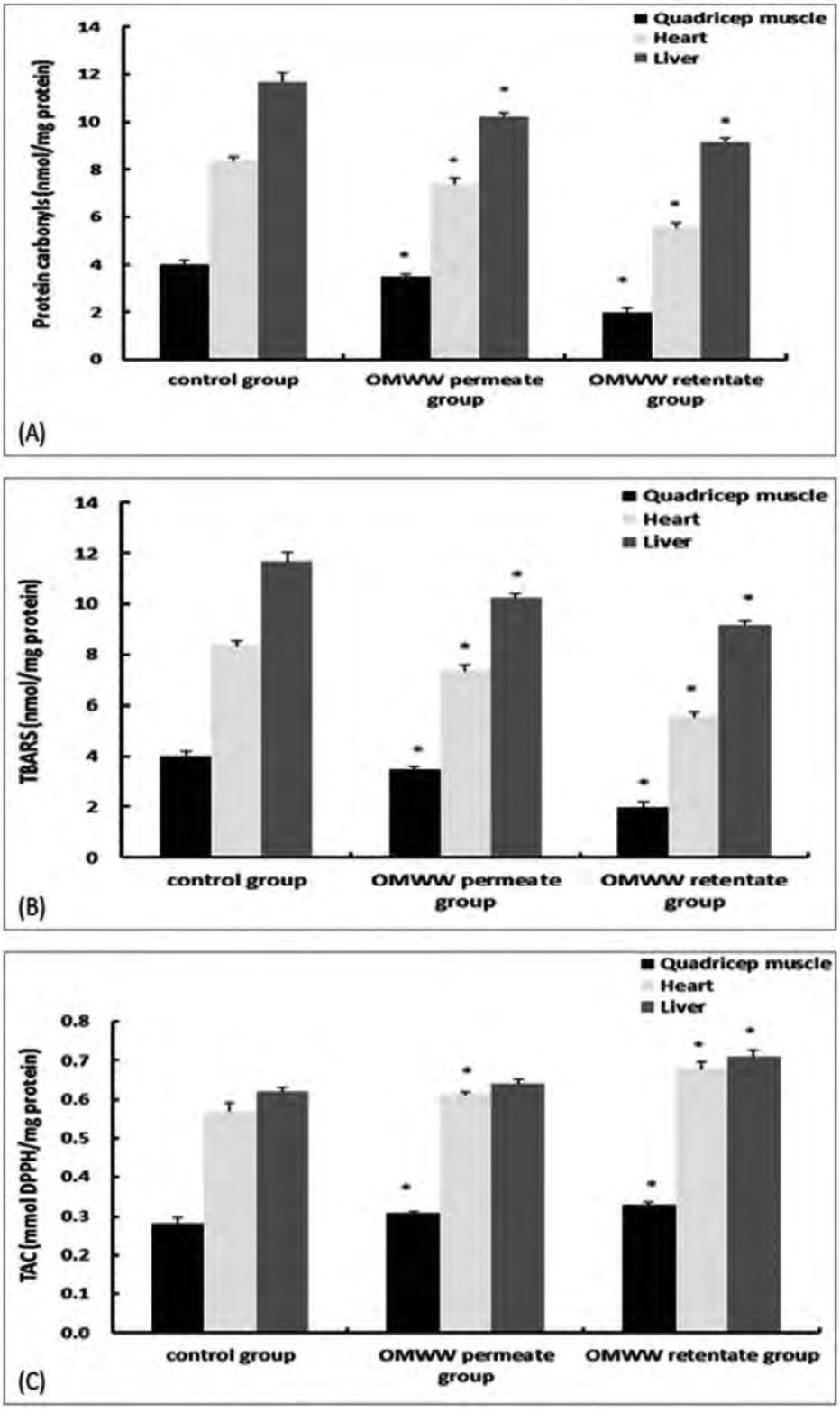

Fig. 4. Effects on (A) protein carbonyl levels, (B) TBARS levels and (C) TAC levels in tissues of chickens after administration for 37 d of feed containing standard ration (control group) or OMWW permeate or OMWW retentate. *Significantly different from the values of control group at the same sampling time ($p < 0.05$).

retentate and permeate administration reduced GSH levels in all tested tissues at 37 d after feeding initiation. An explanation for these results may be that at 17 d after feeding initiation (i.e. in the early age of 30 days old) the broilers had low GSH levels, and so OMWW retentate or permeate increased broilers' GSH levels. However, in older age (i.e. 27 and 37 d after feeding initiation), the broilers' organism produced high GSH levels by itself. Thus, the biochemical mechanisms responsible for GSH synthesis not only were not increased further after OMWW retentate or permeate consumption, but they were also decreased. Moreover, Chen et al. (2013) have also

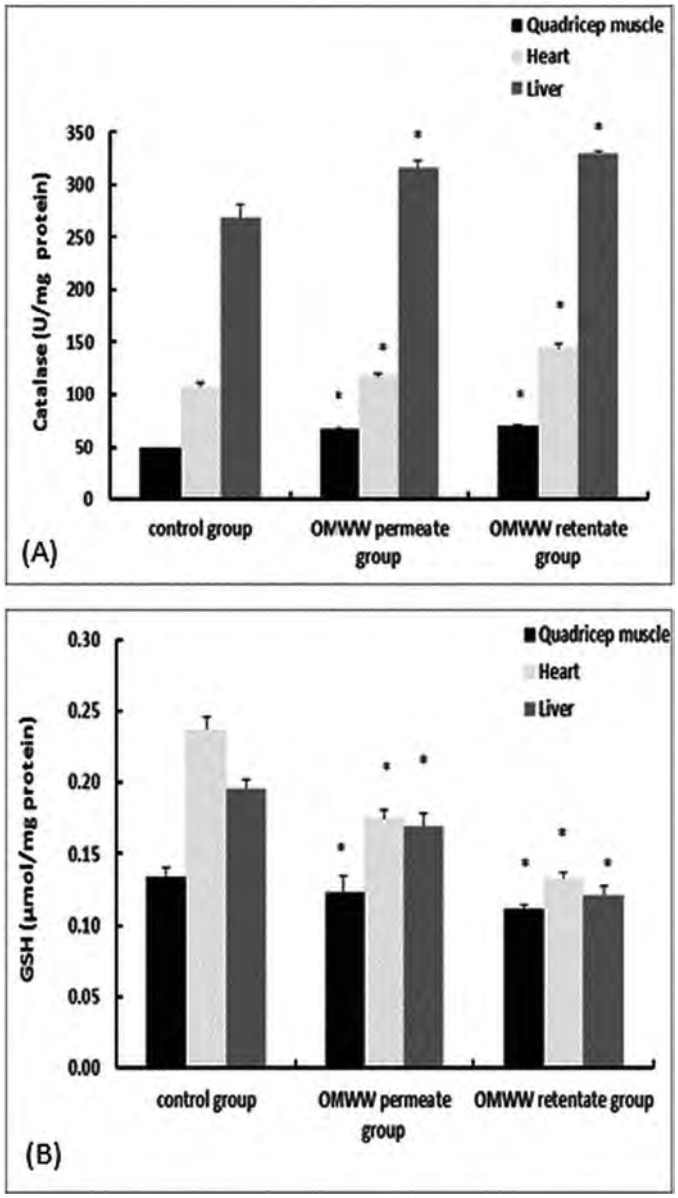

Fig. 5. Effects on (A) catalase activity and (B) GSH levels in tissues of chickens after administration for 37 d of feed containing standard ration (control group) or OMWW permeate or OMWW retentate. *Significantly different from the values of control group at the same sampling time ($p < 0.05$).

shown that supplemental methionine, a major precursor of GSH synthesis, increased GSH levels in broilers with low GSH concentration but decreased GSH levels in broilers with high GSH. The effects of OMWW retentate and permeate on GSH were possibly mediated through modulation of the main enzymes which are responsible for GSH synthesis such as γ-glutamylcysteine ligase (GCL) and GSH synthetase (Aquilano et al., 2014). The regulation of expression of these enzymes is mediated through the antioxidant response element (ARE), a cis-acting enhancer sequence that regulates the transcription of various antioxidant genes (Kumar et al., 2014). Thus, compounds of OMWW retentate or permeate may increase GSH levels by inducing the expression or the activation of these enzymes in broilers with low GSH. On the contrary, OMWW retentate or permeate may decrease the expression or inhibit the activity of GCL or GSH synthetase in broilers with high GSH. Importantly, DeLeve and Kaplowitz (1990) have reported that high GSH levels can inhibit its synthesis through feedback inhibition of GCL. Of course, more experiments are needed to confirm this hypothesis. However, if this hypothesis is correct, then it emphasizes the need for adding antioxidant compounds in broilers' feed especially in very early age when the organism is not mature enough to produce antioxidant molecules such as GSH by itself.

As mentioned above, the observed enhancement of broilers' antioxidant mechanisms by feed supplemented with OMWW products is mainly attributed to its polyphenolic content. Studies have demonstrated that OMWW contains antioxidant polyphenolics such as tyrosol, hydroxytyrosol, verbascoside, isoverbascoside (an oxidized form of verbascoside) and a number of higher molecular weight phenolics arising from oxidative polymerization of mainly hydroxytyrosol and caffeic acid (Cardinali et al., 2010, 2012). Interestingly, the results from all the oxidative stress biomarkers showed that OMWW retentate was more potent than OMWW permeate. This difference can be explained by the higher polyphenolic levels of the former compared to the latter, since a large part of polyphenols had been removed from the latter after passing it through resin columns. Also, OMWW retentate contained 1% v/v olive oil and consequently its respective antioxidant compounds, while OMWW permeate did not.

The above findings from all the tested oxidative stress biomarkers suggest that addition of byproducts (especially OMWW retentate) from OMWW processing using ceramic membrane microfiltration into broilers' feed improves their redox status. These results present particular interest, since different pathological conditions of farm animals are thought to be associated with oxidative stress (Lykkesfeldt and Svendsen, 2007). Thus, the findings of this study suggest for the first time that feed supplemented with OMWW compounds could be used for enhancing the redox status of broiler chickens by reducing oxidative damage of biological molecules (i.e. protein oxidation, lipid peroxidation) and increasing antioxidant mechanisms (i.e. catalase activity, GSH and TAC levels). Based on these results, OMWW retentate or permeate could also be used as supplements in other farm animal feeds or even in human foods. This usage would also be a good solution for the environmental problems caused by OMWW. Moreover, it would be interesting to examine a possible transfer of the antioxidant polyphenolic compounds or their metabolites from feed to broiler meat and subsequently to humans.

Conflict of interest

The authors declare that there are no conflicts of interest.

The Transparency document associated with this article can be found in the online version.

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